Gut Pathogens

BRIEF REPORT Open Access

Significant abundance of bacterial flagellin and expression of its surface localized receptor toll-like receptor 5 and cytokine interleukin-22 in South African infants with poor oral rotavirus vaccine take

Nontlantla J. Haindongo[1], Mapaseka Seheri[1] and Cliff A. Magwira[1,2*]

Abstract
Bacterial flagellin, a potent intestinal innate immune activator, prevents murine rotavirus (RV) infection independent of adaptive immunity and interferons. The flagellin-induced immunity is mediated by Toll-like receptor (TLR5) and Nod-like receptor C4 (NLRC4), which elicit the production of interleukins 22 (IL-22) and IL-18, respectively. Here, we assessed whether a high abundance of flagellin at the time of vaccination would negatively affect the oral RV vaccine take. Fecal samples were collected from infants a week after first dose of Rotarix vaccination to establish vaccine shedders ($n=50$) and non-shedders ($n=44$). The abundance of flagellin and expression of flagellin-encoding fliC, TLR5 and NLRC4, IL-22 and IL-18 genes was determined by qPCR. There were no differences in the abundance of flagellin between vaccine shedders and non-shedders ($p=0.15$). However, the expression of FliC was increased 7.5-fold in non-shedders versus shedders ($p=0.001$). Similarly, TLR5 ($p=0.045$), and not NLRC4 ($p=0.507$,) was significantly expressed in non-shedders versus shedders. The expression of IL-22 ($p=0.054$), and not IL-18 dependent NLRC4 ($p=0.650$), was increased 3.4-fold in non-shedders versus shedders. Collectively, our observations suggest a possible negative impact of the abundance of viable flagellated bacteria at the time of vaccination on the replication and therefore the performance of RV vaccines.

Keywords Bacterial flagellin, Toll-like receptor 5, Nod-like receptor C4, Interleukin-22, Rotavirus vaccine, Shedding

*Correspondence:
Cliff A. Magwira
cmagwira@gmail.com; cliff.magwira@smu.ac.za
[1]Diarrheal Pathogens Research Unit (DPRU), Department of Virology, Sefako Makgatho Health Sciences University, Ga-rankuwa, Pretoria, South Africa
[2]Department of Medical Virology, School of Medicine, Sefako Makgatho Health Sciences University, Molotlegi St, Ga-Rankuwa 0208, Pretoria, South Africa

Introduction

Rotavirus (RV) remains one of the principal causes of severe dehydrating diarrhea in infants in low to middle income countries (LMIC) [1]. Vaccines for severe RV diarrhea are available but do not perform satisfactorily in these countries [2] due to a variety of factors [3–6]. Increasing evidence suggests the composition of certain genera or species of gut microbiota may also play a role in how infants respond to RV vaccines [7–9]. However, the vaccine response may not rely solely on distinct bacterial genera or species but also on the functional levels of bacterial components in the periphery such as flagellin [10].

Bacterial flagellin is a potent innate immunity activator in the intestines [11] and has been shown to prevent and cure murine RV infection when repeatedly administered intraperitoneal [10]. The protection was achieved independent of type I and II interferons (IFNs), which play a crucial role in antiviral immunity [12, 13] as the flagellin' s antiviral effect was found to be fully maintained in mice lacking receptors for the two IFNs [10]. The flagellin-elicited intestinal immunity required the activation of surface localized Toll-like receptor 5 (TLR5) and cytosolic Nod-like receptor C4 (NLRC4) on dendritic cells and intestinal epithelial cells [10]. The flagellin activation of TLR5 and NLRC4 elicits the production of cytokines interleukin-22 (IL-22) and IL-18, respectively, whose administration to mice reportedly recapitulated fully the capacity of flagellin to protect against RV infection [10].

Several members of the human intestinal commensal bacteria including members of phylum Proteobacteria express flagella [14], from which a structural subunit flagellin is constantly released into the lumen. Changes in the abundance of flagella-producing bacteria in the gut may influence the luminal concentration of flagellin, consequently altering the threshold of intestinal immune activation. However, the extent to which optimal intestinal immune activation by flagellin could impact human oral live attenuated RV vaccine infection/replication, and therefore RV vaccine response, has not been tested. Here, we report differences in abundance of bacterial flagellin as well as expression of flagellin-encoding fliC, flagellin-receptors TLR5 and NLRC4, and cytokines IL-22 and IL-18 genes between South African infants who shed the RV vaccine in fecal samples and their non-shedding counterparts.

Methodology

Study design, participants and ethics

This cross-sectional study used fecal samples collected from infants who reported at a clinic, north of Pretoria for routine rotavirus immunization programme between 2021 and 2022. The specimens were collected a week after the first dose of Rotarix (GlaxoSmithKline, Rixensart, Belgium) vaccination after written and informed consent from the guardians. Only physically health infants weighing ≥ 2 kg and not on antibiotic therapy within 3 weeks prior to enrolment were included in the study. Study infants were divided into RV vaccine shedders and non-shedders based on the detection of the Rotarix vaccine strain in fecal material. The study was approved by Sefako Makgatho Health Sciences University Research and Ethics Committee, ethics number SMUREC/M/01/2023: PG.

Sample collection and storage

Fecal samples were collected from the infant's diapers by scooping the fecal material into sterile plastic containers with sterile spoons and immediately frozen in a − 20 °C freezer located at clinic. Frozen specimen were transferred to the laboratory in cooler boxes containing ice blocks and stored at − 20 °C.

Fecal viral RNA isolation and vaccine virus shedding assay

Viral RNA was isolated from a 10% fecal suspension (w/v) using a QIAamp RNA Stool Mini Kit (Qiagen, Germany) as recommended by the manufacturer. Rotarix vaccine shedding in fecal samples was detected by real-time qRT-PCR of fecal RNA samples using NSP2 primers (NSP2-F: GAA CTT CCT TGA ATA TAA GAT CAC ACT GA, NSP2-R: TTG AAG ACG TAA ATG CAT ACC AAT TC) and probe (NSP2-P: FAM-TCCAATAGATTGAAGTC AGTAACCCA-BHQ1) that target the NSP2 gene of the Rotarix vaccine strain [15] and Luna one-step RT-PCR kit (New England BioLabs, Ipswich, MA, USA) following the manufacturer's instructions. The PCR amplification was performed in Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, California) using the conditions described by [15].

Fecal genomic DNA isolation and detection of bacterial flagellin

Genomic DNA was isolated from the fecal material using QIAamp Fast DNA Stool Mini Kit (Qiagen, German) following the manufacturer's instructions. Primers for bacterial flagellin were designed from fliC gene that codes for the flagella subunit. Several fliC sequences from different flagellated bacteria were retrieved from National Center for Biotechnology information (NCBI) and aligned using Molecular Evolutionary Genetics Analysis (MEGA) software. Primers (fliC F1: CGC CAA CGA CGG TAT CTC, fliC R1: GAT TTC GTC CTG GAT GGA, fliC F2: CGT AAC GCT AAC GAC GGT AT, fliC R2: GCC GTT GAA CTG AGT CTG) were designed from the conserved regions and optimized as described previously [16]. The specificity of primers was verified by conventional PCR of genomic DNA isolated from flagellated *Escherichia coli* against genomic DNA from several non-flagellated bacteria.

Detection of bacterial flagellin in fecal genomic DNA samples

Bacterial flagellin was detected in fecal genomic DNA samples by qPCR using fliC primers (This study). Briefly, a 10 µL qPCR reaction mixture was made up of 1X Luna Universal qPCR Master Mix (New England BioLabs, Massachusetts, USA), 0.5 µM each of the forward and reverse primers, 3 µL DNA template and PCR grade water. The qPCR assay was performed in a Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, California) under the following conditions: 2 min of initial denaturation at 95 °C, 45 cycles of denaturation at 95 °C for 15 s and extension at 60 °C for 30 s. A melting curve analysis was performed at 65 °C for 0.05 s and 95 °C at 0.5 s to differentiate between primer dimers and true positives. A Ct value of less than 40 was regarded as being positive for the bacterial flagellin.

Total RNA isolation and cDNA synthesis

Total human and bacterial RNA were extracted from fecal samples using a NucleoSpin RNA kit (Macherey-Nagel, Duren, Germany) following the manufacturer's instructions with minor modifications [17]. The isolated RNA was converted to complementary DNA (cDNA) using a Tetro cDNA synthesis kit (Bioline Meridian Bioscience, United Kingdom) and performed in a GeneAmp PCR System 9700 (Applied Biosystems, Waltham, MA, USA) under the following conditions: incubation of samples at 25 °C for 10 min, followed by reverse transcription at 42 °C for 30 min and incubation at 85 °C for 5 min.

Expression assay for fliC, TLR5, NLRC4, IL-18 and IL-22 genes

The expression of fliC, TLR5, NLRC4, IL-18 and IL-22 genes in fecal cDNA samples was assayed by qPCR using the following primers: fliC (this study); TLR5F-TTG CTC AAA CAC CTG GAC AC and TLR5R-TTG GCA ATG CGG TTT TCT CC [18]; NLRC4F- CGC AGT CCC CTC ACC ATA GAA G and NLRC4R- GCC TCA AGT TAC CCA AGC TGT CAG [19]; IL-22 F- CACGGAGTC AGTATGAGTGAG and IL-22R-CAAATGCAGGCATT TCTCAGAGA [20]; IL-18 F-GCT TCC TCT CGC AAC AAA C and IL-18R-CAC TTC ACA GAG ATA GTT ACA GCC [21]). Briefly, the 10 µL PCR reaction mixture consisted of 5 µM SYBR green master mix (New Lab Bio, England), 0.2 µM each of the forward and reverse primers, 2 µl of the cDNA with nuclease free water making up the remainder. The amplification was done under the following conditions: 2 min of initial denaturation at 95 °C, 45 cycles of denaturation at 95 °C for 15 s and extension at 60 °C for 1 min, with a melt curve insertion (65 °C to 95 °C: Increment 0.5 °C for 0:05 s). Gene expression was normalized with the housekeeping glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) primers (GAPDHF-GAG TCA ACG GAT TTG GTC GT and GAPDHR- GAC AAG CTT CCC GTT CTC AG [22] as done previously [23].

Statistical analysis

Differences in demographics and other characteristics between vaccine shedders and non-shedders was determined by the Chi-square test. Abundance of bacterial flagellin (Ct values) was transformed into logarithmic form and descriptive statistics were presented as median and interquartile ranges (IQR), and displayed as scatter plots (GraphPad Prism 9.2.0, GraphPad Software, San Diego, CA, USA). Difference in abundance of flagellin between vaccine shedders and non-shedders was determined by Mann Whitney U test. Fold-change gene expression was calculated using delta Ct as described by Schmittgen and Livak [23]. An unpaired t test was used to measure the differences in mean gene expression between the two groups. Abundance of flagellin was used as a continuous variable to evaluate its association with vaccine shedding (yes or no) using logistic regression. In all statistical tests, $p \leq 0.05$ was regarded as statistically significant.

Table 1 Demographic and other baseline characteristics of study infants

Characteristic	Overall	Shedders	Non-shedders	*p* value
	n = 94 (%)	*n* = 50 (%)	*n* = 44 (%)	
Age				
7 weeks	94 (100)	50 (100)	44 (100)	1.00
Sex				
Female	53 (56)	28 (56)	25 (57)	0.47
Male	41 (44)	22 (44)	19 (43)	
Ethnicity				
Black	94 (100)	50 (100)	44 (100)	1.00
Birth delivery				
Vaginal	66 (70)	36 (72)	30 (68)	0.34
C-section	28 (30)	14 (28)	14 (32)	
Feeding type				
Breast milk (BM)	54 (57)	33 (66)	21 (48)	0.04
BM and Formula	40 (43)	17 (34)	23 (52)	

Results

Demographics

A total of 94 RV vaccinated infants, 50 vaccine shedders and 44 non-shedders, were recruited and eligible for the study. Of these, 56% (53/94) were females and 44% (41/94) male. The majority of the infants (70%, 66/94) were born naturally while 30% (28/94) were born through C-section. About 57% (54/94) infants were fed exclusively with breast milk and the rest (43%, 40/94) were fed with both breast and formula milk. Except for mode of feeding, there were no significant differences in demographics and other baseline characteristics between vaccine shedders and non-shedders (Table 1).

Abundance of bacterial flagellin not significantly different between vaccine shedders and non-shedders

Alignment of fliC sequences from various flagellated bacteria did not result in a consensus conserved region from which one could design a single primer pair able to detect all the flagellated bacteria. The fliC genes aligned into two groups based on the conserved regions: Group1 was composed of species such as *Escherichia coli*, *Yersinia pestis Hafnia paralvei*, *E. albertii* and others while Group 2 consisted of *Enterobacter kobei*, *Citrobacter werkmannii*, *Klebsiella aeruginosa* and others. Two sets of primers (fliC1and fliC2) were designed and PCR with genomic DNA from different flagellated and non-flagellated bacteria indicated the primers were specific to fliC. This was confirmed by sanger sequencing in which the randomly selected sequences generated by the primers from fecal genomic DNA were 99.9% identical to fliC genes.

A total of 94 fecal genomic DNA samples from vaccine shedders and non-shedders were assayed for bacterial flagellin. The flagella subunit was detected in fecal genomic DNA samples of both study groups using the two set of primers. There were no significant differences in the average abundance of flagellin (fliC1 and fliC2 Ct values combined) between vaccine shedders and non-shedders ($p = 0.15$) (Fig. 1a). When the abundance was stratified according to the feeding type, infants fed exclusively on breastmilk had significantly high counts of bacterial flagellin compared to those fed with both breast and formula milk ($p = 0.042$) (Fig. 1d). There was no significant difference in the abundance of flagellin between male and female infants ($p = 0.573$) (Fig. 1b), and infants born naturally and those born through C-section ($p = 0.158$) (Fig. 1c).

FliC and TLR5, not NLRC4 highly expressed in non-shedders

A total of 40 fecal samples, 20 from vaccine shedders and the rest from non-shedders were randomly selected and assayed for fliC, TLR5 and NLRC4 gene expression. Unlike the abundance, the average expression of fliC was increased 7.5-fold in non-shedders (mean 2-Δ Ct = 57.76 ± 16.92) compared to vaccine shedders (mean 2- delta (2Δ) Ct = 7.69 ± 2.56), $p = 0.001$ (Fig. 2a). Consistent with fliC, the expression of surface localized TLR5 was increased 3.4-fold (mean 2-ΔCt = 2.13 ± 0.63) in non-shedders compared to shedders (mean 2-ΔCt = 0.63 ± 0.19), $p = 0.045$ (Fig. 2b). In contrast, the expression of cytosolic NLRC4 was increased 1.45-fold in vaccine shedders (mean 2-ΔCt = 0.29 ± 0.12) versus non-shedders (mean 2-ΔCt = 0.20 ± 0.05), but did not reach statistical significance ($p = 0.507$) (Fig. 2c).

Cytokine IL-22, not IL-18 gene, significantly expressed in non-shedders

We also investigated whether the expression of flagellin-signaling TLR5 and NLRC4 were consistent with the expression of cytokines IL-22 and IL-18, respectively, in stool mRNA samples. Like TLR5, the expression of cytokine IL-22 gene in fecal mRNA samples of non-vaccine shedders was increased 4.4-fold (mean 2-ΔCt = 0.61 ± 0.23) compared to vaccine shedders (mean 2-ΔCt = 0.14 ± 0.06), $p = 0.054$ (Fig. 3a). On the other hand, there was no significant difference in the expression of NLRC4-dependent IL-18 between vaccine shedders and non-shedders ($p = 0.650$) (Fig. 3b).

Association between expression of FliC and RV vaccine shedding

Infants with higher expression of fliC were less likely to shed the vaccine in stool samples (OR: 0.95, 95% CI: 0.0.911–1.012), but did not reach statistical siginificance ($p = 0.056$). Similarly, high levels of TLR5 expression were protective of vaccine shedding (OR: 0.7, 95% CI: 0.461–1.05, $p = 0.061$). On the other hand, infants with elevated levels of NLRC4 expression were 2.77 likely to shed the vaccine in stool samples (95% CI = 0.147–52.04) but was not statistical significance ($p = 0.492$). The expression of NLRC4-dependant IL-18 had no effect on shedding of the vaccine ($p = 0.634$).

Discussion

RV vaccine shedding indicates the vaccine virus replicated in the enterocytes and thus stimulating the immune system to generate protection against RV infection. Although RV vaccine shedding in stool samples has not yet been validated as measure of vaccine response, a recent study reported a 92% and 55% RV shedding rate a week post first dose vaccination in United Kingdom and Malawi, respectively [24]. These figures mirror the reported vaccine efficacy in these two countries [25, 26], suggesting that RV shedding could be used as an additional measure to estimate vaccine response. Here, we used RV shedding as a proxy of vaccine response to study its relationship with the abundance of bacterial flagellin. The study found no significant difference in the abundance of bacteria flagellin between RV vaccine shedders and non-shedders. However, the qPCR assay used to quantify the abundance of bacterial flagellin provided an estimate of fliC gene copy numbers in a sample, regardless whether the bacteria was viable or not. Hence, we performed the fliC expression assay to estimate the number of viable flagellated bacteria. The expression of fliC was increased 7.5-fold in non-vaccine shedders versus shedders, suggesting a possible effect of the abundance of viable flagellated bacteria on RV vaccine replication and shedding. Given that the bacterial flagellin is a potent

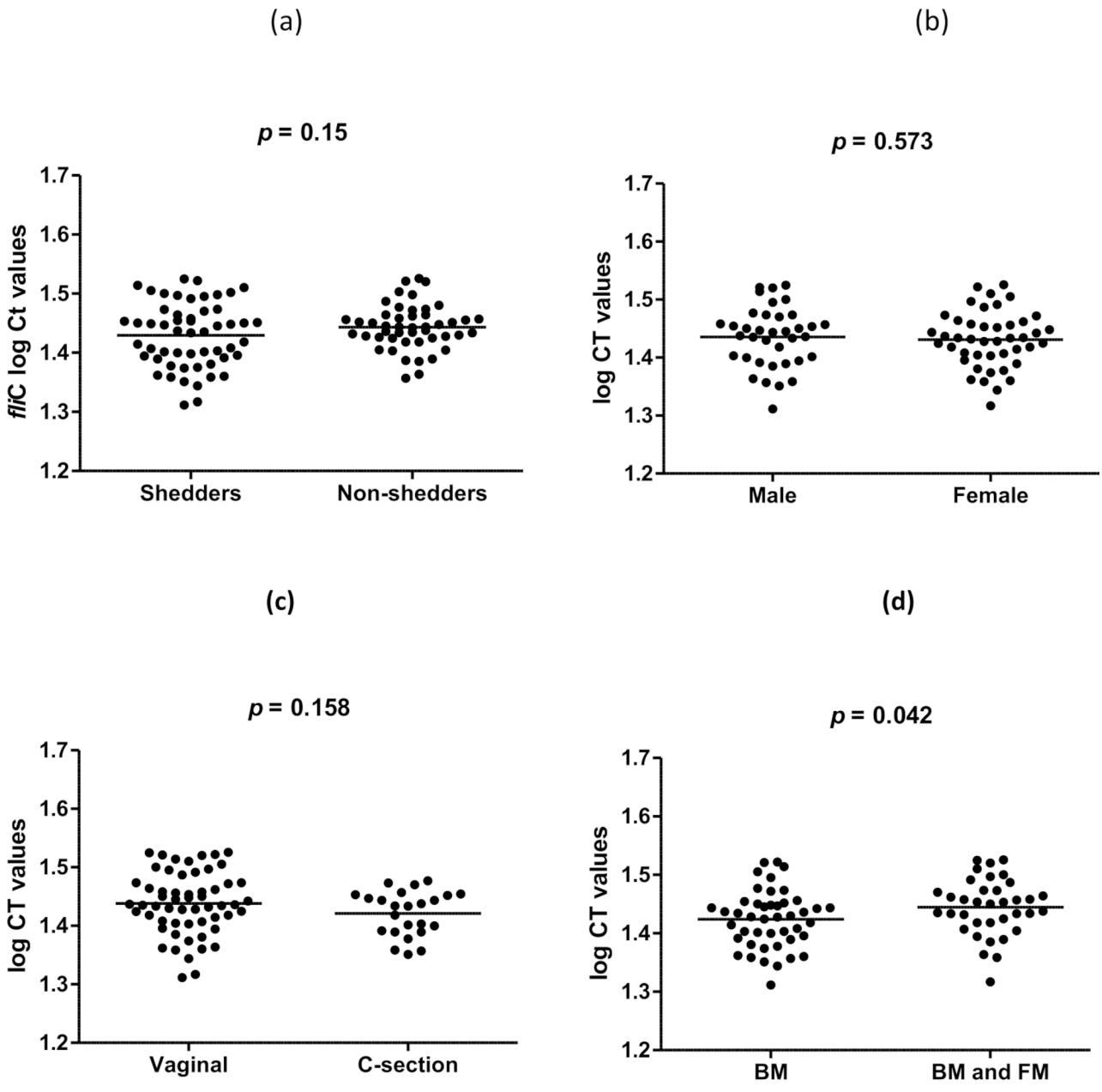

Fig. 1 Average abundance of bacterial flagellin (**a**) rotavirus vaccine shedders versus non-shedders (**b**) male versus female infants (**c**) naturally versus C-section born infants (**d**) breastmilk versus breast/formula milk fed infants

intestinal immune activator [11], we hypothesize that the presence of high counts of flagellin-producing bacteria in non-shedders may have optimally activated the intestinal immunity [27], thereby preventing the RV vaccine from infecting and replicating in the enterocytes hence no shedding. Recently, a multi-country study involving Malawi, India and UK failed to recapitulate the relationships that have been reported between the composition of bacterial microbiota and oral RV vaccine outcome. For example, while the higher abundance of Bacteroidetes among infants without seroconversion to RV vaccine was reported in Ghana, similar findings were not found in infants in Malawi and India [24]. Perhaps this suggest that the influence of bacterial microbiota may not rely on specific genera or species but functional level of the microbiota such as bacterial flagellin [10] or lipopolysaccharides 17].

Signaling of bacterial flagellin occurs via TLR5 and NLRC4 [10]. Hence, we tested whether the high level of expression of fliC in non-shedders was consistent with the level of expression of the two flagellin receptors. Indeed, the expression level of TLR5 in non-shedders

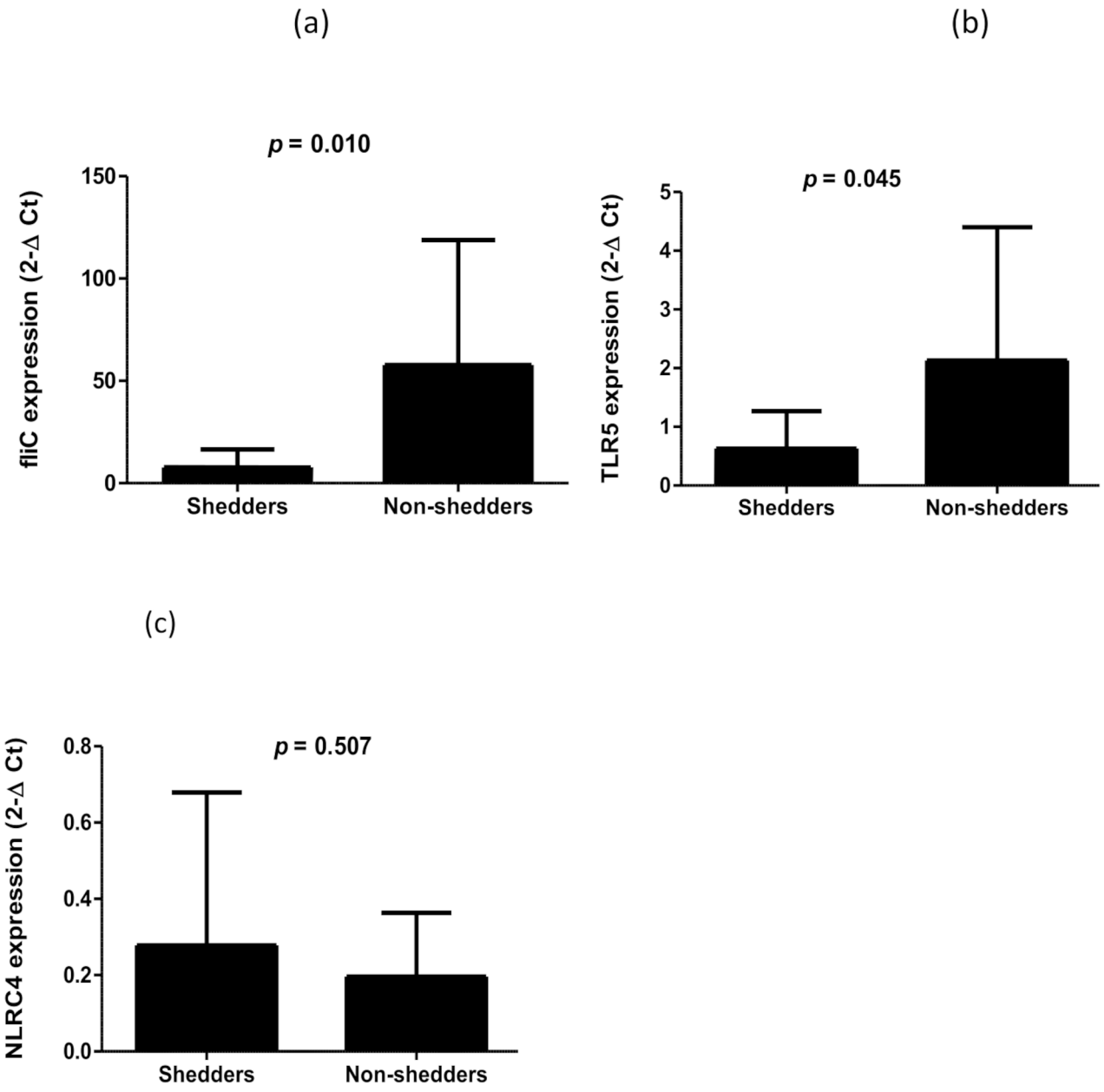

Fig. 2 Average expression of (**a**) flagellin-encoding fliC (**b**) surface-localized flagellin receptor TLR5 and (**c**) cytosolic flagellin-receptor NLRC4 in fecal samples of RV vaccine shedders versus non-shedders

was consistent with the expression level of FliC. In contrast, the expression of NLRC4 did not correspond with the expression of FliC, suggesting that TLR5, not NLRC4, could have been the mediator of the flagellin-activated intestinal immunity. This is consistent with findings of a murine study which found TLR5, and not NLRC4, to be responsible for intestinal immunity that prevented RV infection [10].

The flagellin activation of TLR5 and NLRC4 induces the production of pro-inflammatory cytokines IL-22 and IL-18, respectively. Thus, we assessed whether the levels of expression of the two cytokines in non-shedders was in line with the level of expression of their inducers. We observed the expression of IL-22 was consistent with the expression of TLR5 and FliC. In contrast, the expression of IL-18, like the NLRC4 expression, was not consistent with expression of FliC. The two cytokines were reported to recapitulate the flagellin's cure or prevention of RV infection in mice [10]. IL-22 inhibits the virus from entering the target cells, whereas IL-18 removes existing rotavirus from infected cells. The fact that IL-22 was significantly expressed in non-shedders suggest that the

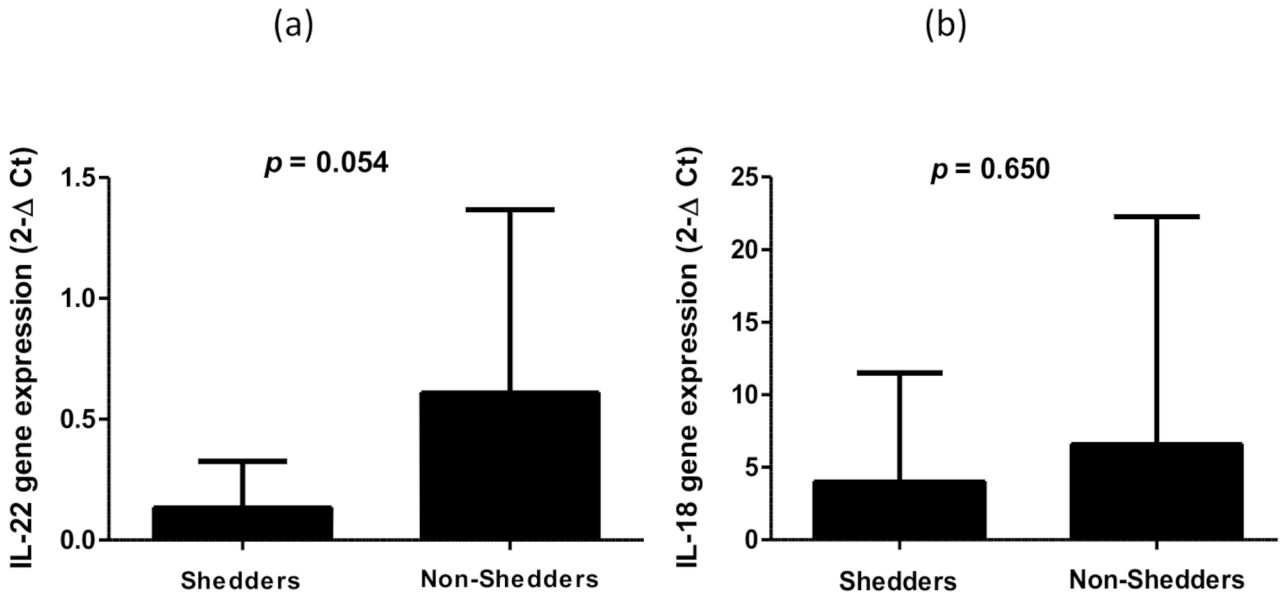

Fig. 3 Expression of cytokine (**a**) IL-22 and (**b**) IL-18 in fecal cDNA samples between RV vaccine shedders and non-shedders

Table 2 Association between expression of flagellin-encoding FliC and shedding of RV vaccine in stool samples

Variable	Odds ratio	95% Confidence interval	*p* value
FliC expression	0.92	0.850–1.005	0.056
TLR5 expression	0.70	0.462–1.051	0.061
NLRC4 expression	2.77	0.147–52.04	0.492
IL-22 expression	0.07	0.002–2.202	0.059

flagellin-mediated intestinal immunity is more dependent on TLR5 than the NLRC4 inflammasome [28, 10]. The expression of both TLR5 and NLRC4 inflammasome at the same time was interesting as it is inconsistent with reports that NLRC4-mediated signaling of flagellin downregulates the TLR5-mediated signaling of the subunit [29]. It is not known whether some of the study infants had asymptomatic flagellated intracellular pathogens like Salmonella, but some flagellin from extracellular bacteria have been shown to internalize into epithelial cells and activates NLRC4 [30], and this may explain why TLR5 and NLRC4 were both activated. Previous murine studies suggest the two do indeed work together albeit with different pathways. IL-18 induces signaling pathway that lead to a speedy reduction in RV load while IL-22 reprograms the epithelial cell gene expression that results in resistance to RV infection [10].

The study had limitations. Due to unavailability of blood, we were unable to measure the levels of cytokines IL-22 and 18 to validate the expression levels of the two. We also used vaccine shedding as a measure of vaccine response although it has not been validated yet as measure of correlate of protection for RV vaccination. However, the RV vaccine shedding rates appear to resemble that of RV vaccine efficacy [24]. Further studies using both serum IgA and shedding as markers of vaccine response are required to validate the findings of the current study.

In summary, we have shown differences in abundance of viable flagellated bacteria between RV vaccine shedders and non-shedders. The expression of flagellin-encoding fliC, its surface localized receptor TLR5, and not cytosolic NLRC4, were significantly high in non-shedders. In addition, the expression of TLR5-elicited IL-22 and not NLRC4-dependant IL-18, was significantly high in non-shedders versus shedders. Collectively, our observations suggest that flagellin-mediated intestinal immunity could negatively influence RV vaccine take in infants harboring high abundance of the flagellated bacteria. This calls into question suggestions of including bacterial flagellin as adjuvants, especially in rotavirus vaccines.

Author contributions

N.G.H. performed the laboratory assays, initial data analysis and drafted the manuscript. C.A.M. conceptualized, supervised, analyzed data and revised the manuscript. M.L.S. sourced funds, reviewed and approved the manuscript. All authors have read and agreed to the published the manuscript.

Funding

The study was supported by funding from the National Research Foundation (NRF) and Poliomyelitis Research Foundation (PRF) (awarded to N.G.H.), Sefako Makgatho Health Sciences University, # D134 (C.A.M.) and South African Medical Research Council, RDA (L.P.K.).

Data availability

Data is available from corresponding author on request.

Declarations

Conflict of interest

The authors declared no conflicts of interest.

Received: 20 October 2024 / Accepted: 24 December 2024
Published online: 17 January 2025

References

1. Du Y, Chen C, Zhang X et al. Global burden and trends of rotavirus infection-associated deaths from 1990 to 2019: an observational trend study. Virol J. 2022; 19(166).
2. Patel MM, Glass R, Desai R, Tate JE, Parashar UD. Fulfilling the promise of rotavirus vaccines: how far have we come since licensure? Lancet Infect Dis. 2012;12(7):561–70.
3. Emperador DM, Velasquez DE, Estivariz CF, Lopman B, Jiang B, Parashar U, Anand A, Zaman K. Interference of Monovalent, Bivalent, and trivalent oral Poliovirus vaccines on Monovalent Rotavirus Vaccine Immunogenicity in Rural Bangladesh. Clin Infect Dis. 2016;62(2):150–6.
4. Chen MY, Kirkwood CD, Bines J, Cowley D, Pavlic D, Lee KJ, Orsini F, Watts E, Barnes G, Danchin M. Rotavirus specific maternal antibodies and immune response to RV3-BB neonatal rotavirus vaccine in New Zealand. Hum Vaccin Immunother. 2017;13(5):1126–35.
5. Kazi AM, Cortese MM, Yu Y, Lopman B, Morrow AL, Fleming JA, McNeal MM, Steele AD, Parashar UD, Zaidi AKM, Ali A. Secretor and Salivary ABO Blood Group Antigen Status Predict Rotavirus Vaccine take in infants. J Infect Dis. 2017;215(5):786–9.
6. Magwira CA, Kgosana LP, Esona MD, Seheri ML. Low fecal rotavirus vaccine virus shedding is significantly associated with non-secretor histo-blood group antigen phenotype among infants in northern Pretoria, South Africa. Vaccine. 2020;38(52):8260–3.
7. Harris VC, Armah G, Fuentes S, Korpela KE, Parashar U, Victor JC, Tate J, de Weerth C, Giaquinto C, Wiersinga WJ, Lewis KD, de Vos WM. Significant correlation between the infant gut microbiome and Rotavirus Vaccine Response in Rural Ghana. J Infect Dis. 2017;215(1):34–41.
8. Harris VC, Ali A, Fuentes S, Korpela K, ha M, Tate J, Parashar U, Wiersinga WJ, Giaquinto C, de Weerth C, de Vos WM. Rotavirus vaccine response correlates with the infant gut microbiota composition in Pakistan. Gut Microbes. 2018;9(2):93–101.
9. Parker EP, Ramani S, Lopman BA, Church JA, Iturriza-Gómara M, Prendergast AJ, Grassly NC. Causes of impaired oral vaccine efficacy in developing countries. Future Microbiol. 2018;13(1):97–118.
10. Zhang B, Chassaing B, Shi Z, Uchiyama R, Zhang Z, Denning TL, et al. Viral infection. Prevention and cure of rotavirus infection via TLR5/ NLRC4-mediated production of IL-22 and IL-18. Science. 2014;346:861–5. https://doi.org/10.1126/science.1256999
11. Vijay-Kumar M, Gewirtz AT. Flagellin: key target of mucosal innate immunity. Mucosal Immunol. 2009;2(3):197–205.
12. Broquet AH, Hirata Y, McAllister CS, Kagnoff MF. RIG-I/MDA5/MAVS are required to signal a protective IFN response in rotavirus infected intestinal epithelium. J Immun1ol. 2011;186:1618–26. https://doi.org/10.4049/jimmunol.1002862
13. Ingle H, Peterson ST, Baldridge MT. Distinct effects of type I and III interferons on enteric viruses. Viruses. 2018;10(1):46. https://doi.org/10.3390/v10010046
14. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability, and resilience of the human gut microbiota. Nature. 2012;489(7415):220–30.
15. Gautam R, Esona MD, Mijatovic-Rustempasic S, Ian Tam K, Gentsch JR, Bowen MD. Real-time RT-PCR assays to differentiate wild-type group a rotavirus strains from Rotarix(®) and RotaTeq(®) vaccine strains in faecal samples. Hum Vaccin Immunother. 2014;10(3):767–77.
16. Green MR, Sambrook J. Optimizing primer and probe concentrations for Use in Real-Time polymerase chain reaction (PCR) assays. Cold Spring Harb Protoc. 2018;2018(10).
17. Kgosana LP, Seheri ML, Magwira CA. Abundance of Selected Lipopolysaccharide-Rich Bacteria and Levels of Toll-like Receptor 4 and Interleukin 8 Expression Are Significantly Associated with Live Attenuated Rotavirus Vaccine Shedding among South Africans Infants. Vaccines. 2024, 12, 273.
18. Falah AA, Ghalib FR, Hasan SH. Role of toll-like receptor 5 in the development of Ulcerative Colitis. Arch Razi Inst. 2021;76(6):1639–43.
19. Nasiri E, Kariminik A. Up-regulation of AIM2 and TLR4 and down-regulation of NLRC4 are associated with septicemia. Indian J Med Microbiol. 2021;39(3):334–8.
20. Rudloff I, Bachmann M, Pfeilschifter J, Mühl H. Mechanisms of Rapid induction of Interleukin-22 in activated T cells and its modulation by Cyclosporin A. J Biol Chem. 2012;287(7):4531–43.
21. Lorey SL, Huang YC, Sharma V. Constitutive expression of interleukin-18 and interleukin-18 receptor mRNA in tumour derived human B-cell lines. Clin Exp Immunol. 2004;136(3):456–62.
22. Colantonio AD, Epeldegui M, Jesiak M, Jachimowski L, Blom B, Uittenbogaart CH. IFN-α is constit2utively expressed in the human thymus, but not in peripheral lymphoid organs. PLoS ONE. 2011;6(8):e24252.
23. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101–8.
24. Parker EPK, Bronowski C, Sindhu KNC, Babji S, Benny B, Carmona-Vicente N, Chasweka N, Chinyama E, Cunliffe NA, et al. Impact of maternal antibodies and microbiota development on the immunogenicity of oral rotavirus vaccine in African, Indian, and European infants. Nat Commun. 2021;12(1):7288.
25. Walker JL, Andrews NJ, Atchison CJ, Collins S, Allen DJ, Ramsay ME, Ladhani SN, Thomas SL. Effectiveness of oral rotavirus vaccination in England against Rotavirus-confirmed and all-cause acute gastroenteritis. Vaccine X. 2019;1:100005.
26. Madhi SA, Cunliffe NA, Steele AD, Witte D, Kirsten M, Louw C, et al. Effect of human rotavirus vaccine on severe gastroenteritis in African infants. N Engl J Med. 2010;362:289–98.
27. Tran HQ, Ley RE, Gewirtz AT, Chassaing B. Flagellin-elicited adaptive immunity suppresses flagellated microbiota and vaccinates against chronic inflammatory diseases. Nat Commun. 2019;10(1):5650.
28. Lopez-Yglesias AH, Zhao X, Quarles EK, Lai MA, Vandenbos T, Strong RK, et al. Flagellin induces antibody responses through a TLR5- and inflammasome-independent pathway. J Immunol. 2014;192:1587–96.
29. Li W, Yang J, Zhang E, et al. Activation of NLRC4 downregulates TLR5-mediated antibody immune responses against flagellin. Cell Mol Immunol. 2016;13:514–23.
30. Chebly H, Marvaud J-C, Safa L, Elkak AK, Kobeissy PH, Kansau I, Larrazet C. Clostridioides Difficile Flagellin activates the intracellular NLRC4 Inflammasome. Int J Mol Sci. 2022;23(20):12366.

Publisher's note