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Molecular Detection of Group A Rotavirus in Children under Five in Urban and Peri-urban Arusha, Tanzania

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Authors' contributions

This work was carried out in collaboration between all authors. Author EG co-designed the study, collected data, co-analyzed the data and co-wrote the original draft of the manuscript. Author JB co-supervised the implementation of all phases of the study and co-analyzed the data. Author PP co-supervised the implementation of all phases of the study and co-wrote the original draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background: Rotavirus gastroenteritis is the leading cause of severe diarrhea in children under the age of five years worldwide. However, very little information is available on Rotavirus status in Tanzania.
Aims: The project aimed at investigating Rotavirus infection in Tanzanian children to reflect prevalence post introduction of the Rotarix® virus, which occurred in late 2012.

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Methods: The study considered prevalence in an urban and peri-urban context in Arusha, Tanzania for children under five. The study involved molecular detection of rotavirus in stool samples using PCR targeting Group A Rotavirus as well as a questionnaire to determine possible contributing factors, such as vaccination status, age, and exclusive breastfeeding to infection.
Results: Out of a total of 100 stool samples collected, 37% were positive for Rotavirus. The Fisher's Exact Test was used to relate conventional PCR test results and various factors associated with Rotavirus positive samples. Household practices of boiling water, as well as parents'/guardians' knowledge on the Rotavirus vaccine and child vaccination status were significantly ($p<0.05$) associated with Rotavirus infection.
Conclusion: The findings of this study should inform further studies to address the molecular epidemiology of the disease and associated risk factors. In this study we undertook surveillance for molecular detection and characterization of Rotavirus while considering the impact of prevention and control measures, such as vaccinations and uptake of safe practices (i.e., boiling water) on prevalence.

Keywords: Rotavirus; low income countries; Tanzania; molecular detection; childhood vaccines; diarrheal diseases.

1. INTRODUCTION

Annually, Rotavirus claims the lives of more than 450 000 children, with more than half of these deaths occurring in Africa [1-3]. These numbers are further accentuated in sub-Saharan Africa where rates of more than 300 deaths per 100 000 are evidenced [2,4]. This highly contagious condition is passed through contact, primarily through oral-fecal route or via contaminated fomites, not pharmaceutically treatable, nor significantly managed through water/sanitation/ hygiene measures. However, the ultimate solution is prevention, which is currently available in the form of a vaccination which has been shown to be highly effective (reducing severe cases by more than 60%) and cost-effective against severe Rotavirus infections [5,6]. Two oral vaccines are available – Rotarix® and RotaTeq® - and have been recommended by the World Health Organization [7] as part of national immunization strategies. Through the Global Alliance for Vaccine and Immunization (GAVI), over 20 African countries, including Tanzania, have included this vaccine in their programs [8].

Rotavirus is one of the major enteric pathogens which is communicable between humans and animals, therefore a zoonosis [9]. Rotavirus strains are not only distributed in species-specific pattern (human- or animal- specific), but accumulating evidence indicates that animals may act as a source of virus and/or of genetic material for diversification of human Rotavirus [10-12]. The trilaminar Rotavirus particle has an outer capsid comprising two proteins (VP4 and VP7) responsible for antibody production and protective immunity; an intermediate layer formed by VP6; and an inner layer of VP2, which encompasses two other proteins (VP1 and VP3), as well as the viral genome consisting of 11 segments of double-stranded RNA [11]. This latter element encodes six structural and six non-structural proteins [11], each with distinct functions [13]. It has been found that the outer capsid proteins bear G and P serotype specificities, with 12 G serotypes and 15 P serotypes in humans [1,14-16]. Due to the variability patterns in Rotavirus serotypes, a binomial typing identifies strains. At this time, G1P, G2P, G3P, G4P, and G9P are closely associated with disease occurrence (resulting in more than 90% of cases) [14-18]. Of note, the serotype of the Rotavirus does not correlate with the severity of presentation or the seasonal patterns of the disease [1,16].

Rotaviruses are classified into seven groups (labelled A to G) based on antigenic specificity conferred by the VP6 structural protein [14]. To date, Group A, B and C Rotaviruses have been found in both humans and animals [17], whereas viruses in the Group D, E, F, and G have been found only in animals, specifically E in pigs and D,F and G in birds [11,16,18]. Group A Rotaviruses (GARV) are, however, the most important common causative agent in diarrhea in children. As a result, GARV are the main target of currently available Rotavirus vaccines for children [19,20].

To date, no known study has specifically addressed Rotavirus prevalence in Tanzania since the introduction of Rotarix® within the Extended Program of Immunization (EPI) introduced in late 2012. A number of studies

were conducted prior to this initiative including studies in Dar es Salaam [21-24], in Mwanza [25-27], and in Ifakara [28]. In one study conducted in Dar-es-salaam, an unexpectedly high proportion (81.6%) of G9 serotype was found, which was higher compared to other African countries [23]. Furthermore, the complete absence of serotypes G2 and G4, the most common, in this study group was in marked contrast to the global situation [29-31]. So, there is a need to study the prevalence of the disease after the introduction of the vaccine, which may be a further indication of the serotypes of the virus present in the community, as some are not responsive to the current vaccines. In this study, we will detect post-vaccine period prevalence of Rotavirus in children under five years of age in urban and peri-urban Arusha, using the molecular method, as well as determining prevalence of known risk factors. This study responds to prior research recommendations on assessing the impact of the recently rolled out vaccine on subsequent Rotavirus prevalence [24,27].

2. MATERIALS AND METHODS

2.1 Study Setting and Sample

According to the 2012 Tanzanian Population and Housing Census [2], Arusha has a general population of 1,694,310 people, of approximately 15% (249,957) are children under five years of age. The Arusha District is economically the wealthiest in the country with a range of activities including livestock and farming, as well as tourism. This area has continually shown a shift from markedly rural to a much more urban/peri-urban area. There is a preponderance of inhabitants who keep livestock in the living spaces, which is a potential vector of animal to human infections, as well as for fecal contamination of water, food and/or fomites.

This study was conducted in three major Arusha hospitals (Mt. Meru District and Referral Hospital, St. Elizabeth Hospital, and Selian Lutheran Hospital). The study sample included children under 5 years of age admitted to one of the participating hospital with diarrheal symptoms during the study dates of May through August, 2014. A confirmation of symptoms meant that the child had experienced three or more watery stools within a 24 hour period [32]. The control group consisted of children admitted or visiting the hospitals' outpatient departments with symptoms not inclusive of diarrhea. Consent of the parents/guardians was required in both groups.

A total of 100 stool samples were analysed, comprising 77 patients with diarrhea and 23 patients without diarrhea. Recruitment was done during medical ward rounds and visits to the outpatient clinics.

2.2 Ethical Approval

Research ethical approval was received from the National Medical Research Institute through which an ethical clearance certificate was issued by the Medical Research Coordinating Committee of Tanzania. Permission was also sought and granted by the Regional Medical Officer, as well as from the respective hospital authorities where the study recruitment occurred.

2.3 Data Collection and Management

2.3.1 Stool sample collection and processing

One stool sample was collected from each child included in the study in a sterile wide-mouthed container then transferred to a sterile cryovial. The sample was then stored in liquid nitrogen and transferred to the laboratory where it was stored at -80°C until analysis.

Prior to RNA extraction, 10% (wt/vol) stool suspension was prepared in Phosphate Buffered Saline (PBS) with a pH 7.4 and stored at 4°C. The suspension was spun at 1 600 (×g) for 5 minutes in an Eppendorf™ centrifuge (Eppendorf AG, Germany) and the supernatant was used for RNA extraction. RNA was extracted from the previously prepared 10% stool suspension, by withdrawing 250 µl of the stool supernatant and transferring it to a sterile1.5 ml Eppendorf™ tube. Extraction of dsRNA was done using the Trizol method as previously described by the WHO [16]. This RNA was directly used for cDNA synthesis.

Preceding RNA detection using PCR, cDNA synthesis was done using Maxima H Minus First Strand cDNA Synthesis™ kit with the technique described by the manufacturer (Thermo Scientific™, Denmark). Conventional PCR was then done for the detection of Group A Rotavirus (GARV) as described previously [33] with a

universal primer set NSP3-F with nucleotides 963 to 982 (ACCATCTACACATGACCCTC) and NSP3-R with nucleotides 1049 to 1034 (GGTCACATAACGCCCC) using Thermo Scientific™ reagents according to the manufacturer's instructions. For the conventional PCR, the following conditions were applied: A preheating step at 94°C for 3 minutes for initial denaturation, followed by 40 PCR cycles at 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 1 minute and a final extension at 72°C for 10 minutes and then the samples were held at 4°C until they were removed from the C1000 Touch™ Thermo Cycler (Bio-Rad Laboratories Inc, USA).

These PCR products were loaded on 5% agarose gel stained with GelGreen nucleic stain and using Ultra Low Range (ULR) nucleic ladder, gel electrophoresis was ran for one hour with 100 voltage. A Bio-Rad Gel DocTM EZ Imager™ (Bio-Rad Laboratories Inc, USA) was used for imaging.

2.3.2 Questionnaire

A standardized questionnaire was used for both positive and control cases. The questionnaire captured demographic data (age), Rotavirus vaccination status, parent/guardian knowledge of Rotavirus vaccine availability, exclusive breastfeeding for the first six months of life, sibling(s) with diarrhea, attending kindergarten, and proximity to livestock (i.e., cattle and/or swine operation. The questionnaire was administered verbally by the researcher after obtaining informed consent from the parent/guardian.

2.4 Data Analysis

Data analysis was done using the Statistical Package for Social Sciences (SPSS) (Version 20). Using this software, Fisher's Exact Test was done to test association of Rotavirus positive samples and possible pre-disposing factors. This test was considered appropriate given the small sample size. A p value <0.05 was considered statistically significant.

3. RESULTS

Samples were collected from Mt. Meru Hospital (28 samples), St Elizabeth Hospital (66 samples) and Selian Hospital (6 samples). Out of the 100 samples tested, 37% were positive for Rotavirus from the PCR results analysed in agarose gel electrophoresis. A representative sample of the gel electrophoresis is shown Fig. 1. The age distribution of participants and respective Rotavirus status is shown in Table 1. It is noted that all positive Rotavirus cases (37) were from within the 77 diarrhea symptomatic participants, representing slightly more than 48% of these sampled individuals.

Table 1. Age categories and rotavirus status of participants

Age (Months)	Number of children	Rotavirus status (Positive)
0-6	20	7 (35%)
7-12	35	15 (43%)
13-18	6	3 (50%)
18-24	3	1 (33%)
25-30	8	5 (62.5%)
31-36	4	2 (50%)
37-42	9	2 (22%)
43-48	6	1 (16.7%)
49-54	5	1 (16.7%)
55-59	4	0 (0%)
Total	**100**	**37 (100%)**

Due to the small number of positive pools, Fisher's Exact Tests were used to relate Conventional PCR test and various factors associated with the incidence of Rotavirus. Our findings revealed showed that household compliance with advanced boiling of drinking water (P=.017) and parent/guardian knowledge on vaccination efficacy were positively associated with Rotavirus status (P<.042).

All children in our sample, except one, were over 10 weeks which placed in them in the window of opportunity for completion of the two doses of Rotarix™ regime delivered at 6 and 10 weeks. As shown in Fig. 2, 37% of our sample were unvaccinated (i.e., 36% were outside the expected Rotarix™ protocol). Of the Rotavirus positive individuals, 8 (21.6%) were vaccinated. Vaccination status was shown to be positively associated with Rotavirus status (P<.05) (see Fig. 2). However, age category, exclusive breastfeeding for the first six months of life, residing near a cattle and/or swine operation, diarrheal status, sibling(s) with confirmed diarrhea, and attending kindergarten were not significantly associated with Rotavirus status (P>.05).

4. DISCUSSION

Rotavirus is the leading cause of severe diarrhea in children under the age of five years in both developed and developing countries. Although there have been major strides globally to introduce a vaccination program for Rotavirus, there continues to be a high incidence of this disease. In Tanzania, the number of deaths reported due to diarrheal diseases in this group in 2010 was 11,391 (representing 9% of all childhood deaths) of which 8, 171 were attributed to Rotavirus [1,3]. In December 2012, the Government of Tanzania introduced the Rotavirus vaccination to their EPI palette.

This study is one of the early assessments of the effectiveness of this campaign, although it focuses only on a small population in Arusha, Tanzania. The results of this study showed that over one-third (37%) of the children tested were positive for Rotavirus using PCR. This prevalence is higher than most results reported in prior pre-vaccination era studies, including a range of 18.1% to 43.4% in Dar es Salaam [26-29]; 9.8% to 49.4% [25-27] in Mwanza; and

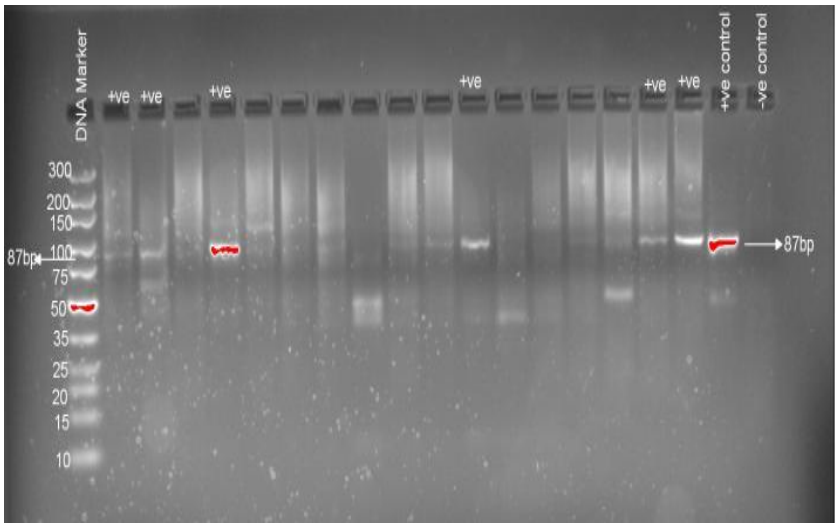

Fig. 1. Agarose gel electrophoresis picture with an ultra low range DNA marker

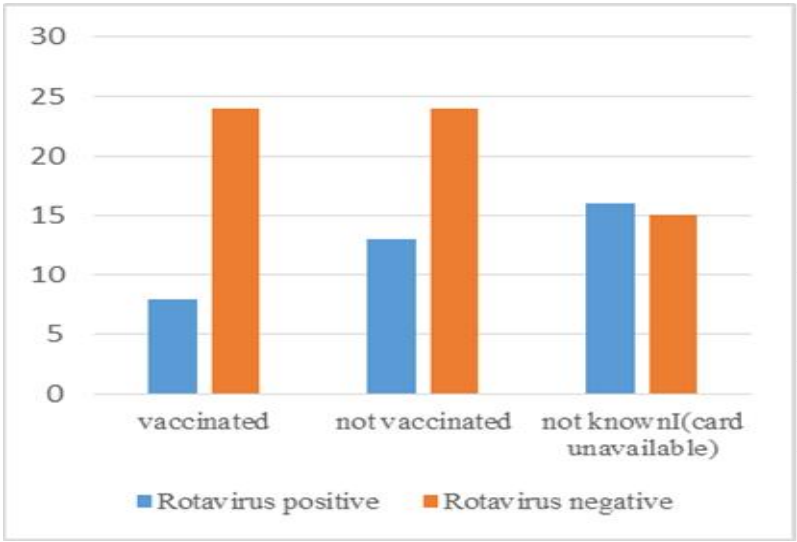

Fig. 2. Rotavirus detection and reported vaccination status

23% [28] in Ifakara. Globally, vaccination campaigns have been found to reduce rotavirus related hospitalizations by 49-92% and deaths by 22-50% [34]. Multi-country efficacy of the vaccination has been demonstrated by reduction in gastroenteritis related hospitalizations and gastroenteritis related deaths as reflected in Table 2 [35]. This result suggests a need to further study the efficacy of the Rotavirus vaccination initiative to determine whether there is a positive shift in the pathogenic cause of diarrheal diseases in this population.

Table 2. Exemplars of global effectiveness of rotavirus vaccination

Country	Reduction in hospitalizations	Reduction in deaths
Mexico	40%	46%
Panama	30%	50%
Venezula	19-26%	57-64%

Additional findings on risk factors for Rotavirus showed that household advanced boiling of drinking water practices, vaccination status, and parent/guardian knowledge on Rotavirus vaccine were positively associated with Rotavirus status ($P<0.05$). Our results concur with previous findings on effect of boiling drinking water relative to reducing risks of Rotavirus infection, which showed that heating water at 65°C for five minutes is effective in damaging the structural components and disrupting the viral essential life processes like denaturing the proteins [36-38]. In this study, parents'/guardians' knowledge on Rotavirus vaccination was significantly associated with Rotavirus incidence, yielding a lower risk of acquiring Rotavirus. This could have transformed to the fact that, these parents/ guardians understood the importance of vaccination and also the route of Rotavirus transmission. According to a study done in Canada on maternal awareness on Rotavirus vaccine, it was shown that the knowledge of these mothers positively contributed to their intention to have their children vaccinated [39]. From our study, vaccination against Rotavirus significantly reduced risks of Rotavirus infection. The primary public health intervention for Rotavirus recommended is vaccination. Rotarix® is the form of Rotavirus vaccine being administered in Tanzania's EPI in alignment with the WHO recommendation for embedding of the Rotavirus vaccine in all national immunization programs. Tanzania became the 42nd country worldwide to amend their EPI in collaboration with GAVI to include a Rotavirus vaccine [8]. Clinical trials on Rotarix® in South Africa and Malawi found that the vaccine significantly reduced severe diarrhea episodes and that the infection was preventable by vaccination [6].

In this study, other risk factors, such as age category, exclusive breastfeeding for the first six months of life, residing near a cattle and/or swine operation, diarrhea status, sibling(s) with diarrhea, and attending kindergarten were not significantly associated ($P>0.05$). Our results further contribute to the debate on exclusive breastfeeding and Rotavirus. The animal-human transmission potential was found to be unrelated to the Rotavirus occurrence in this sample; however, the population is drawn from a primarily urban population using urban health care services which may impact this finding. Further studies are recommended in more rural/remote and pastoral areas of the country to determine actual impacts. The lack of significance respecting sibling and/or peer (kindergarten attendance) co-infections was again based primarily on verbal reports which are limited. However, this relationship should be further explored in order to determine whether handwashing and sanitation strategies are actually invoked and impact on the mitigation of human to human transmission in this population.

The major limitations of this study include its cross-sectional approach, limited sample size, larger demographic age groupings, and non-tracking of days from onset of symptoms and prior diarrheal episodes (which may contribute to the capture of Rotavirus prevalence). The lack of baseline data limited comparisons. Our study did not consider the issue of seasonal variability in the occurrence of Rotavirus which may have implications for the findings [40]. Finally, the heavy reliance on self-reporting by the parents/ guardians may have skewed reporting of vaccination history and positive behaviors, such as water boiling practices and exclusive breastfeeding.

5. CONCLUSION

This study showed that slightly more than 48% of the diarrheal symptomatic participants were Rotavirus positive (i.e., 37/77). Risk factors which were found to reduce the risk of Rotavirus presence in this population were adherence to advanced boiling of drinking water, Rotavirus vaccination compliance, and increased parent/ guardian knowledge on Rotavirus vaccine. Other

factors such as age, exclusive breastfeeding for the first six months of life, residing near a cattle and/or swine operation, sibling(s) with diarrhea, and attending kindergarten were not significantly associated.

This study opens the dialogue on the effectiveness of the introduction of the Rotavirus vaccine in Tanzania by providing a small prevalence study in Arusha. As a result, the researchers recommended replication studies, retrospective studies, as well as consideration of monitoring and disease surveillance for Rotavirus to assess effectiveness of the national immunization strategy.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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