**­­Duration and density of faecal rotavirus shedding in vaccinated Malawian children with rotavirus gastroenteritis**

Aisleen Bennett1,2,, Louisa Pollock1,2, Khuzwayo C. Jere1,2, Virginia E. Pitzer3, Benjamin Lopman4, Naor Bar-Zeev1,5, Miren Iturriza-Gomara1,2,6, Nigel A. Cunliffe1,2

1.Malawi-Liverpool-Wellcome Trust Clinical Research Programme, College of Medicine, University of Malawi, Blantyre, Malawi.

2. Centre for Global Vaccine Research, Institute of Infection & Global Health, University of Liverpool, Liverpool, UK

3. Department of Epidemiology of Microbial Diseases, Yale School of Public Health, Yale University, New Haven, USA.

4. Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, USA

5. International Vaccine Access Center, Bloomberg School of Public Health, John Hopkins University, Baltimore, USA

6. NIHR Health Protection Research Unit in Gastrointestinal Infections, University of Liverpool, Liverpool, UK

**Corresponding author details:** Dr Aisleen Bennett. Institute of Infection and Global Health, The Ronald Ross Building, University of Liverpool, 8 West Derby Street, Liverpool

 Email abennett@sgul.ac.uk

Running head: Rotavirus shedding dynamics

Word count abstract: 100

Word count text: 1990

**Abstract**

Quantifying rotavirus shedding among vaccinated individuals will aid understanding of rotavirus vaccine indirect effects. Serial stool samples were collected from 196 children who presented with rotavirus gastroenteritis to health facilities in Blantyre, Malawi, and tested for rotavirus using a VP6 semi-quantitative real-time PCR. Median duration of faecal shedding was 28 days(95% CI 19, 28). Median copy numbers for peak shedding were 1.99x107(IQR 3.39x106, 6.37x107). Faecal viral load was positively associated with disease severity and negatively associated with serum anti-rotavirus IgA. High and persistent rotavirus shedding among vaccinated children with breakthrough disease may contribute to ongoing rotavirus transmission in this setting.

**Key words: rotavirus, transmission, vaccine effectiveness, shedding**

**Introduction**

Rotavirus vaccine has been introduced in over 90 countries worldwide, including 45 low-income or GAVI eligible countries(1). However, rotavirus vaccine effectiveness (VE) is reduced in low-income countries (LIC)(2), and in several countries rotavirus remains the commonest cause of hospitalised gastroenteritis in children <5 years despite high vaccine coverage(3). Vaccine effectiveness is higher against severe rotavirus disease than milder disease(4), but the impact of vaccination on faecal shedding (and therefore infectiousness) amongst vaccinated individuals with breakthrough rotavirus disease is unknown. Differences in the intensity of faecal-oral transmission of rotavirus between settings (“force of infection”) may contribute to the observed variation in vaccine performance, but few data exist on individual level faecal shedding – one of the primary mechanisms of rotavirus transmission.

The dynamics of faecal shedding of rotavirus in unvaccinated populations has been examined in children with both asymptomatic rotavirus infection and with clinical rotavirus disease using electron microscopy, enzyme-immune assay and PCR(5). Faecal shedding has been shown to extend beyond symptom resolution, persisting for a median of 10 days since symptom onset in Australia and 24 days in India using PCR based assays(6,7). Data from India demonstrated a positive relationship between faecal viral load and disease severity(5).

There is a lack of shedding data from vaccinated infants in any setting, including infants from sub-Saharan Africa in whom differences in intestinal integrity, nutritional state, co-morbidities, and immune response may lead to variation in shedding with concomitant impact on transmission. We aimed to describe patterns of wild-type rotavirus shedding over time and identify factors associated with faecal viral load in vaccine-age-eligible children with symptomatic rotavirus disease in Malawi, a LIC in southern Africa. The live oral monovalent rotavirus vaccine was introduced into Malawi’s national immunisation programme on 29th October 2012 with doses at 6 and 10 weeks of age.

**Methods**

Vaccine-age-eligible children (born on or after 12th September 2012 and >6 weeks old) presenting with acute gastroenteritis to Queen Elizabeth Central Hospital (QECH) and to three government health centres in Blantyre, Malawi between February 16th 2015 to 11th November 2016 were tested for rotavirus using immunochromatographic tests on a faecal sample. Rotavirus positive children had demographic data collected, 2mls of serum collected at recruitment to measure anti-rotavirus IgA titres and a second bulk stool sample obtained 48 hours after presentation (primary cohort). A subset of children had more intensive stool sampling carried out for 28 days (intensive cohort); samples were collected daily from time of presentation for the first 7 days after symptom onset, twice weekly from 7 until 14 days and weekly from day 14 until day 28.

**Data collection**

Disease severity was defined using the 20-point Vesikari score (<7 indicates mild, 7-10 indicates moderate and ≥11 indicates severe disease). Severe acute malnutrition (SAM) was defined as any of weight-for-height Z-score (WHZ)<-3 SD, mid-upper-arm circumference (MUAC)<115mm, or nutritional oedema(8). Weights were adjusted for percentage dehydration prior to calculating anthropometric indices. Data on HIV status and vaccine status were collected from government issued health passports. HIV testing was performed by the government health system. HIV infection was defined as a positive HIV rapid test (≥12 months of age) or positive HIV DNA PCR (<12 months)(9). Children of HIV positive mothers were defined as HIV exposed.

**Laboratory procedures**

Stool samples were tested for wild-type rotavirus using a real-time semi-quantitative reverse transcription PCR (qRT-PCR)(5). An additional qRT-PCR targeting a distinct rotavirus gene (NSP3) was run on each sample with a cycle threshold (C­T) value in the range 35-40 as a confirmatory assay(10). Due to lack of reproducibility in samples with very low viral loads samples were defined as rotavirus positive if >=100 viral copy numbers and positive on NSP3 assay.

Serum anti-rotavirus IgA titres were measured using a semi-quantitative sandwich ELISA(11). Results were calculated on a minimum of two values per sample with a coefficient of variation <20% and were expressed as geometric mean titres (IU/ml)*.*

**Statistical analysis**

Viral load did not follow a normal distribution so was log-transformed for analysis. Risk factors for faecal viral shedding density were investigated using multivariable linear regression, where the outcome variable was peak log-viral load (the largest value obtained from the two samples) in children from the primary cohort. Variables achieving a Wald test p-value of ≤0.1 on univariate analysis were selected for evaluation in the multivariable model. Nested models were compared using F tests. Variables which improved model fit (p ≤0.05) were retained in the final model.

Data from the intensive cohort were used to evaluate change in viral load over time using linear mixed models with a random intercept to account for within-child clustering; for this analysis all data were included regardless of viral load. Polynomial terms (quadratic and cubic) were included to account for the non-linear relationship between faecal viral load and time.

Time-to-event analysis was used to estimate the duration of viral shedding in children from the intensive cohort. The event of interest was defined as cessation of shedding and the start time for analysis was the onset of symptoms. Cessation of shedding was defined as the last time-point from which rotavirus could be detected until censoring. Thus, an individual with no detectable rotavirus at a given analytical time-point but who was shedding rotavirus in subsequent samples was classified as having ongoing shedding at the time-point of analysis. Follow up was limited to 28 days.

**Results**

We recruited 196 index children from whom 374 faecal samples were collected. 21 of these children were also recruited into the intensive cohort and had a further 136 samples collected. Median age of all children was 11.5 months (IQR 8.8, 15.2). A total of 25 (13%) children were HIV exposed. 0f children with documented HIV test results, 3/89 (3.4%) were HIV infected. The majority of children had severe rotavirus gastroenteritis (168/193, 86.5%). Median anti-rotavirus IgA titres at presentation were 4 IU (range 0-831), and 27% (45/164) children met the traditional threshold for seroconversion (>20 IU)(11). Two dose rotavirus vaccine coverage was 194/196 (99.0%). Details of recruited children are presented in Table S1.

**Predictors of viral load (primary cohort)**

Median copy numbers for peak shedding were 1.99x107 (IQR 3.39x106, 6.37x107). The two unvaccinated children had peak viral loads comparable to the vaccinated children (median peak shedding 9.64x106). On multivariable analysis, a positive association between peak shedding density and Vesikari score and a negative association with IgA titres were identified (Table 1). The reference group used for Vesikari (mild disease) contains only 3 observations, however the relationship between Vesikari score and viral load persisted when Vesikari was coded as a continuous variable or as 3 approximately equal groups (Table S2) There was also weak evidence of a negative association with WHZ.

**Change in viral load over time and duration of viral shedding (intensive cohort)**

Viral load ranged from 21 to 1.91x109 copies. It declined significantly with time from symptom onset (regression coefficient for relationship between log copy numbers and time in days since symptom onset: -1.68, 95% CI -2.51, -0.85) (Fig 1a, Table S3). Viral load was significantly higher when children were symptomatic (regression coefficient: 6.94, 95% CI 5.15, 8.73). This effect was reduced when adjusted for time from symptom onset (regression co-efficient: 1.87, 95% CI 0.25, 3.49). The proportion of children shedding rotavirus declined over time, from 100% at the first visit to 20% at the end of follow-up (Fig 1b). Median duration of shedding based on survival analysis was 28 days (95% CI 21, 28).

**Discussion**

In a cohort of vaccinated Malawian children with rotavirus gastroenteritis, children shed rotavirus in high density at the time of initial symptom-onset, and continued to shed rotavirus for an extended period of time after symptom resolution. Faecal viral shedding density was positively associated with disease severity on presentation and negatively associated with anti-rotavirus IgA titres.

The pattern of rotavirus shedding observed among symptomatic rotavirus cases is similar to that observed among unvaccinated children from India(7). In both Malawi and India, high viral titres on presentation rapidly declined over the first 10 days after symptom-onset and then plateaued, with a median duration of shedding of approximately 4 weeks (28 days in Malawi (95% CI 21, 28) , 24 days in India). This is substantially longer than the median duration of shedding reported from Australia (10 days)(6). While this could reflect differences in sensitivity of the assay used, the immune response to both natural rotavirus infection and rotavirus vaccine is reduced in LICs compared to higher-income settings(12). The extended duration of shedding could therefore result from delayed clearance of replicating virus as a result of sub-optimal mucosal immunity, or very high rates of re-infection. High frequency of asymptomatic rotavirus shedding has been described in young children from Malawi(13), and this may be partly explained by prolonged faecal shedding of rotavirus following symptomatic infection. Due to logistical restraints, the duration of follow-up in this study was limited, and the reported duration of shedding represents a minimum estimate. This is particularly true considering our definition of cessation of shedding – it is possible that some of the children near the end of follow-up were misclassified as having stopped shedding.

Children who develop severe rotavirus disease despite being vaccinated shed large quantities of virus. This may contribute to ongoing community transmission of rotavirus and provide one explanation for the persisting high burden of disease in LICs, with limited evidence of indirect (herd) protection despite high vaccine coverage(4). However, it is notable that children recruited into this study are biased towards those with severe disease presenting to health-care facilities. In our cohort, disease severity was significantly associated with viral load; children with moderate and severe disease had significantly higher viral loads than those with mild disease. Immunity following rotavirus vaccination mimics that following natural rotavirus exposure, which generates incremental protection against rotavirus gastroenteritis of increasing severity(12). It is therefore plausible that vaccination could reduce the severity of rotavirus gastroenteritis episodes and thereby decrease the total shedding burden of rotavirus in the community. The association between disease severity and viral load is most striking between mild and moderate/severe disease; however, numbers of children recruited into this study with mild disease were small, presumably because most children with mild disease do not present to health-care facilities. The analysis is sensitive to the presence of children with mild disease, but robust to different analytical approaches. Further studies are required to ensure that children with mild disease are appropriately represented, and to formally evaluate the potential for vaccination to reduce viral shedding density at the population level.

Increasing anti-rotavirus IgA titres at presentation were negatively associated with faecal viral load, independent of disease severity. Serum anti-rotavirus IgA titres are known to correlate with intestinal IgA titres(14), and it is plausible that higher levels of intestinal IgA could reduce viral replication and thereby faecal viral load. However, only a quarter of the vaccinated children in our cohort reached the criteria for seroconversion, and it is known that anti-rotavirus IgA titres are an imperfect correlate of protection, particularly in low-income settings(15).

We found that rotavirus viral shedding density is negatively associated with WHZ, implying that increasingly malnourished children shed more virus. Careful interpretation is required, as the standard deviation for our anthropometric measurements are outside the WHO range for data quality assessment purposes(16). Nevertheless, the negative association between WHZ and viral load is corroborated by weak evidence of a negative association between MUAC and viral load (Table 1). It is biologically plausible that children with poorer nutritional states could shed more rotavirus due to reduced ability to mount mucosal immunity, differences in intestinal microbiome and mucosal integrity, and a tendency for more severe rotavirus disease in the presence of malnutrition(17).

**Conclusions**

Children in Malawi shed large quantities of rotavirus for an extended period of time following an episode of moderate and severe diarrhoea, despite prior vaccination. Persistently high faecal virus shedding may contribute to high prevalence of asymptomatic infection in young children in the community and to ongoing rotavirus transmission. While reduced disease severity in Malawian children was associated with lower viral shedding density, the potential impact of this on population-level rotavirus transmission remains to be determined.

**Funding**

This study was supported by two Wellcome Trust Clinical PhD Fellowships [grant numbers 102466/Z/13/A to AB and 102464/Z/13/A to LP] , a Wellcome Trust Programme Grant [grant number 091909/Z/10/Z], the MLW Programme Core Grant Strategic Award [grant number 101113/Z/13/Z] and by the U.S. National Institutes of Health [grant number R01-AI112970 to VEP]. K.C.J. is supported by an International Wellcome Trust Training Fellowship (grant number: 201945/Z/16/Z). The authors received no financial support or other form of compensation related to the development of the manuscript.

**Acknowledgements**

We thank all infants and their families who participated and all members of the RotaRITE study team. We are grateful for the support of the Malawi Ministry of Health and clinical staff at the recruitment sites.

**Potential conflicts of interest**

LP, AB, BL: no conflict. NB-Z and K.C.J have received research grant support from GlaxoSmithKline Biologicals for work on rotavirus vaccines. MI-G has received research grant support from GlaxoSmithKline Biologicals and Sanofi Pasteur MSD for work on rotavirus. NAC has received research grant support and honoraria for participation in rotavirus vaccine advisory board meetings from GlaxoSmithKline Biologicals. V.E.P. is a member of the WHO Immunization and Vaccine-related Implementation Research Advisory Committee (IVIR-AC) and has received reimbursement from Merck for travel expenses to attend a Scientific Input Engagement unrelated to rotavirus vaccines

**Previous presentations**

Aspects of this work have been presented at the European Society for Paediatric Infectious Diseases (ESPID) meeting, June 2018, Sweden.

**Disclaimer**

MIG is affiliated to the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Gastrointestinal Infections at University of Liverpool in partnership with Public Health England (PHE), in collaboration with University of East Anglia, University of Oxford and the Quadram Institute. MIG is based at The University of Liverpool. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, the Department of Health or Public Health England.

**Corresponding author details:** Dr Aisleen Bennett. Institute of Infection and Global Health, The Ronald Ross Building, University of Liverpool, 8 West Derby Street, Liverpool

 Email abennett@sgul.ac.uk

**References**

1. Global Introduction Status | Rota Council [Internet]. [cited 2019 Oct 16]. Available from: http://rotacouncil.org/vaccine-introduction/global-introduction-status/

2. Parashar UD, Johnson H, Steele AD, Tate JE. Health Impact of Rotavirus Vaccination in Developing Countries: Progress and Way Forward. Clin Infect Dis. 2016 May 1;62(suppl 2):S91–5.

3. Platts-Mills JA, Amour C, Gratz J, Nshama R, Walongo T, Mujaga B, et al. Impact of Rotavirus Vaccine Introduction and Postintroduction Etiology of Diarrhea Requiring Hospital Admission in Haydom, Tanzania, a Rural African Setting. Clin Infect Dis. 2017 May 29;62:S213-9.

4. Bar-Zeev N, Jere KC, Bennett A, Pollock L, Tate JE, Nakagomi O, et al. Population Impact and Effectiveness of Monovalent Rotavirus Vaccination in Urban Malawian Children 3 Years after Vaccine Introduction: Ecological and Case-Control Analyses. Clin Infect Dis. 2016;62(Suppl 2):S213–9.

5. Kang G, Iturriza-Gomara M, Wheeler JG, Crystal P, Monica B, Ramani S, et al. Quantitation of group A rotavirus by real-time reverse-transcription-polymerase chain reaction: correlation with clinical severity in children in South India. J Med Virol. 2004/03/26. 2004;73(1):118–22.

6. Richardson S, Grimwood K, Gorrell R, Palombo E, Barnes G, Bishop R. Extended excretion of rotavirus after severe diarrhoea in young children. Lancet. 1998/07/04. 1998;351(9119):1844–8.

7. Mukhopadhya I, Sarkar R, Menon VK, Babji S, Paul A, Rajendran P, et al. Rotavirus shedding in symptomatic and asymptomatic children using reverse transcription-quantitative PCR. J Med Virol. 2013/06/19. 2013;85(9):1661–8.

8. World Health Organisation. WHO child growth standards and the identification of severe acute malnutrition in infants and children. WHO/UNICEF joint statement. 2009.

9. Ministry of health Malawi. Clinical Management of HIV in Children and Adults. 2011.

10. Freeman MM, Kerin T, Hull J, McCaustland K, Gentsch J. Enhancement of detection and quantification of rotavirus in stool using a modified real-time RT-PCR assay. J Med Virol. 2008/06/14. 2008 Aug 1;80(8):1489–96.

11. Bernstein DI, Sack DA, Rothstein E, Reisinger K, Smith VE, O’Sullivan D, et al. Efficacy of live, attenuated, human rotavirus vaccine 89-12 in infants: a randomised placebo-controlled trial. Lancet. 1999;354(9175):287–90.

12. Angel J, Franco MA, Greenberg HB. Rotavirus immune responses and correlates of protection. Curr Opin Virol. 2012;2(4):419–25.

13. Bennett A, Bar-Zeev N, Jere KC, Tate JE, Parashar UD, Nakagomi O, et al. Determination of a viral load threshold to distinguish symptomatic versus asymptomatic rotavirus infection in a high-disease-Burden African population. J Clin Microbiol. 2015;53(6):1951–4.

14. Hjelt K, Grauballe PC, Schiotz PO, Andersen L, Krasilnikoff PA. Intestinal and serum immune response to a naturally acquired rotavirus gastroenteritis in children. J Pediatr Gastroenterol Nutr. 1985/02/01. 1985;4(1):60–6.

15. Premkumar P, Lopman B, Ramani S, Paul A, Gladstone B, Muliyil J, et al. Association of serum antibodies with protection against rotavirus infection and disease in South Indian children. Vaccine. 2014/08/06. 2014;32 Suppl 1:A55-61.

16. Mei Z, Grummer-strawn LM. Standard deviation of anthropometric Z-scores as a data quality assessment tool using the 2006 WHO growth standards: a cross country analysis. Bull World Health Organ. 2013;85(6):1–7.

17. Mondal D, Minak J, Alam M, Liu Y, Dai J, Korpe P, et al. Contribution of enteric infection, altered intestinal barrier function, and maternal malnutrition to infant malnutrition in Bangladesh. Clin Infect Dis. 2012;54(2):185–92.

**Figure legends**

Figure 1. A) Rotavirus shedding over time in symptomatic children. Dots represent the raw data on log(viral load), and the red fitted line represents the regression line including a quadratic term to account for the non-linear nature of viral decay. Confidence bounds represent the 95% confidence limit on either side of the fitted value. Regression coefficients are given in Table S3. (B) Kaplan Meier Plot of time to cessation of shedding in index children. Analysis time is in days since symptom onset.