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Epidemiology and Genetic Diversity of Rotavirus in Children Under Five Years, Before and After Vaccine Introduction in Maputo, South Mozambique

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Abstract: Mozambique introduced the Rotarix monovalent vaccine (RIX4414 Rotarix^{MT}) into its childhood immunization schedule in September 2015. We aimed to assess the impact of the vaccine in the prevention of infection and in the diversity of RVA genotypes before and after vaccine introduction. A descriptive cross-sectional study in children under five years of age hospitalized with acute diarrhea in the Pediatric Services of Maputo Central Hospital between 2013 and 2018 was conducted. eight hundred and two stool samples were collected during the pre-vaccination period (2013 - 2015) and 196 in the post-vaccination period (2016 - 2018), where a median reduction of 76% of cases of hospitalization for diarrhea was observed, decreasing from 28.7% (82/284) in the pre-vaccine period to 15.50% (10/66) in the post-vaccination period, ($p < 0.05$) in all age groups. Similarly, there was a median reduction of 53.89% in the proportion of hospitalization for rotavirus diarrhea in children <12 months of age, from (28.29%) in the pre-vaccination period to 13.04% in the post-vaccinal. After the introduction of the vaccine, there was a change in the usual seasonal peaks. The most prevalent combinations of rotavirus genotypes in the pre-vaccine period were G2P[4] in 2013 62/86 (72%), G1P[8] 31/48 (65%) in 2014, G9P[8] 48/70 (69%) in 2015 and in the post - vaccination period were: G1P[8] 5 (50%) in 2016 and 5/8 (62.5%) in 2017; G3P[8], G3P[4], G4P[6] 1/3 (33%) in 2018. After vaccine introduction a reduction of diarrhea hospitalization and rotavirus associated were observed and a wide variety of rotavirus strains circulated in the pre- and post-introduction periods of the vaccine. The results of this study support evidence of the impact of rotavirus vaccination highlighting the importance of program continuity as well as monitoring studies of rotavirus genotypes following the introduction of the vaccine in Mozambique.

Keywords: epidemiology; rotavirus; vaccine; HCM; mozambique

1. Introduction

Acute diarrheal disease remains one of the major public health problems in the world. Approximately, 20.8% deaths among children under five years old due to diarrheal disease caused by Rotavirus A (RVA) are reported annually, most of them in low-income countries [1].

Rotavirus belongs to the genus Rotavirus, family Reoviridae, whose genome consists of 11 segments of double-stranded RNA [2]. Genotypes are classified according to a binary system through the determination of gene sequences that encode the VP7 (G types) and VP4 (P types) proteins[3].

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However, a more complete classification system was recently suggested, based on the sequence of all genomic segments of the virus [4]. The most common VP7 (G) and VP4 (P) combinations worldwide are: G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and G12P[8] [5].

There are two Rotavirus vaccines licensed, the pentavalent RotaTeq vaccine and monovalent Rotarix vaccine [6,7]. Both have the ability to reduce the severity of Rotavirus diarrheal disease in children under five years of age [7]. The Global Enteric Multicenter Study (GEMS) has shown that Rotavirus is one of the major causative agents of diarrheal disease in Mozambique. Given the impact of rotavirus diarrhea on pediatric morbidity and mortality, it was evident that a vaccine was needed to reduce the severity of diarrheal disease[8]. Therefore, in September 2015, Mozambique introduced the Rotarix monovalent vaccine (RIX4414 Rotarix^{MT}) in its Extensive Vaccination Program (PAV) [9].

A previous study during first 2 years post-rotavirus vaccine introduction in Mozambique showed a decline of 12.2% and 13.5% in 2016 and 2017, respectively among rotavirus hospitalizations [10]. However, the frequency of circulating strains was not evaluated. Previous studies in sub-Saharan Africa have shown that there is wide variability of rotavirus strains circulating between the period before and after vaccine introduction. Highlighting the importance of program continuity as well as monitoring studies of rotavirus genotypes following the introduction of the vaccine. Therefore, this study aimed to determine the impact of vaccine in the prevention of infection and diversity of RVA genotypes in Maputo city.

2. Materials and Methods

A cross sectional study in children aged < 5 years old hospitalized from diarrheal disease at Pediatrics Department of of Maputo Central Hospital "Hospital Central de Maputo – Mozambique (HCM)" over five-year period, April 2013 to December 2018. HCM is located in Maputo city in the south of the country. HCM is the largest and principal quaternary care referral hospital in Mozambique, with 2000 beds. Over 50% of inpatients are referred from peripheral Hospitals.

2.1. Selection Criteria of the Participants

Children were included into the study if there were under aged five years, hospitalized with acute diarrhea as primary illness of less than 7 days. Diarrhea was defined as three or more loose of watery stools within a 24-hour period. Children with other symptoms, such as stools with visible blood presentation were excluded [11].

2.2. Data Collection

Data were actively collected 5 days per week from all inpatient admitted in pediatric medical wards with acute diarrhea. Children who met the eligibility criteria and whose parents or legal guardian consented were enrolled in the study. Demographic and clinical information were recorded in a case report form. The information included date of admission, duration of the hospitalization, residence, age, history of diarrhea, fever and vomiting, physical examination, treatment and outcomes.

2.3. Retrospective Data Review

It was not possible to enroll all children aged < 5 years old who were treated to acute diarrhea during study period. Therefore, it was also retrospectively reviewed inpatient hospital registration April 2013 to December 2018. Monthly counts of acute diarrhea admissions were extracted from the hospital registers books.

2.4. Monovalent Rotavirus Vaccine (RV1) Coverage

We considered an infant fully immunized against rotavirus if she had received 2 doses of RV1. These data were captured directly from a child's health card. There were taken pictures of health

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cards for confirmation. RV1 vaccination coverage was kindly provided by the Ministry of Health's expanded vaccination program.

The period from April 2013 to December 2015 was defined as the pre-vaccine period and the period from January 2016 to December 2018 was defined as the post-vaccine period.

2.5. Ethical Consideration

Written informed consent was obtained from the parents and guardians of all study participants. The study protocol and the informed consent was reviewed and approved by the National Bioethical Committee for Health from Mozambique (IRB00002657, reference Number: 172/CNBS/12).

2.6. Stool Sample Collection and Laboratory Procedures

Stool samples were collected from diapers in sterile polystyrene tubes, within 48h of admission. The tubes were labeled and kept refrigerated in cooler boxes, then transported on the same day to the Virology laboratory in the National Institute of Health in Maputo. Samples were stored at – 40ºC until testing was performed. In case of watery diarrhea, a portion of the diaper was eluted in saline solution (0.9%). Enzyme Immunoassay EIA for Rotavirus A (RVA) (Prospects Rotavirus Kit, Oxoid, Ltd, United Kingdom) was used to screen stool samples for Rotavirus infection following manufacturer recommendations.

To genotype the rotavirus strains, we extorted dsRNA from 10% stool suspensions using QIAamp Viral RNA extraction method (Qiagen, Hilden, Germany) following manufacturer's instructions. The reverse transcriptase (RT) for cDNA synthesis was performed with 5 µl of extracted RNA template using Invitrogen™ SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Amplification was carried out by semi-nested PCR using probes for a conserved nucleotide sequence of the VP7 (1,062 bp) and VP4 (877 bp) genes and consensus primer pairs Con2/Con3 and sBeg/End9 as described by Das et al [12] and Gouvea et al [13] respectively. The second round was performed by semi-nested PCR using specific primer including RVG9 to identify G-type, G1-G4, G8-G10 and G12 respectively (4). While for P- type specific primer with Con3, P[4], P[6],P[8],P[9],P[10],P[11] and P[14] strains [13]. G and P genotypes were detected by electrophoresis of PCR products in a 1.5% agarose gel, examining the migration patterns.

2.7. Nucleotide Sequencing and Phylogenetic Analysis

The sequencing was performed by the PDTIS / Fiocruz Sequencing Platform and Phylogenetic Analysis Specimens that could not be confirmed the genotypes by semi-nested PCR were subjected to nucleotide sequencing using individually the consensus G and P primers. First round products of RT-PCR (consensus amplicons) were purified with QIA quick™ PCR Purification Kit (QIAGENTM, Valencia, CA), according to the manufacturer's instructions and sequenced using the Big Dye Cycle Sequencing reaction kit™ (Applied) Biosystems, Foster City, CA) and ABI Prism 3730 Genetic Analyzer™ (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences obtained were aligned and edited using the Bio-Edit Sequence Alignment Editor (version 7.2.5) [14] then compared with corresponding sequences of selected rotavirus strains available in the Gen Bank data base. The phylogenetic analysis was performed using (Mega 7.0) [15] and the neighbor-joining method with distances calculated by the Kimura-2 parameter method. Statistical support was assessed by bootstrapping with 2,000 replicates.

2.7. Data Analysis

Data were analyzed using IBM SPSS version 20 and R version 3.5.2. statistical software. Trends and seasonal patterns analyses of rotavirus hospitalizations for pre- and post-vaccine periods were performed. There were also compared hospitalizations due to rotavirus before and after the vaccine was introduced. We compared decline of diarrhea hospitalization and rotavirus positive samples by

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age group in the pre and post vaccine periods. There were also examined total hospital admissions for diarrhea pre and post–vaccine introduction. The median number of tests and number of positive tests pre–vaccine and post vaccine introduction were performed. We examined the percentage of decline in the total number of samples tested and the number of positive rotavirus cases before and after the vaccine was introduced. We also calculated the percentage of decline and genotypes observed before and after the vaccine was introduced. A significance level of p= 0.05 was used in all analyses.

3. Results

3.1. Frequency of Diarrhea Observed in Pre and Post Vaccine Introduce

During study period a total of 4.147 children less than 5 years with diarrhea were hospitalized in the study site. From these, 1.151 (27.7%) were enrolled in the study and 998 (86.7%) stool samples were collected and tested for Rotavirus infection. Eight hundred and two cases (80.4%) in the pre-vaccine period and 196 (19.6%) in the post-vaccine period (P <0.001). Of the total cases included 701 (70.2%) were younger than 12 months (P <0.001) (Table 1).

3.2.Frequency of Rotavirus a Detected by EIA

Rotavirus (RVA) infections were detected in 267/998 (26.7%) of analyses stool samples. From these, a total of 239/802 (30%) were collected in the pre-vaccine period, while 28/196 (14.2%) were from samples collected in the post-vaccine period. The annual frequency of RVA cases in the pre-vaccine period was: 107/324 (33.0%) in 2013; 50/194 (25.7%) in 2014 and 82/284 (28.9%) in 2015 and of 12/70 (17.1%) in 2016; 10/66 (15.2%) in 2017 and 6/60 (10.0%) in 2018 in the post-vaccination period (Figure 1).

3.3. Diarrhea and Rotavirus a Hospitalization Trends

Overall a gradual decline of the number of children tested for rotavirus A (RVA) and correspondent number of tested positive and negative for rotavirus was observed (Figure 1). There was a significant decline in the median tested positive for RVA from 28.8% to 15.5% (p =0.028) Children observed prior to the per vaccine period had 57% less chance to be infected than from post-vaccination period; (OR= 0.43; IC,95%: 0.21-0.99) in the pre and post vaccine period respectively in children < 5 years old.

In children of the ranged (0-11 moths) there was a median reduce in positivity from 28.3% to 13,04% (p =0.049) and 62.0% low chance to be infected by rotavirus than post vaccine (OR=0.38; IC,95%: 0.15-0.96). However, there was no statistical difference in the other age groups 12-23 months (p = 0.66); they had 62% low chance to be infected by rotavirus than post vaccine (OR = 0.38; CI 95% 0.298-2.5) and 24-59 months (p = 1.0, OR = 0.71 CI 95% (0.062-8.15) (Table2).

Similarly decline was observed in diarrhea hospitalization cases over the years. In this study, there was a decline in the median of the total number of the cases tested by 76.7% (75.35 - 78.87%) and also a reduction of 87.7% (85.37 - 92.68 %) of the number of positive cases for RVA in all age groups.

In addition, a reduction of 47.5% (40.63 - 65.37%) in the proportion of RVA positive in all age groups of children < 5 years from 2013 to 2018 was observed Table 1. We also observed that the introduction of rotavirus vaccine has resulted in a reduction of rotavirus-associated hospitalizations in all older age groups mainly in children <12 months of age (Table 2).

The median age of RVA infection increased from 9 months to 11 months at intervals of 8 to 11 months and 7.5 to 11 months between the pre and post vaccination periods respectively.

Regarding rotavirus severity, children from pre vaccine period were 90% more likely to develop fever than those observed in the post vaccination period (OR = 1.90; CI = 1.36-2.65; p = 0.001), and the chances of present with vomit was 15% higher in the pre-vaccination period than in the post-

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vaccination period (OR = 1.15, CI = 0.83-1.60 p = 0.37). Children hospitalized in the pre-vaccination period were 34% more likely to have dehydration than those in the post-vaccination period (OR = 1.34, CI = 0.82-2.11 p = 0.22).

3.4. Temporal Rotavirus Distribution in Pre and Post Vaccine

Prior to the introduction of the vaccine, rotavirus diarrhea exhibited consistent seasonality with rotavirus-associated peaks of diarrhea during the cold and dry months (May - July). However, in the post-vaccine era there was a substantial shift with peaks of rotavirus associated diarrhea during the months of August-September (Figure 2).

3.5. Detection and Genotype Characterization

A total of 267 rotavirus EIA-Positive samples with adequate quantity were submitted to molecular characterization by polymerase chain reaction (RT-PCR). From these, 205 stool samples (90,7%) were performed in the pre vaccine period and 21 (9,3%) in post vaccine. Among VP7 G genotypes, the most frequent were: G2 96/205 (46,8%), G9 53/205(26,0%) in the pre-vaccine period and in the post-vaccine period G1 11/21 (52.4%) was more frequent followed by genotypes G3 4/21 (19.0%) and G9 2/21 (9.5%).

Regarding to VP4 P type, P [8] 106/205 (51.7%) was the most frequent; followed by the genotypes P[4] 70/205 (34.1%) and P[6] 25/205 (11.5%).

The post-vaccine period was characterized by persistence of the predominance of genotype P[8] 13/21 (61.9%), followed by genotypes P [4] 6/21 (28.6%) and P [6] 2 / 21 (9.5%). The genotype results demonstrated high rotavirus strains diversity during pre-vaccine and post vaccine period. The most common binary G and P combinations per year were G2P [4] 62/86 (72%)- 2013; G1P [8] 31/48(65%)- 2014; G9P [8] 48/70(69%)- 2015; G1P [8] 5/10(50%)- 2016; G1P [8] 5/8(62,5%)-2017 e 1/3 (33%) G3P [4], G4P [6], G3P [8] 2018

During the pre-vaccine period the most common rotavirus combination in pre-vaccine was G2P [4] 67(32,7%) followed by G9P[8] 23(23,4%) and G2P[6] 21(10,2%). Other genotypes detected were G2P [8] 8(3,9%), G12P [8] 3(1.5%), G1P[4] 2(1.0%), G4P[8] 2(1.0%) G2P[6] 2(1.0%), G9P[6] 2(1.0%), G9P[8]P[6] 2(1.0%), GNTP[8] 4 (2%) and G3P[8], G1P[6], G8P[4], G9P[NT];GNTP[4], with 1(0.5%). After vaccine introduced the G1P[8] 10(47,6%) was the most predominant followed by G3P[4] and G3P[8] 2(9,5%) others genotypes detected in low frequency were G1P[4], G2P[4], G4P[6],G9P[4],G9P[8], G9G3P[6], G12G3P[4] with 1(%) respectively (Figure 3).

Phylogenetic analysis was based on partial nucleotide sequence (896 bp) of the gene coding for VP7 protein characterized in G1, G3 and G9 grouping in three clusters. In the G1 genotype cluster, it is possible to observe that the study samples showed a close genetic relationship with the circulating strains from India. In cluster G3, the study samples are grouped with the samples from Pakistan. And in the G9 genotype cluster it can be observed that the study samples are related to the Republic of Zimbabwe samples, Italy and Japan (Figure 4).

4. Discussion

In Mozambique, rotavirus vaccine was introduced in the National Immunization program in September 2015 through Extended Vaccination Program (PAV) During 3 years after vaccine introduction, we observed dramatic reduction in all diarrhea cases and rotavirus related hospitalization at the large referral hospital in Maputo, when compared with previous years. Active surveillance found that proportion of rotavirus positive stools specimens from children < 5 years of age reduced by more than half. This reduction was notable in age group of 0-11 and 12-23 months of age, however the data among 23-59 were based on small number. These results are in agreement with those previously reported in Mozambique [16] and by other African countries where rotavirus vaccine was already introduced which continuously reported a reduction in hospitalization for diarrhea in children under five years after vaccine introduced [17].

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The reduction observed in diarrhea and rotavirus hospitalization during the years of active surveillance is probably due to the large reduction in incidence in age group targeted by the vaccination, and the gradual increase in rotavirus of vaccine coverage in Mozambique, from 76% in 2016 to 89% in 2017 (National Immunization Program, 2017. Data not published), these findings were also observed in Zambia, Zimbabwe and Malawi [18,19] and support the hypothesis that the observed reduction in rotavirus infection is probably related to the introduction of the rotavirus vaccine in the routine immunization program, and probably can be attributed to the basically worldwide diarrheal disease treatment campaigns with oral rehydration therapy (ORT), zinc supplementation and improved hygiene, optimization of nutrition[20].

However, data among unvaccinated children are more variable and based on smaller numbers, it's believed that the indirect effect from vaccine (herd immunity) has contributed in the reduction of rotavirus associated diarrhea hospitalizations in all age group[21]. We confirmed consistent rotavirus distribution all year round with epidemic peaks in the colder and drier months. Similar findings were previously reported in Mozambique[22,23]. It is believed that local climatic factors and geographic are associated with the seasonality of rotavirus infection. Some factors may be related to the seasonal cycle of infection, and correlations between epidemic cycles and variations in temperature, humidity, atmospheric pressure, rainfall, and wind have been observed[15]. During the post-vaccine period, a substantial shift in temporal pattern of rotavirus infectious (August-September) was observed, this is in agreement with previous studies conducted in Mozambique and other neighboring countries which reported similar events after the introduction of vaccine [10,18,23,24].

The genotyping results observed during the pre-vaccine period 2013-2015 was characterized with high rotavirus strain diversity compared with the post vaccine period. This study illustrates the natural fluctuation of rotavirus genotypes circulating in Mozambican children pre and post vaccine introduced.

We observed oscillation of rotavirus genotypes distribution round the years. In 2013 G2P [4] was the most predominant genotype, whereas G1P[8] was most frequent in 2014, and G9P[8] in 2015.

Similar results were previously reported in Mozambique [25] and in studies conducted in Africa countries prior vaccine introduction [26]. However, there is no sufficient evidence to support that genotype diversity and rotavirus strain oscillation has a specific impact on vaccine efficacy in Africa, or several factors that may be related. This emphasizing the need of monitor rotavirus strains throughout the year in the country and evaluate the vaccine efficacy in Mozambique.

In Mozambique, the G2P [4] genotype has been reported in Manhiça [25] during the same period, 2013 . More study will be required in order to better understand the dynamics of this strain diversity[26]. G1P[8] was the most frequently detected strain in circulating in HCM in 2014.

The frequency of detection of G2P [4] and G9P [8] decreases suddenly within three years of vaccine introduction. In contrast to results observed in some Latin American countries where they report the heterotypic predominance of G2P[4] after vaccine introduction [27]. This fact has generated a great debate in the scientific community because less is known about if this finding is directly related to the effect of the vaccine or reflects a natural fluctuation of the rotavirus strains [28,29].

On the other hand, an increase of the rarely detected G3 genotype in Mozambique was observed in combination with P[4], P[6], P[8], but in low proportions. These findings contradicts with those from a study in Madagascar where G3P[8] or G1P[8] were not detected in post vaccination period [30].

At first observation, it appears that the genotype G1 was not affected by the vaccine. However, these results should be interpreted with care, as it is known that there are different genotype G1 strains that can co-circulate at the same station, or oscillate over different seasons [31].

It is believed that some G1 strains may be selected due to selective vaccine pressure, which may result in the divergence of some G1 strains that may not be affected by the vaccine strain, to better understand this scenario, further phylogenetic studies and complete rotavirus genome analysis are needed.

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Different studies have been reporting reduction in the occurrence of G12 after the rotavirus vaccine introduction. However, further studies are needed to prove whether if this finding is associated with the introduction of the vaccine or the natural oscillation of the strains described above [32–34].

The emergence of G9P[4] in Mozambique was intriguing. Increased documentation of this strain has been observed in many countries following the introduction of the monovalent vaccine [30,34,35]. It is not clear yet if its increase is due to the introduction of the vaccine. Report from Ghana admit that its increase is probably related to the increase of the recombination process in the community [36].

The increased tendency of detection of mixed genotypes after vaccine introduction is of crucial importance as there is little evidence regarding the ability to induce heterotypic protection against these G12 strains for both vaccines [33,37]. In Maputo city and province, where most of the isolated came from, have been experiencing major sanitation deficit, which may probably justify the emergence of mixed strains in post vaccine period[38,39].

The distribution of RVA genotypes is known to fluctuate over time and across different geographic regions. However, the mechanisms involved in this phenomenon are still unclear [40].

When comparing the nucleotide sequence data from post vaccine introduction corresponding to genotypes G1, G3 and G9 with the prototypes obtained from GenBank, the results show that the study prototypes group in 99-100% with the prototypes of other countries and not with those circulating in Mozambique. These results suggest the need for further studies to monitor the evolution of RVA strains post vaccine introduce in Mozambique. With this consideration, continued surveillance of rotavirus strains post rotavirus vaccine introduce is of crucial importance for the identification of emerging strains as well as for the evaluation of vaccine efficacy in different regions of the country.

5. Conclusions

The data from this study reinforce the importance and impact of RV1 vaccine by reducing cases of diarrhea and gastroenteritis hospitalization in Mozambique caused by RVA in all age groups.

There was a change in prevalence by age group represented by a tendency of increased cases of hospitalization for diarrhea attributed to RVA in children older than 12 months.

Reducing the severity of diarrheal disease in post-vaccination compared to pre-vaccine (RV1) hospitalized children shows the importance of maintaining and improving Mozambique's immunization program.

There was a change in the temporal distribution profile of RVA infection.

A variation in the profile of rotavirus strains was observed over the years of this study.

A reduction in genotypes P [4], P [6] and the most common combinations G2P [4] and G9P [8] was observed;

Despite the reduction in the number of positive samples, the diversity of circulating RVA strains and the introduction of new genotypes remain a major challenge for vaccine efficacy.

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