PREVALENCE AND GENETIC DIVERSITY OF GROUP A ROTAVIRUS IN CHILDREN UNDER FIVE YEARS, BEFORE AND AFTER VACCINE INTRODUCTION AT MUKURU INFORMAL SETTLEMENTS, NAIROBI COUNTY, KENYA

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Prevalence and Genetic Diversity of Group A Rotavirus in Children Under Five Years, before and after Vaccine Introduction at Mukuru Informal Settlements, Nairobi County, Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

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DEDICATION

To my parents, Johnson Njeru and Lydia Njoki for the inspiration and encouragement you gave, always wishing me the best in this work to enable me achieve my goal.

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ABBREVIATIONS AND ACRONYMS

ABI	Applied Bio systems
AVL	Viral Lysis Buffer
DNA	Deoxyribonucleic acid
dNTPs	Deoxy nucleoside Tri phosphate
EB	Elution Buffer
EIA	Enzyme Immuno assay
ELISA	Enzyme-linked immunosorbent Assay
GAVI	Global Alliance for Vaccines and Immunization
GE	Gastroenteritis
H.I.V	Human Immunodeficiency Virus
KEMRI	Kenya Medical Research Institute
MAFFT	Multiple Alignment using Fast Fourier Transform
MEGA	Molecular Evolution Genetic Analysis
MMM	Medical Missionaries of Mary (Mukuru kwa Njenga)
NCBI	National Center for Biotechnology Information
OD	Optical density
PB	Phosphate Buffer

PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
RM	Reuben Medical Center Clinic (Mukuru kwa Reuben)
RNA	Ribonucleic acid
RT	Reverse transcriptase assay
RT-PCR	Reverse transcriptase polymerase chain reaction
SERU	Scientific Ethical & Review Unit
SPSS	Statistical Package for the Social Sciences
SDGs.	Sustainable Development Goal 3
TBE	Tris Boris EDTA
µl	Microliters
USA	United State of America
VP	Viral protein
WHO	World Health Organization

ABSTRACT

Rotavirus vaccines have been shown to be a lifesaving and cost-effective public health intervention in Africa and have resulted in reduced rotavirus mortality. In Kenya, rotavirus diarrhea causes 19% of hospitalizations and 16% of clinic visits among children <5 years of age and causes 4471 deaths and 8,781 hospitalizations per year. The aim of this study was to compare the prevalence and molecular characterization of Group A rotavirus in pre- and post-vaccine period in children less than five years attending Mukuru informal settlements clinic Nairobi County. A total of 270 stool samples (150 pre-vaccine and 120 post-vaccine) were tested for rotavirus using ELISA Prospect kit (Oxoid Ltd UK), sequenced partially for VP4 and VP7 and data analyzed using SPSS version 20. Sequences were assembled using Sequencer software, aligned using MAFFT and phylogenetic trees were constructed using MEGA version 5.2. For the vaccine coverage, data proportions for the children vaccinated was retrieved from immunization clinics record books and entered into excel for analysis. The results showed that rotavirus was more prevalent before vaccine introduction in children <12 months 6/36 (16.7%) compared to after vaccine introduction ($P<0.05$; $\chi^2=6$; df =1) and 13-24 months 4/42 (9.5%) ($P<0.05$; $\chi^2=9.6$; df =1). In children 25-36 months and 37- 48 months however, there was no significance difference in prevalence of rotavirus infection pre-and post-vaccine introduction ($P>0.05$; $\chi^2=0.5$; df =1) and ($P>0.05$; $\chi^2=0.184$; df =1) respectively. The prevalence of rotavirus infection detected in female was 8/75 (10.7%) compared to male 7/75 (9.3%) for the period before vaccine however, this was not statically significant. For the period after vaccine introduction cases in male were higher 4/64 (6.3%) compared to female 2/56 (3.6%). Overall, rotavirus prevalence was 10% (15/150) and 5% (6/120) in pre-vaccine and post-vaccine samples respectively. The major genotype combination both before vaccine and after vaccine was G1P [8] followed by G9P[8]. Other uncommon strains circulating in Mukuru informal settlement after vaccine introductions include G2 [P4], G3[P4], G4[P4]. The proportion of children vaccinated in Mukuru with rotavirus vaccine was 80.3% in the first year of vaccine

introduction. There was significant statistical difference in rotavirus prevalence for cases less than 12 months of age, 13-24 months and over 49 months pre-and post-vaccine introduction. However, no significant difference in prevalence for age category 25-36 and 37- 48 months thus the vaccine proved to have a significant protection in the most vulnerable group of children. In conclusion, this study showed a reduction in prevalence of Group A rotavirus in Mukuru selected clinics one year after vaccine introduction into National Immunization program in Kenya.

CHAPTER ONE

INTRODUCTION

1.1 Background

Rotavirus infections is a leading cause of diarrhea in children under five years. It is estimated to cause 527,000 deaths globally, with 232,000 (85%) of these deaths occurring in South Asia and Sub-Saharan Africa in children under five years (WHO 2009; Mwenda *et al.,* 2010). A report by WHO in 2012 on rotavirus surveillance from four sites in Kenya: Embu, Siaya, Kilifi county hospitals and Kenyatta referral hospital showed the prevalence of rotavirus infections to be 27% (WHO 2012, Nokes *et al*., 2008). Safe and effective rotavirus vaccines are now available. Kenya introduced Rotarix® (GSK) into the routine immunization programme in July 2014 in all government health facilities for children aged below one year. Two doses are administered at the 6^{th} and 10^{th} weeks.

WHO recommends local disease surveillance studies prior to introduction of new vaccines. As part of this surveillance, circulating strains and genotypes are monitored. In Africa, the rotavirus vaccine has shown protection against diverse circulating rotavirus infections strains in clinical trials and in routine immunization programmes in selected countries like Ghana, Malawi and South Africa (Christabell *et al.,* 2014; Veerle *et al*., 2013; Madhi *et al.,* 2010). In Kenya (Siaya) rotavirus vaccine trial showed efficacy of 63.9% through two years of follow up in 1308 infants (Felkin *et al*., 2012).

Emergence of new strains has been seen over the recent years as shown by the characterization of rotavirus G12 serotype (Armah *et al*., 2003; Cunliffe *et al*., 2009; Page *et al.*, 2009;). It would be worthy determining any emerging genotypes that may not be covered by the vaccine currently in use. Having baseline surveillance for prevalence of rotavirus infections and trends before vaccine introduction is important in monitoring the effects of the vaccine and for the country to get an understanding of epidemiology of rotavirus. This study aimed at determining the number of rotavirus infections before and after vaccine introduction, the emerging genotypes and the vaccine coverage in Mukuru informal settlements in Nairobi County and its environs.

1.2 Statement of the Problem

In Kenya, rotavirus infection causes 19% of hospitalizations and 16% of clinic visits for diarrhea among children <5 years of age and causes 4471 deaths and 8781 hospitalization per year. Nationally, rotavirus disease costs the health care system $10.8 million annually. Routine vaccination with a 2-dose rotavirus vaccination series would avert 2467 deaths (55%), 5724 hospitalizations (65%), and 852,589 clinic visits (59%) and would save 58 disability-adjusted life-years (DALYs) per 1000 children annually (Tate *et al.*, 2009).

New genotypes that are not covered by the vaccine may slowly render the vaccine ineffective as a result of selection for viral strains able to survive and evade the host responses.

There is limited data on outpatient populations in Kenya and moreover in urban slums. This study contributed to the ongoing local inpatient WHO Rotavirus sentinel surveillance study in Kenya and better informs the decision makers (Ministry of Health & GAVI) and international donor agencies on the performance of the rotavirus vaccine in the real-world setting.

1.3 Justification

The introduction of free vaccine in 2014 in all Kenyan government facilities was meant to control rotavirus infections and reduce diarrheal cases in children under five years of age. The efficacy of this vaccine depends on the ability of the vaccine to cover the whole range of rotavirus circulating strains. However, it has not been established if all rotavirus genotypes are effectively covered by the vaccine or if there are any new/emerging genotypes capable of evading the vaccine induced host immune responses. There is no published data on the trends of rotavirus infections and circulating genotypes post licensure of the vaccine in Kenya. This study therefore evaluated the prevalence of rotavirus infections by comparing the pre- and post-vaccine introduction. It also provided data on the genetic diversity of rotavirus infections and assessed the uptake of rotavirus vaccine in selected clinics in Mukuru informal settlements.

1.4. General Objective

To determine the prevalence and genetic diversity of Group A rotavirus in pre- and post-vaccine period in children under five years attending Mukuru informal settlements selected clinics.

1.5. Specific Objectives

1. To determine the prevalence of Group A rotavirus infections in children under five years for pre-vaccine (2013-2014)) and post-vaccine (2014-2015) periods
2. To determine the rotavirus genotypes isolated from children under five years during pre- and post-vaccine period using molecular techniques
3. To assess the uptake of rotavirus vaccine in children attending Medical Missionaries of Mary (MMM) and Reuben Medical health center within Mukuru informal settlements between July 2014 to July 2015.

1.6 Research Questions

1. What is the prevalence of Group A rotavirus infections in children under five years attending Mukuru selected clinics before and after vaccine introduction?
2. What genotypes of rotavirus infections are circulating in children under five years attending Mukuru selected clinics before and after vaccine?

3. What is the estimated vaccine uptake of rotavirus vaccine in children attending Medical Missionaries of Mary (MMM) and Reuben Medical health center within Mukuru informal settlements between July 2014 to July 2015?

CHAPTER TWO

LITERATURE REVIEW

2.1 Rotavirus Infections Disease Burden and Vaccine Coverage

Enteric viruses are recognized as the most important etiological agents of gastroenteritis causing 50-70% of diarrhea worldwide. Viral diarrhea leads to majority of childhood morbidity and mortality (WHO 2012). Rotavirus infections is the single most important etiological factor, causing approximately 527,000 deaths per year worldwide, 25 million clinic visits and two million hospitalizations in children under 5years annually worldwide (WHO 2007; Parashar *et al*., 2003; Sabrina *et al*., 2007).

In Africa rotavirus infections is responsible for 230,000 deaths in children under five each year accounting for more than 60% percent of global total rotavirus deaths (WHO report, 2009). Previous studies in Kenya found that rotavirus infections caused 19% of hospitalizations and 16% of clinic visits for diarrhea among children <5 years of age and the overall annual mortality in Kenya was estimated to be 68 deaths per 100,000 children (Nokes *et al.,* 2008; Tate *et al.*, 2009).

Rotavirus is contracted through the oral faecal route. It is highly contagious and spreads easily from person to person through contaminated hands and objects following contact with feacal matter. Children with severe rotavirus infections have frequent diarrhea and vomiting leading to dehydration and often need to be rehydrated with intravenous fluids, Oral rehydration solution (ORS) and zinc tablets or risk dying (WHO 2009).

In developing countries, this type of urgent health care is inaccessible and unavailable, making the rotavirus prevention through vaccination critical to saving children's lives. Other measures that would be combined in preventing diarrhea are improvement of water quality, hygiene and prevention of bacterial and parasites infections (Navaneethan & Giannella 2008).

Following clinical trials in America and Europe that showed efficacy of over 85% against severe rotavirus infections, there was recommendation of introduction of rotavirus vaccine trials programs in numerous countries in Africa and Asia (WHO 2007). Studies on rotavirus vaccine trials in South Africa and Malawi found an efficacy of 61 and 44% respectively (Madhi *et al.,* 2010) while Mali and Ghana showed an efficacy of 83% in the first year of life (Armah *et al.,* 2010). In Kenya (Siaya) rotavirus vaccine trial showed efficacy of 63.9% through two years of follow up in 1308 infants (Felkin *et al.,* 2012).

WHO extended the recommendation after review of clinical trials data from Africa to post licensure data from America for introduction of national immunization programs of Africa countries (WHO 2009; Jiang *et al*., 2010). Through Global Alliance for Vaccines and Immunization (GAVI), 19 countries in Africa have introduced the rotavirus vaccine in their immunization programs, with Kenya being the latest in July 2014. Rotarix® vaccine is now available for free in all government facilities for children below 1 year in two doses administered at the six and the tenth week.

Changes of strain patterns have been seen in some countries after vaccine introduction. Established baseline surveillance for rotavirus infections and strain prevalence before and after vaccine will help in successful monitoring of vaccine impact in Kenya and understanding epidemiology of rotavirus (Kirkwood *et al.*, 2011; Hull *et al.,* 2011). This would then inform the health policy makers including the Ministry of health Kenya in collaboration with GAVI.

2.2 Rotavirus Genome

Rotaviruses are members of the *Reoviridae* family (Matthews *et al*., 1979) and are characterized by their non-enveloped icosahedral structure of 70nm in diameter. It has 11-segmented double-stranded RNA genome that is surrounded by three protein shells: a core, an inner capsid, and outer capsid **(Figure 2.1).** A total of 6 structural (VP1-VP4, VP6, VP7) and 6 non-structural protein(s) (NSP1-NSP6) are encoded by the rotavirus genome. With the exception of gene segment 11, which encodes two proteins, the remaining gene segments encode a single protein (Estes & Cohen 1989).

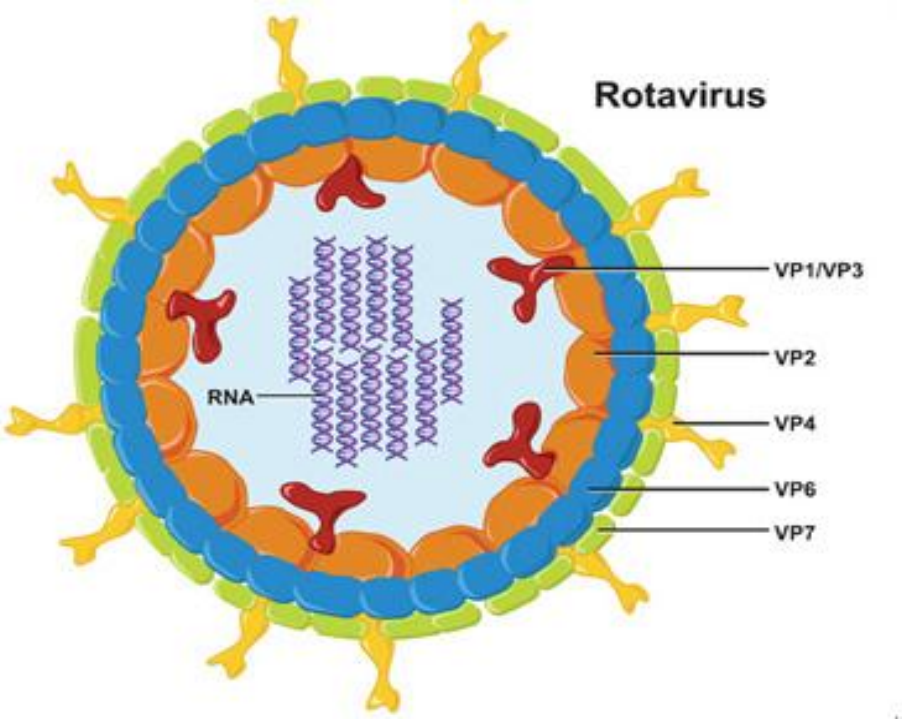

Figure 2.1: Rotavirus structure,

adapted from http://www.niaid.nih.gov (Accessed on January 17th 2015)

2.3 Rotavirus Genotypes

Two structural proteins form the outer shell, VP7 (a glycoprotein—G protein) and VP4 (a protease-cleaved protein—P protein), and are the prime antigenic targets for neutralizing antibody responses (Estes and Cohen, 1989). Because the two gene segments that encode VP4 and VP7 can segregate independently during genomic re-assortment, a typing system consisting of both P and G types has been developed.

Characterization of the P types by traditional methods of virus neutralization or EIAs with serotype-specific antibodies are difficult due to lack of serotype-specific non-cross-reactive antibodies. Therefore, molecular methods have been used to define the P and G genotypes based on PCR and sequencing. To date, 27 G –types and 35 P genotypes have been characterized in human rotaviruses (Matthijnnssens *et al.,* 2011). Gene re-assortment could theoretically lead to more than 100 different G and P protein combinations. However, only 5 strains are most commonly detected worldwide and include: P [8], P[4], G1, G2 , G3, G4, and G9 (Santos *et al.,* 2005).

In Africa, there is not only predominance of the G1 P[8], G1 P[4], G2 P[8], G3 P[8], G4, G9 genotypes but also appearance of novel strains such as G8P [6], G10 and G12P [6] (Armah *et al.,* 2003; Mwenda *et al.,* 2010). In Kenya and Tanzania, detection and characterization of P and G genotypes has been reported with G9, G1, G3, P [8], P [6], P [4] dominating (Sabrina *et al.,* 2007, Nyang'ao *et al.,* 2010).

A study previously conducted in Mukuru informal settlements reported the main genotypes as G1, G2, G3, G4 and G9 and P [8], P [4], P [6] and P [9] (Gikonyo *et al.,* 2010). This current study aimed to add a more recent picture of the data before vaccine and immediately after vaccine introduction as well as adding to the existing data on molecular typing of rotavirus in addition to finding out the vaccine coverage of children under 5years in Mukuru area in Nairobi.

2.4 Pathogenesis

The virus enters the body through the mouth. Viral replication occurs in the villous epithelium of the small intestine. Recent evidence indicates that up to two-thirds of children with severe rotavirus gastroenteritis show the presence of rotavirus antigen in serum (antigenemia). Infection may result in decreased intestinal absorption of sodium, glucose, and water, and decreased levels of intestinal lactase, alkaline phosphatase, and sucrose activity, and may lead to isotonic diarrhea (Ulrich and Hans, 2011).

The immune correlates of protection from rotavirus infections are poorly understood. Serum and mucosal antibodies against VP7 and VP4 are probably important for protection from disease. Cell-mediated immunity probably plays a role in recovery from infection and in protection. Recovery from a first rotavirus infection usually does not lead to permanent immunity. After a single natural infection, 38% of children are protected against any subsequent rotavirus infections, 77% are protected against rotavirus diarrhea, and 87% are protected against severe diarrhea. Reinfection can occur at any age. Subsequent infections confer progressively greater protection and are generally less severe than the first (Lintao *et al.,* 2013*).*

2.5 Clinical Features

The incubation period for rotavirus diarrhea is short, usually less than 48 hours. The clinical manifestations of infection vary and depend on whether it is the first infection or reinfection. The first infection after 3 months of age is generally the most severe.

Infection may be asymptomatic, may cause self-limited watery diarrhea, or may result in severe dehydrating diarrhea with fever and vomiting.

Up to one-third of infected children may have a temperature greater than 102°F (39°C). The gastrointestinal symptoms generally resolve in 3 to 7 days. The clinical features and stool characteristics of rotavirus diarrhea are nonspecific, and similar illness may be caused by other pathogens. As a result, confirmation of a diarrheal illness as rotavirus infections requires laboratory testing (Lintao *et al.,* 2013).

2.6 Rotavirus Vaccine (Rotarix®)

The Rotarix® vaccine works by provoking the body's immune response to the rotavirus organism, without actually causing illness. It is an oral vaccine against rotavirus infections which contains a live, weakened form of human rotavirus. The rotavirus causes inflammation of the linings of the stomach and intestine (gastroenteritis), resulting in sickness and diarrhea. In young children, this can lead to dehydration. When the body is exposed to foreign organisms, such as viruses and bacteria, the immune system produces antibodies against them. Antibodies help the body recognise and kill the foreign organisms. They then remain in the body to help protect the body against future infections with the same organism which is mostly referred to as active immunity. (Lintao *et al.,* 2013*).*

The immune system produces different antibodies for each foreign organism it encounters. This establishes a pool of antibodies that helps protect the body from various different diseases. These altered forms of the organisms stimulate the immune system to produce antibodies against them, but don't actually cause disease themselves. The antibodies produced remain in the body so that if the organism is encountered naturally, the immune system can recognise it and attack it, thus preventing it from causing disease.

Each virus stimulates the immune system to produce a specific type of antibody. This means that different vaccines are needed to prevent different diseases. Rotarix® vaccine stimulates the immune system to produce antibodies against rotavirus. It can be given to young children to prevent gastroenteritis caused by infection with this virus. Children who have had this vaccination are known to excrete the virus in their stools after the vaccination for at least 14 days, but especially around the seventh day. As the virus is a weakened form it shouldn't cause any problems to healthy people. In clinical trials, efficacy was demonstrated against gastro-enteritis due to rotavirus of most common genotypes G1 P[8], G2 P[4], G3 P[8], and G9 P[8] (Armah *et al*., 2003)

However, it could pose a risk to people with suppressed immune system, for example those taking high-dose steroids or having chemotherapy. People looking after a child who has recently been vaccinated should make sure they wash their hands after changing the child’s nappies to avoid contracting or spreading the virus.

A fully effective vaccine may not provide protection against rotavirus infections in all children who receive it (Lintao *et al.,* 2013*).*

2.7 Laboratory Diagnosis of Rotavirus Infections

2.7.1 Cell Culture

Successful propagation of rotaviruses has been achieved by use of Vero. Other culture methods employ requirement of calcium and 5% chicken serum to aid in efficient multiplication of the virus. Nevertheless, different rotavirus strains vary in their capacity to grow in cell cultures and growth of rotavirus from clinical faeces is very difficult and does not work for every sample (Weilin *et al.,* 2017).

Bacterial contamination can be prevented by centrifugation and use of antibiotics in the culture throughout during infection. Cyto-pathogenic effects may also not be visible following infection in African Green Monkey Kidney Cells (AGMK). In addition, the faeces may be cytotoxic and dilution required before infection. Rhesus Monkey Kidney (MA104) cells are commonly used for propagation and characterization of both animal and human rotaviruses. Despite this knowledge about factors necessary for virus propagation the rate of isolation of rotavirus in cell culture is very minimal hence this is not a suitable method for rotavirus detection.

2.7.2 Rotavirus Electron Microscopy

Electron microscopy was traditionally used for rotavirus detection. This technique is advantageous because of high speed, simplicity, high resolution and good preservation of three dimensional structures of virus particles.

However, this method is insensitive as it requires 10^8 particles concentration for virus detection (Iturriza *et al.,* 2008). In addition, this technique is not only labour intensive for detection of large numbers of stool specimens but the technique also requires highly trained personnel. This technique cannot distinguish between different groups of rotavirus. Moreover, electron microscope is a very expensive instrument to purchase (Cárcamo *et al.,* 2005).

2.7.3 Immunological Techniques for Rotavirus Infections Detection

Antigen detection methods like enzyme immunoassays (EIA) latex agglutination, lateral flow immunoassays and immunochromatography have been employed. They detect protein antigens on rotavirus particles in stool specimens. ELISA uses rotavirus antibodies to capture antigens onto wells of microtitre plates. A second rotavirus antibody coupled to an enzyme is widely used which absorbance is read using a colorimeter. ELISA is very sensitive, specific and is important in detecting large volumes of stool samples (World Health Organization, 2009). It is more preferably used because it is convenient, reliable and inexpensive.

The major disadvantage of this assay is the inability to be able to detect non-group A rotaviruses. Latex agglutination utilizing latex particles coated with anti-rotavirus antibodies can be used for rotavirus antigen detection. The Latex agglutination technique has lower sensitivity than EIA.

Immunochromatographic shows high sensitivity and results comparable to those achieved with EAI, and is rapid and technically very simple (World Health Organization, 2009). Immunochromatographic methods are being widely employed for rapid testing.

2.7.4 Molecular Identification of Rotavirus infections

Nucleic acid amplification by PCR of VP7 and VP4 rotavirus genes has been recently developed for detection of rotavirus infections. This method is 1000 times more sensitive than immunoassays in detection of rotavirus infections and other enteric viruses (Iturriza *et al.*, 2001). These two genes in rotavirus genome are very important not only for surveillance studies to determine circulating strains but also for vaccine development (Kapikian *et al.*, 1996). Real time PCR is useful for verifying that RNA extracts contain intact rotavirus RNA. This technique is very expensive, labour intensive and not suitable for routine rotavirus detection studies (World Health Organization, 2009)

2.8 Prevention and Treatment

Rotavirus infections can be treated by drinking boiled water, washing hands before handling the baby food, drinks and improved sanitation. Preventing dehydration is the most important step in treatment of viral gastroenteritis. Patients are advised on high liquid intake in order to correct the water deficit due to vomiting and diarrhea.

This can also be achieved through administering oral rehydration therapy. Administrations of zinc tablets which are commercially manufactured are further recommendations of rotavirus infections treatment that has proved to reduce high rates of mortality. However, adults can be encouraged to drink fluids to curb down dehydration. (Kapikian *et al.*, 1996)

2.9 Introduction of Rotavirus Vaccines in Different Countries

The World Health Organization (WHO) recommends that rotavirus vaccines be introduced into every country's national immunization program, particularly in countries where diarrheal disease is a major health problem. As of 1st May 2016, 81 countries worldwide had introduced rotavirus vaccines in their national immunization programs (i.e., public sector). Other countries, such as Canada, India, Italy, the Philippines, Sweden, and Thailand, have introduced rotavirus vaccines in phased or regional introductions. Rotavirus vaccines are also available in more than 100 countries through the private market **(Figure 2.2).** (www.*path.org/rotavirusvaccine/country-introduction)*

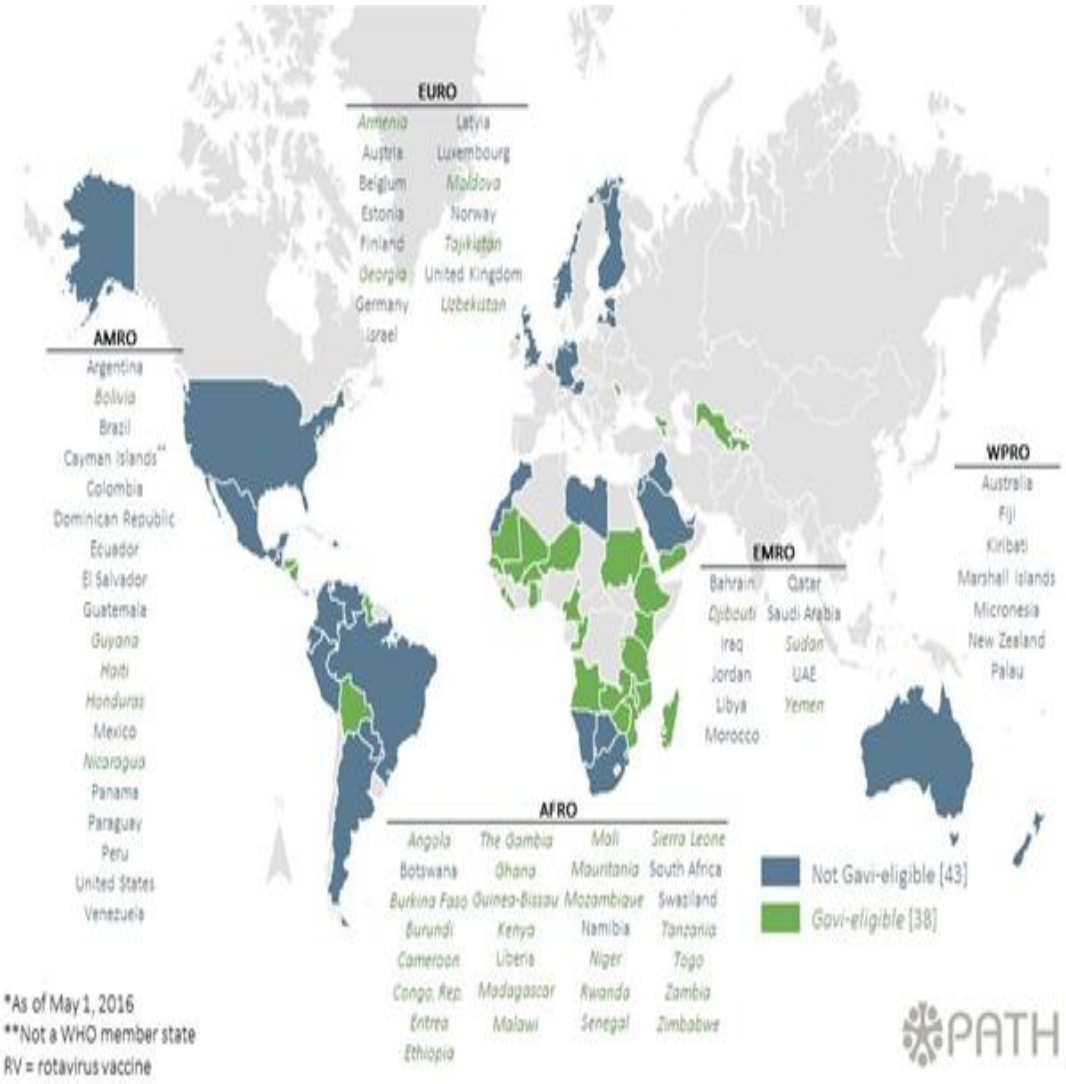

Figure 2.2: Countries that have introduced rotavirus vaccine globally

adapted from *http://www. sites.path.org/rotavirusvaccine/country-introduction-maps-and-spreadsheet/Accessed on 1st August 2016*

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

The study was carried out using samples collected from children with diarrhea seeking treatment at Medical Missionaries of Mary (MMM) and Reuben Medical Center in Mukuru urban informal settlement situated 15 km East of Nairobi city Centre **(Figure 3.1).** This is an urban informal settlement, overcrowded with closely clustered houses, garbage dumps, open drains and inadequate sanitation with only a few residents own homes.

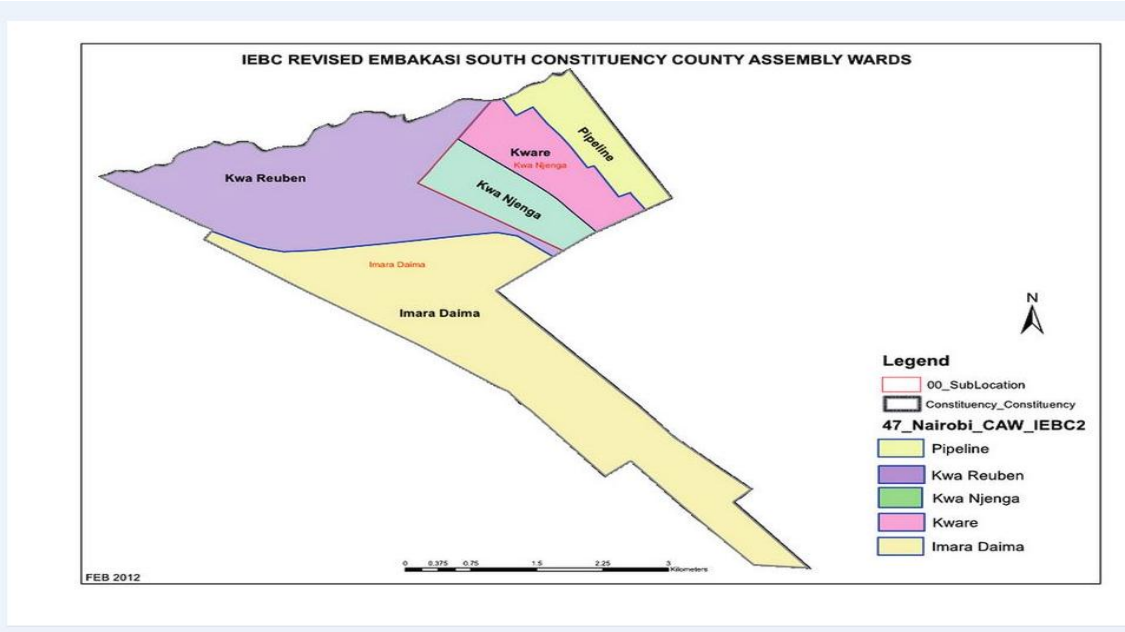

Figure 3.1: Mukuru map. Adapted from ***http://www.IEBC.org/Accessed on 1July 2016***

3.2 Study Population

The area's total population is about 89,258, children who are under 1year (12months) are 2,678, children less than 5 years (60 months) are 11,336 and children under 15yrs are 27,045 (Gikonyo *et al.*, 2010)

3.3 Study Design

The study was descriptive study, archived stool samples from *Salmonella* surveillance study (SSC No.2074) were used. A total of 270 stool samples were randomly selected from 3995 children that had been recruited between July 2013-July 2015. Additional demographic data including age, sex and clinical characteristics were also gathered from case report form.

3.4 Sampling

3.4.1 Diarrhea Case Definition:

Diarrhea was defined as three or more loose or watery stool in the last 24hrs, the WHO recommendation of rotavirus infections case definition (WHO, 2002).

3.4.2 Inclusion Criteria

Archived samples from *Salmonela* study from children aged below 5 years who had presented with diarrhea not exceeding 24 hrs. Children, who visited the clinics, recorded in Child Welfare Chat (CWC) and had complete records of age, sex, date of 1st dose and date of 2nd dose.

3.4.3 Exclusion Criteria

Children who visited the clinics, recorded in Child Welfare Chat (CWC) and had incomplete records of either the age, sex, date of 1st dose and date of 2nd dose.

3.4.4 Sample Size Determination

Rotavirus infections caused gastroenteritis in Kenya has got an estimated prevalence of 22% in children (Nakata *et al.*, 1999). At 95% confidence interval, a minimum estimated sample size of 264 samples was used for this study. The size was obtained at per the statistical formula (Naing *et al*., 2006).

$$n = \frac{Z^2 P (1-P)}{d^2}$$

Where:

n=sample size

Z= Z statistic, 1.96 for 95% confidence level

P= expected prevalence or proportion 22%>0.22

d= precision (in proportion of one 5% >0.05)

$$n = \frac{(1.96 \times 1.96)\ 0.22(1-0.22)}{(0.05 \times 0.05)} = = 263 \text{ samples}$$

The estimated sample size was 264 samples for the year before vaccine and after vaccine. The study sample size was increased to 270 to cater for insufficient samples. 150 stool samples before vaccine and 120 samples after vaccine. For the vaccine coverage, all the children under 1 year who had been vaccinated with one or two doses were included.

3.4.5 Sampling Frame

Stool samples were from ongoing Salmonella surveillance study (SSC No 2074) previously aliquoted and stored at -20^{o}C freezers at Centre for Microbiology Research (CMR) Laboratories, KEMRI. For the purpose of this study stored samples were retrospectively retrieved, aliquoted into 1.8ml cryogenic vial tubes. Pre-vaccine period samples were collected from July 2013 to July 2014, while post vaccine samples were collected from July 2014 to July 2015.

For vaccine uptake, only children who were below one year were vaccinated for rotavirus which was given at 6th week and 10th week were included in the study. Children who visited the clinics were recorded and given unique numbers and all the vaccine details from the mother's immunization book were recorded in Child Welfare Chat (CWC) book capturing the age, sex, date of 1st dose and date of 2nd dose. This data was retrieved retrospectively from July 2014 to July 2015 in the health records to determine the estimates of children vaccinated.

3.4.6 Sampling Flow Chart

Purposive sampling technique was used **(Figure3.2)** to select samples from the sampling frame of archived samples from children who presented with diarrhea. They were screened for rotavirus infections using ELISA and the positives samples were sequenced. Sampling technique for the vaccine coverage included all children under one year, who had attended the clinics and had either rotavirus vaccine one dose or two doses given.

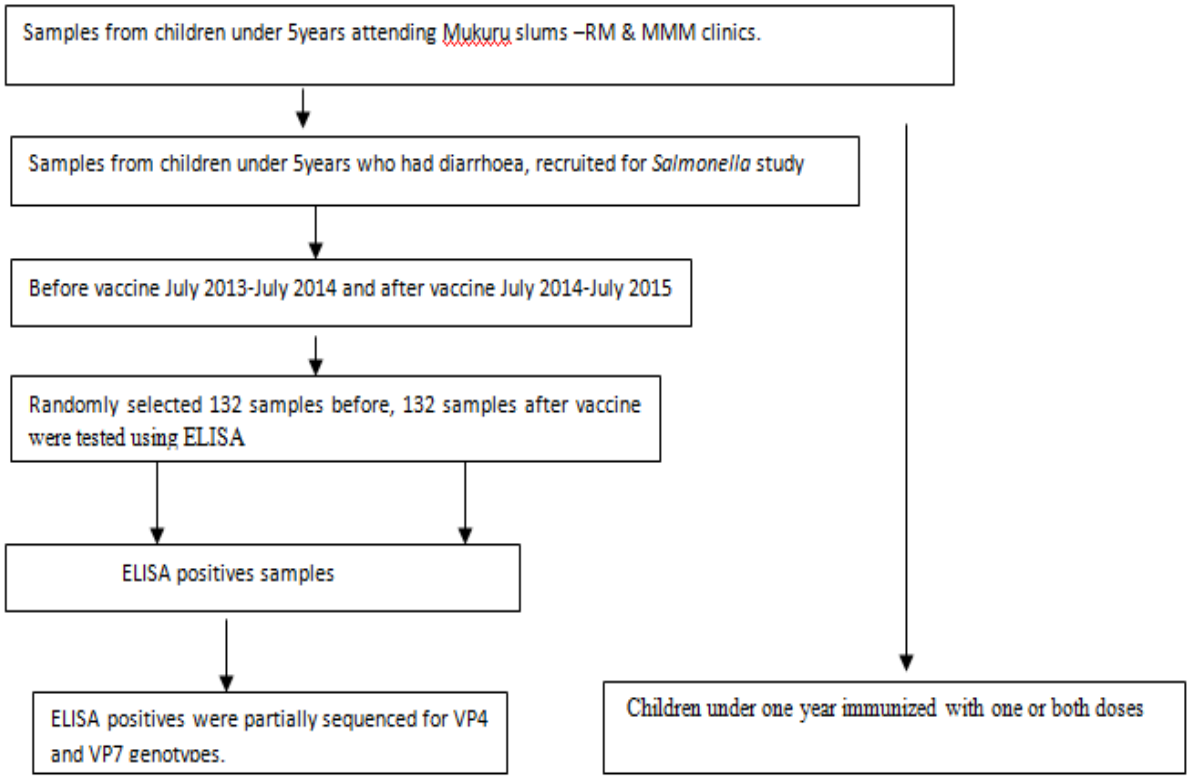

Figure 3.2: Showing the sampling flow chart

3.5 Experimental Procedures

3.5.1 ELISA using ProspectTM Rotavirus kit

Principle: The prospect rotavirus test is a qualitative enzyme immunoassay for detection of rotavirus infections (Group A) in human faecal samples. It utilizes a polyclonal antibody in a solid phase sandwich enzyme immunoassay to detect group specific antigen present in Group A rotavirus infections.

3.5.1.1 Preparation of Feacal Specimens

Sample diluents (Tris Buffered Saline solution) of 1ml was added to suitably labeled tubes of each sample. Approximately 0.1g of solid faeces (small pea-sized portion) or approximately 100ul of liquid stool sample faeces was added and mixed thoroughly. A 10% suspension dilution of faecal specimen was then made from this solution. The sample diluent and the sample were thoroughly mixed and left to settle for 10 minutes prior to testing.

3.5.1.2 Quality Control

At least one PROSPECT^{TM} Rotavirus negative control and one positive control was included with each batch of specimen. Samples were then added in 100 µl in each well. Conjugate (rotavirus specific rabbit polyclonal antibody conjugated to horseradish peroxidase in a buffered protein solution) was added 100µl in each well and mixed gently. Micro-wells with samples were then incubated at 20-30^{o}C for 60+/- 5minutes.

The micro wells were then washed using freshly prepared working strength X1 PBS wash buffer. Two drops (100µl) of the substrate was added to each microcell and incubated at 20-30^{o}C for 10 minutes, 100µl of the stop solution (0.46 mol/L sulphuric solution) was then added to each micro-well. Thorough mixing of the micro wells was done before reading the results.

3.5.1.3 Photometric Reading

The micro-wells were read photometrically using spectrophotometer set at 450nm within 30 minutes after addition of the stop solution. The negative control value was set at less than 0.150 absorbance units and positive control value was greater than 0.500 absorbance units **(Appendix 1).**

3.5.1.4 Calculation of the Cut-off value

The cut-off value was calculated by adding 0.200 absorbance units to the Negative control value. Clinical specimens having absorbance values greater than the cut-off value were positive. Any specimen with absorbance value less than the Cut-off was negative. A result within 0.010 absorbance units of the cut-off value was interpreted as equivocal, and the test repeated.

3.5.2 Characterization of Rotavirus Genotypes using Molecular Techniques

The glycoprotein VP4 and the protease sensitive VP7 which are the structural proteins on the outer capsid, define the virus G and P genotypes respectively. The nucleotide sequences of the VP7 and VP4 encoding segments form the basis of a dual classification

system that defines the G and P genotypes of rotavirus. They remain the target portion when performing PCR and sequencing with general primers designed with conserved regions for each gene but franking the variable region that would change for each new genotype **(Table 3.1., Table 3.2).**

3.5.2.1 RNA Extraction

Precautions: Great care was taken to avoid inadvertently introducing RNases, vinyl gloves were worn when handling samples to prevent contamination from surfaces of the skin. Frequent change of gloves was observed and tubes closed to avoid cross over contamination. The glass wares, surfaces and plastic wares were treated with DNA-OFF™ to remove RNases. To avoid degradation of RNA by endogenous RNases the procedure was performed quickly and the viral RNA stored at -70^{0}C.

RNA was extracted using the Qiamp RNA kit as per the manufacturer's instruction (Qiagen, Hilden Germany), (Shulman *et al*., 2011). A hundred and forty µl of diluted sample leftover from the ELISA test was added to 560µl of the AVL/carrier RNA buffer and mixed thoroughly, then briefly centrifuged for 30 seconds at 8000rpm. This was incubated for 10 min at room temperature. Five hundred and sixty µl of absolute ethanol was added, mixed thoroughly then briefly centrifuged. Six hundred and thirty µl of the sample was then transferred to the QIAMP spin column and centrifuged at 8000rpm for 1 min. The 2ml column was then transferred to a new collection tube and the above step repeated. The samples were washed with 500µl of wash buffer 1 and 2 respectively. RNA was eluted with 60µl of the elution buffer and stored at -70^{0}C.

3.4.2.1 One-step PCR

Table 3.1: Preparation of Master Mix in PCR

QIAGEN one-step kit	Reagent	Per reaction
	Rnase free Water	11.8µl
	dNTPs(10mM)	1µl
	X5 buffer	5µl
	Primers (F&R10 µ mol)	0.5ul
	RNase out	0.5µl
	Enzyme mix	1µl
	Total	20µ l total master mix
	Add 5µl of RNA(template)	

Table 3.2: Primers references and cycling parameters

Primers	Sequences (5'-3')	Cycling Parameters	Amplicon Size
VP7-F	ATGTATGGTATTG AATATACCAC Ituriza *et al*., (2001)	cDNA at 50^0C for 30 min and denaturation at 95^0C for 15 min followed by 40 cycles of 95 ^{0}C for 1min,52^0C for 1 min and 72^0C for 1 min with the final extension step at 72^0C for 7 min.	881 bp
VP7-R	AACTTGCCACCAT TTTTTCC Ituriza et al., (2001)		
VP4-F	TATGCTCCAGTNA ATTGG Simmonds *et al*., (2008)	cDNA at 50^0C for 30 min and denaturation at 95^0C for 15 min followed by 40 cycles of 95^0C for 1min,50^0C for 1 min and 72^0C for 1 min with the final extension step at 72^0C for 7 min.	663 bp
VP4-R	ATTGCATTTCTTTC CATAATG Simmonds *et al*., (2008)		

3.5.2.3 Purifications of PCR Products

This was done using Qiagen purification kit Protocol as described by (Distefano *et al.*, 2005). A hundred µl of buffer PB was added to 20µl of PCR sample mix in ach well. QIAquick spin column was placed into 2ml collections tube. DNA sample binded to the QIAquick column after centrifugation 30-60sec, flow-through was discarded and returned in same collection tube. To wash, 0.75ml buffer PE was added and centrifuged 30-60sec. Flow-through was discarded. The sample was then dry centrifuged and then placed into 1.5ml micro centrifuge tube and 30µl eluted with EB buffer.

3.5.2.4 Cycle Sequencing

This was done using ABI PRISM Big Dye Terminator Cycle Sequencing ready reaction kit **(Table 3.3)** using same primers as first round PCR products as described by Banerjee *et al*., 2007.

Table 3.3: Preparation of master mix for cycle sequencing

	Reagent		Per reaction
	Big dye terminator mix		0.5µl
	X5 buffer		1.75µl
	Primers 10Mmol	VP4 Forward	1µl
		VP4 Reverse	
		VP7 Forward	
		VP4 Reverse	
	DNase free water		4.75µl
	Purified PCR products		2µl
ABI Big dye kit	Total reaction		10µl

Cycle sequencing cycling parameters were 25 cycles of 95^0C for 0.10sec, 50^0C for 0.05 sec and 60^0C for 4 min and 72^0C for 1 min with the final hold at 4^0C for 10min.

3.5.2.5 Sequence Precipitation

The cycle sequenced products were spun briefly into each well, the following were added: 1ul of 125mm EDTA, 1µl of 3M sodium acetate and 25µl of 100% ethanol. The plate was sealed, vortexed four times and incubated at room temperature for 15 minutes. The plates were then centrifuged at maximum speed (3600-4000) at -20^oC for 20 minutes. Gently inverted on paper towel and spun at X400 for one minute. One hundred µl of 70% ethanol was then added to each well and the mixture centrifuged at maximum speed for 5 minutes.

The plates were gently inverted on paper towel, spun X400 for a minute and left to dry at the bench for 30 minutes. Ten ul of Hi-Di Formamide was added and the product denatured at 95^0C for three minutes. Plates were stored at -4^0C. Denatured plates (ready to read plates) were then tightly packed in a box with 4^0C icepacks and transported to International Livestock Research Institute (ILRI) for reading using an automated DNA sequencer (Applied bio systems) ABI 3730.

3.6 Proportions of Children Vaccinated at Mukuru Informal Settlements Clinics

For vaccine uptake, only children who were below one year were vaccinated for rotavirus which was given at 6^{th} week and 10^{th} week. Children who visited the MMM clinics were recorded and given unique numbers. The vaccine details from the mothers immunization book were also recorded in Child Welfare Chat (CWC) book capturing the age, sex, date of 1^{st} dose and date of 2^{nd} dose. This data was retrieved retrospectively from July 2014 to July 2015 to determine the proportions of the children vaccinated, their age and gender.

3.7 Ethical Consideration

Ethical approval for the study was obtained from the Scientific Ethical and Review Unit (KEMRI/SERU/CMR/P0026/3144) and Pwani University (ERC/MSc/043/2015) as the institution authority collaborating with JomoKenyatta University Mombasa Campus **(Appendix 2 and 3).**

No new consent was required from parents/guardians for their children. The participant identities on samples were already coded differently from those given by the health facilities and their names had not been recorded.

3.8 Shipment and Archiving of Materials

An aliquot of neat stool samples stored at -20^{o}C at KEMRI Center for Microbiology laboratories was used for this study. Aliquots of the samples were transported to KEMRI-Welcome trust laboratories in cold (-4^{0}C) for lab analysis. This included ELISA, RNA extraction, PCR, gel electrophoresis, purification, cycle sequencing and precipitation.

3.9 Biosafety

The biosafety rules and regulations were followed while working with stool samples by using the proper protective equipment. Laboratory benches surfaces were cleaned using 70% alcohol, nitrile gloves were used and changed often, laboratory coats and face masks were used to prevent splashes. Used pipette tips and tubes were discarded in 10% jik before disposal and proper disposal of samples was done per KEMRI Bio-safety and Bio-security review Vol1 issue 3, 2016.

3.10 Data processing:

3.10.1 Data Collection Procedure

All the 270 samples were blinded and no personally identifiable information was present on the same labels. The laboratory results (ELISA ODs and genotypes) were entered into excel spread sheet and protected using passwords known to the principal investigator only. Vaccine data was retrieved from hospital records and entered in excels sheets.

3.10.2 Data Analysis

Data analysis was carried out using the SPSS version 20. Prevalence of rotavirus infections, age, gender and vaccine uptake were presented as proportions. Chi square tests were used to compare proportions of rotavirus infections before and after vaccine and to determine significance at 95% CI and $P<0.05$.

Sequence assembly was done using Sequencer software, aligned using MAFFT and Phylogenetic trees constructed using MEGA version 5.2. Assembled sequences were then compared using a web based tool (NCBI blast) to determine the genotype. Genotype distribution was compared with global sequences from GeneBank using phylogenetic trees.

CHAPTER FOUR

RESULTS

4.1 Demographic Distribution of the Study Population

4.1.1 Gender Distribution

In the study period from July 2013-July 2015, 270 samples (150 before vaccine and 120 after vaccine) were used and demographic data regarding age and gender was recorded. Out of the 150 children sampled for the period before vaccine, there was equal distribution of sample size 75 (50%) of each gender. For the period after vaccine introduction 56 (46.7%) were female while 64 (53.3%) were male. There was no significant difference ($P>0.05$; $\chi^2=0.533$; $df=1$) between number of males and females in the sample **(Table 4.1).**

Table 4.1: Distribution of study participants with regard to gender pre-and post-vaccine introduction in Mukuru Informal settlement.

Gender	Female		Male		Total				
	n	%	n	%	n	%	Chi square	df	P-value
Pre-vaccine	75	50	75	50	150	55.6	0	1	1
Post-vaccine	56	46.7	64	53.3	120	44.4	0.533	1	0.465
Total	131	100	139	100	270	100			

4.1.2 Age distribution of study participants in pre and post vaccine in Mukuru area

Age wise distribution of study participants showed significant difference (P=0.001; χ^2=23.667; df=4) in sample distribution among the age categories for pre-vaccine period **(Table 4. 2).** With the 13-24 months age category having the highest proportion 42/150 (28%), compared to other age categories. However, the sample for post vaccine were equally distributed (P>0.05; χ^2=6.583; df=4) in the different age categories.

Table 4.2: Distribution of study participants based on age pre-and post-vaccine introduction in Mukuru Informal settlement

Age range (months)	<12		13-24		25-36		37-48		>49		Total			
	n	%	n	%	n	%	n	%	n	%	n	Chi square	df	P-value
Pre-vaccine	36	24	42	28	39	26	23	15.3	10	6.7	150	23.667	4	0.001
Post-vaccine	18	15	18	15	33	27.5	26	21.7	25	20.8	120	6.583	4	0.160
Total	54	20	60	22.2	72	26.7	49	18.1	35	13	270			

4.2 Prevalence of Rotavirus Infections by Age

Rotavirus infections was seen to be more prevalent before vaccine introduction in children <12 months 6/36 (16.7%) (P<0.05; χ^2=6; df=1) and 13-24 months 4/42 (9.5%) (P<0.05; χ^2=9.6; df=1) **(Table4.3).** This difference in prevalence was seen to be statistically significant.

In children of 25-36 months and 37- 48 months of age, however, there was no difference in prevalence of rotavirus pre and post vaccine introduction [(P>0.05; χ^2=0.5; df=1) and (P>0.05; χ^2=0.184; df=1) respectively.

Table 4.3: Distribution of rotavirus infections pre-and post-vaccine introduction in Mukuru Informal settlement by age

Vaccine	Pre-vaccine			Post vaccine					
	Negative	Positive	Total	Negative	Positive	Total			
	n %	n %	n %	n %	n %	n %	chi-square	df	P-value
Age(Months) <12	30(83.3)	6 (16.7)	36(100)	18(100)	0(0)	18(100)	6	1	0.014
13-24	38(90.5)	4(9.5)	42(100)	16(88.9)	2(11.1)	18(100)	9.6	1	0.002
25-36	35(89.7)	4(10.3)	39(100)	31(93.9)	2(6.1)	33(100)	0.5	1	0.485
37-48	22(95.7)	1(4.3)	23(100)	25(96.2)	1(3.8)	26(100)	0.184	1	0.668
>49	10(100)	0.0(100)	10(100)	24(96)	1(4)	25(100)	6.429	1	0.011
Total	135/150	15/150	150/150	114/120	6/120	120/120			

4.3 Prevalence of Rotavirus Infections Based by Gender

Rotavirus infections was detected higher in female 8/75 (10.7%) and compared to males 7/75 (9.3%) for the period before vaccine **(Table4.4).** For the period after vaccine introduction cases in male were higher 4/64 (6.3%) compared to female 2/56 (3.6%). These differences between the males and females pre-and post-vaccine was however not statistically significant.

Table 4.4: Distribution of rotavirus infections by gender in Mukuru Informal settlement pre-and post vaccine introduction.

Vaccine	Pre-vaccine			Post vaccine					
	Negative	Positive	Total	Negative	Positive	Total			
	n %	n %	n %	n %	n %	n %	chi-square	df	P-value
Female	67(89.3)	8 (10.7)	75(100)	54(96.4)	2(3.6)	56(100)	2.756	1	0.097
Male	68(90.7)	7(9.3)	75(100)	60(93.8)	4(6.3)	64(100)	0.871	1	0.351

4.4 Prevalence of Rotavirus Infections by Vaccine Status

A total of 15/150 (15%) and 6 /120 (5%) cases tested positive for rotavirus infections pre-and post-vaccine introduction periods respectively. The figure 4.1 shows the overall prevalence between pre-and post-vaccine. Pre-vaccine rotavirus prevalence was higher compared to post vaccine, the inferential statistical outcomes were not significant (χ=2.411, df=1 p>0.05) .

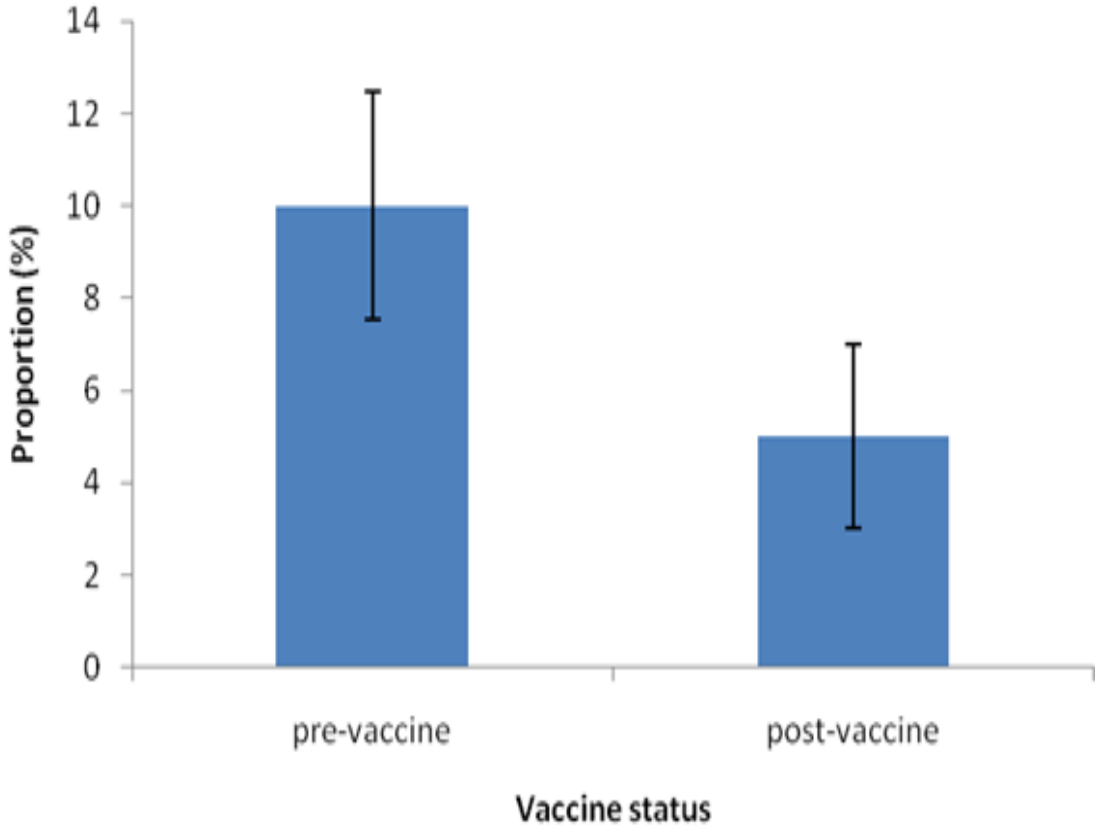

Figure 4.1: Prevalence of rotavirus infections by vaccine group (pre-vaccine and post vaccine) in children under five years in Mukuru informal settlement

4.5 PCR Gel Electrophoresis Images

Plate 4.1 below shows the gel electrophoresis image of PCR products for VP4 rotavirus genotypes. A clear band of 660 base pairs, without smear was considered amplified DNA good to be sequenced.

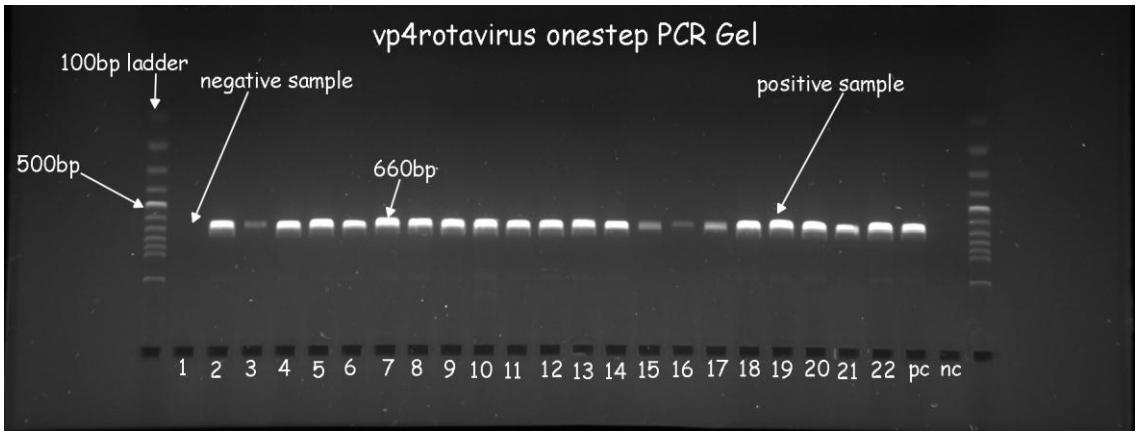

Plate 4.1: Gel electrophoresis photo showing rotavirus genotypes VP4 from the study sample captured using Bio-Rad Gel doc machine.

The PCR products of 830 base pairs of amplified VP7 gene are shown in plate 4.2 below. Products that had clear bands were sequenced.

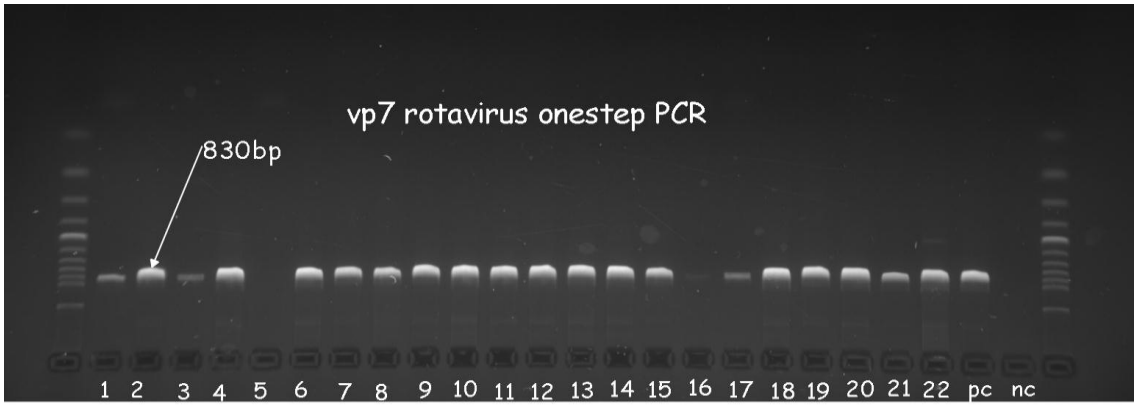

Plate 4.2: Gel electrophoresis photo showing rotavirus genotypes VP7 from the study samples, captured using Bio-Rad Gel doc machine.

4.6 Rotavirus G and P Genotype Combinations

This study detected six G strains G1, G9, G8, G3, G4 and G2 and two P strains P [4] and P[8]. G1P [8] combination was the major genotype combination detected in 53.33% and 37.5% of the sequenced samples before vaccine and after vaccine respectively. Other minor genotypes combination reported circulating in Mukuru were G1P [4], G2P[4], G3P[4], G4P[4], G8P[4] and G9P[8]. These genotypes are as shown in tables 4.5, 4.6 and 4.7 below.

Table 4.5: Genotypes circulating in Mukuru area and the combined genotypes over the entire study period (2013-2015)

	G strains							P strains		
	G1	G9	G8	G3	G4	G2	Total	P[4]	P[8]	Total
n	10	5	4	2	1	1	23	9	16	25
%	43.47	21.73	17.39	8.67	4.34	4. 34	100	36	64	100

Table 4.6: Genotype combinations before and after vaccine

	Genotype combinations distribution							
	G1P [4]	G1P [8]	G2P[4]	G3P[4]	G4P[4]	G8P[4]	G9P[8]	Total
Pre-vaccine(n)	1	8	0	0	0	2	4	15
%	6.67	53.33	0	0	0	13.33	26.67	100
Post vaccine(n)	0	3	1	1	1	2	0	8
%	0	37.5	12.5	12.5	12.5	25	0	100

Table 4.7: Genotype combinations vaccine and non-vaccine type's circulating in Mukuru population after vaccine introduction.

Genotype combination distribution in Mukuru post vaccine period					
Vaccine types	**N**	**%**	**Non–vaccine types**	**N**	**%**
G1P[8]	**(3)**	**37.5**	**G2P[4]**	**(1)**	**12.5**
			G3P[4]	**(1)**	**12.5**
			G4P[4]	**(1)**	**12.5**
			G8P[4]	**(2)**	**25**

4.7 Rotavirus nucleotide sequence of partial P genotype (VP4) phylogenetic tree

4.7.1 VP4 sequences from Mukuru informal settlement

Graphic representation of the phylogenetic analysis of partial VP4 sequences **(Figure 4.2).** The analysis indicated there was no clear separation from the tree between pre-and post-vaccine sequences. However, we observed small clusters that were majority comprised of either pre-or post and one or two taxa from the alternative. Most of pre vaccine sequences were P[8] 16/25 (64%) which were the vaccine target, while the most of the post vaccine sequences were P[4] 9/25 (36%).

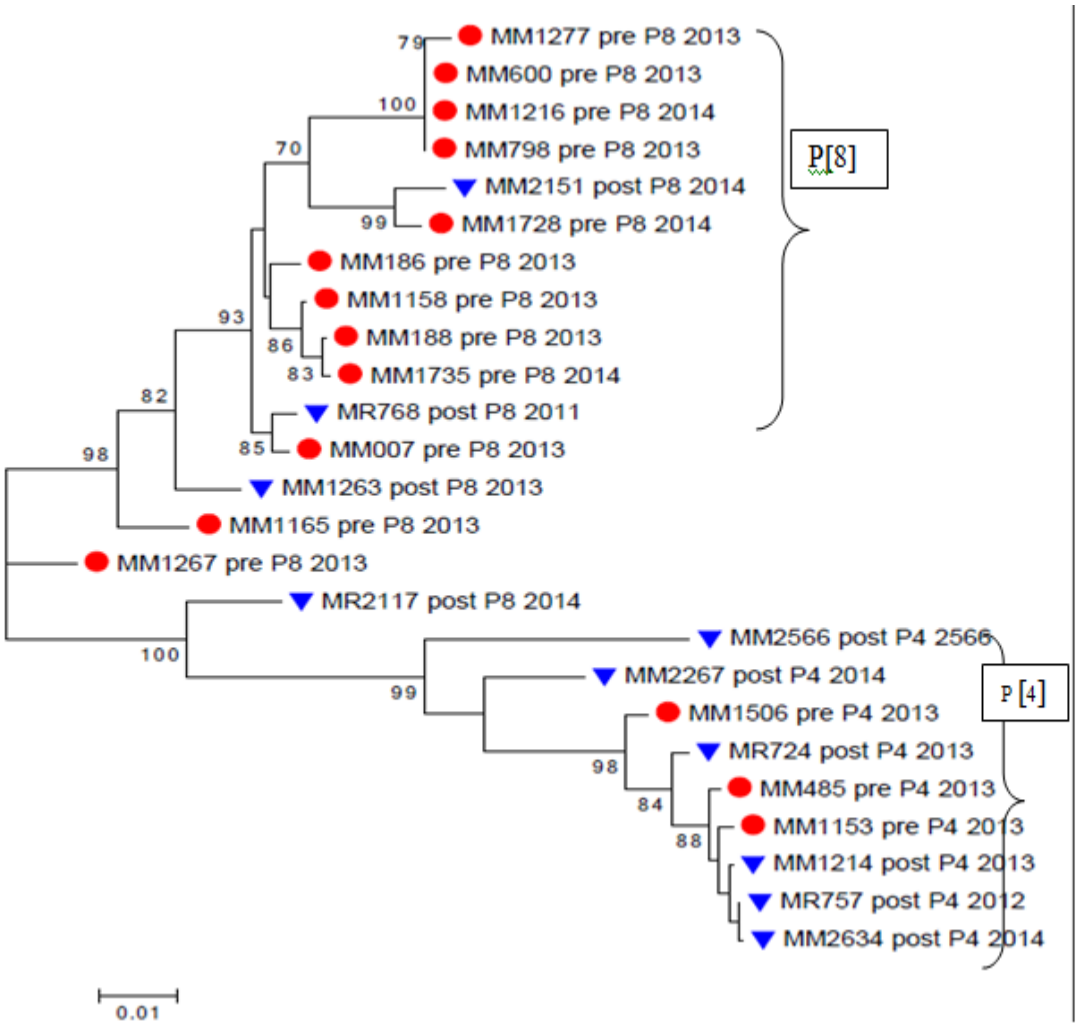

Figure 4.2: Neighbor joining phylogenetic tree of the rotavirus sequences VP4 genotypes detected from Mukuru, Kenya

The blue color triangles represent Mukuru post vaccine VP4 sequences while red colour circles represent VP4 post sequences.

4.7.1 VP4 sequences from Mukuru informal settlement combined with GenBank sequences

VP4 Sequences from Mukuru were combined with sequences at GenBank from other regions **(Figure 4.3).** Of note is that the Mukuru (Kenya) sequences clustered closely with other Kenyans sequences. In one of the clusters a Tanzania sequence clustered within Kenyan sequences. The global sequences clustered separately from Kenyan sequences. Sequences from same country clustered closely indicating strong geographical clustering of rotavirus sequences.

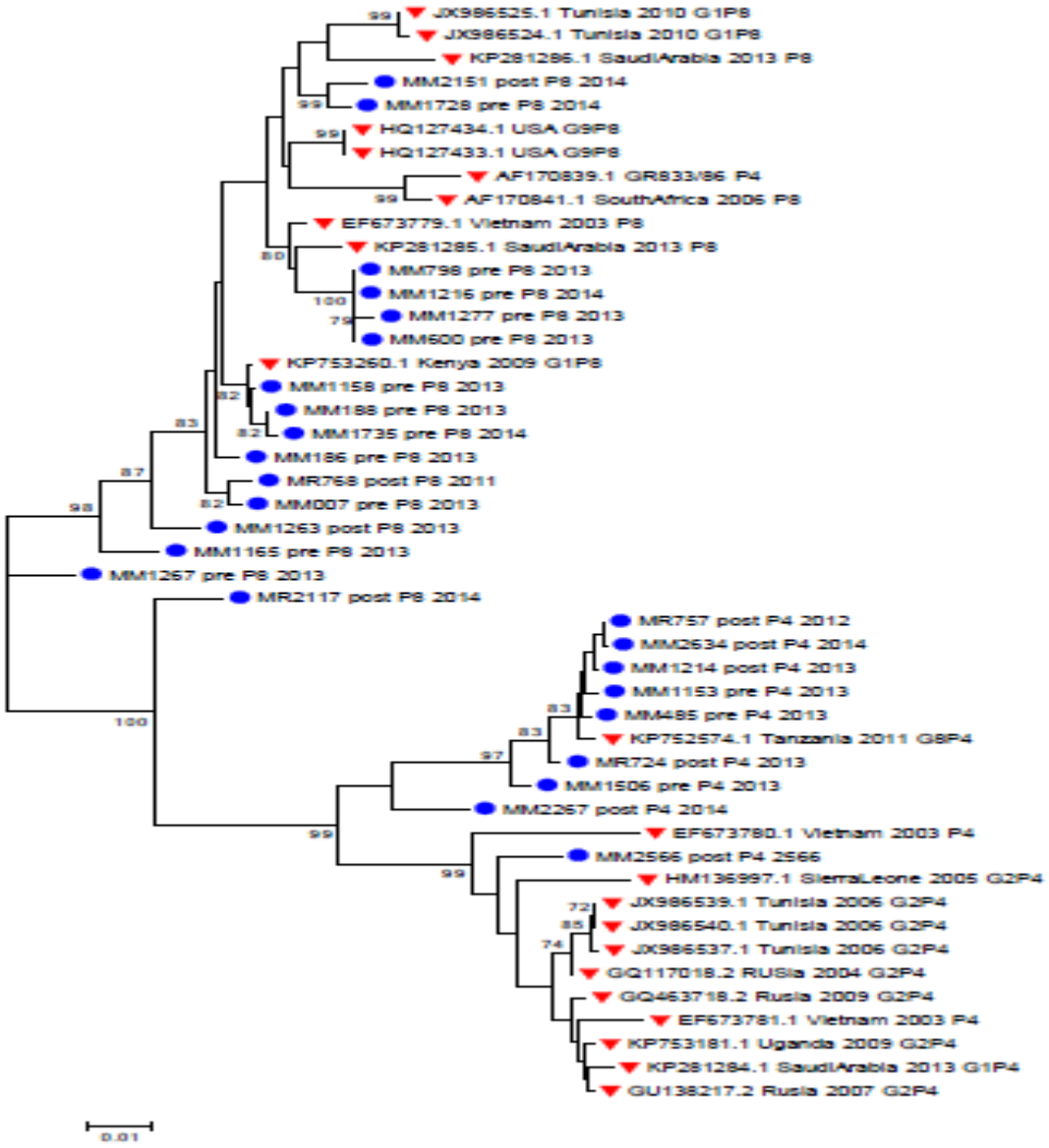

Figure 4.3: Global sequences from GenBank combined with Mukuru genotypes sequences. The blue color triangles represent Mukuru while red color circles represent global.

4.8 Rotavirus nucleotide sequence of partial G genotype (VP7) phylogenetic tree

4.8.1 VP7 sequences from Mukuru informal settlement

Graphic representation of the phylogenetic analysis of partial VP7 sequences **(Figure 4.4)**. The analysis showed three main clusters circulating in Mukuru G1, G8 and G9 which majority were from pre-vaccine period. New genotypes were observed post vaccine including G2, G3 and G4.

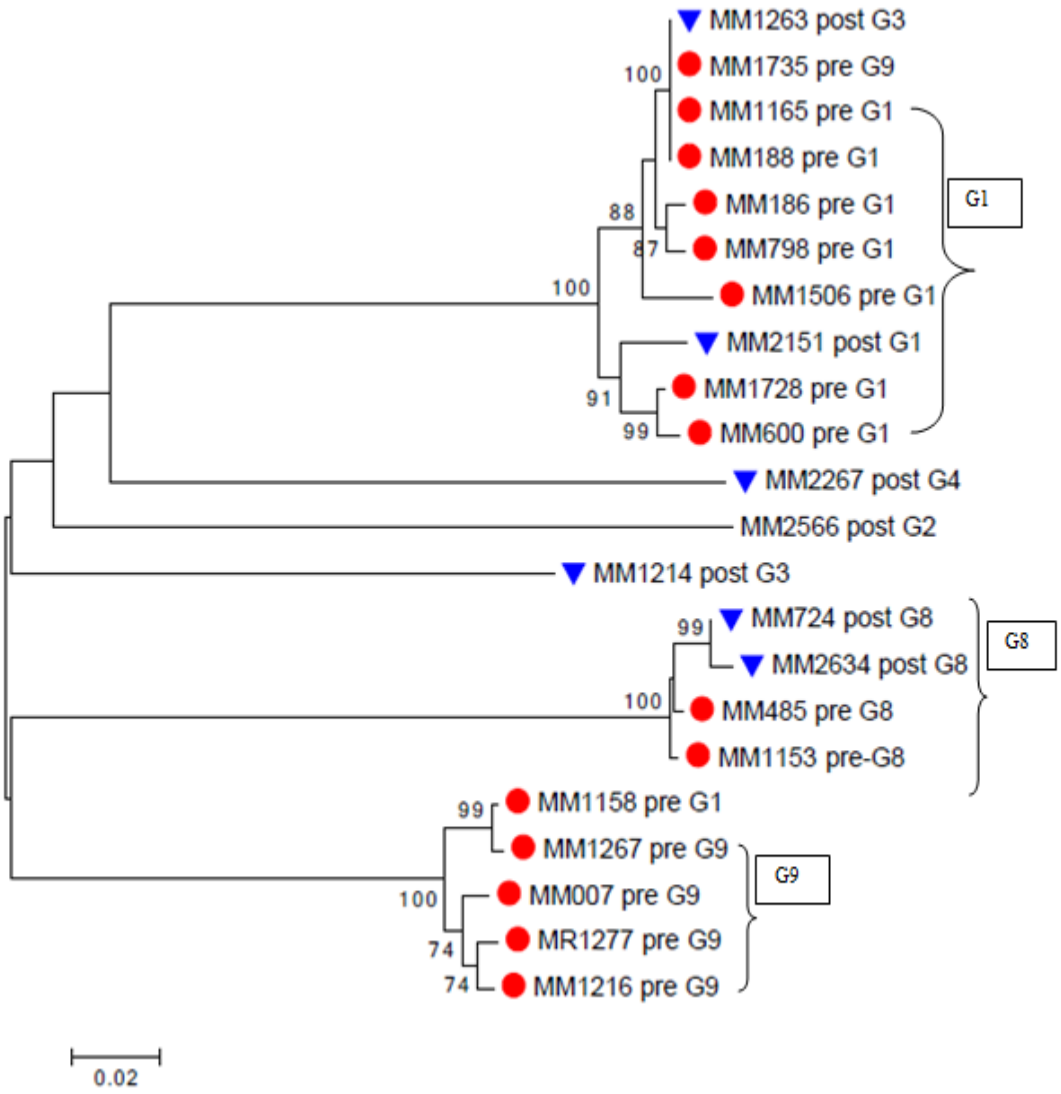

Figure 4.4: Neighbour joining phylogenetic tree of the rotavirus sequences VP7 genotypes detected from Mukuru, Kenya.

The green color triangles represent Mukuru post vaccine VP7 sequences while red colour circles represent VP7 pre-vaccine sequences.

4.8.2 VP7 sequences from Mukuru informal settlement combined with GenBank sequences

VP7 Sequences from Mukuru were combined with sequences at GenBank from other countries. Of note is that the Mukuru genotypes in circulation did not cluster with any published strains **(Figure 4.5).**

Figure 4.5: Global sequences from GenBank combined with Mukuru genotypes sequences. The blue color circles represent Mukuru while red color triangles represent global.

4.9 Vaccine Uptake by Dose

There was a distinct variation of children who received one dose and the one who received two doses. Overall, more than 80% of the children received two doses of the vaccine while 19.75% received one dose. This high uptake demonstrates good distribution and access to the rotavirus vaccine in this urban informal settlement.

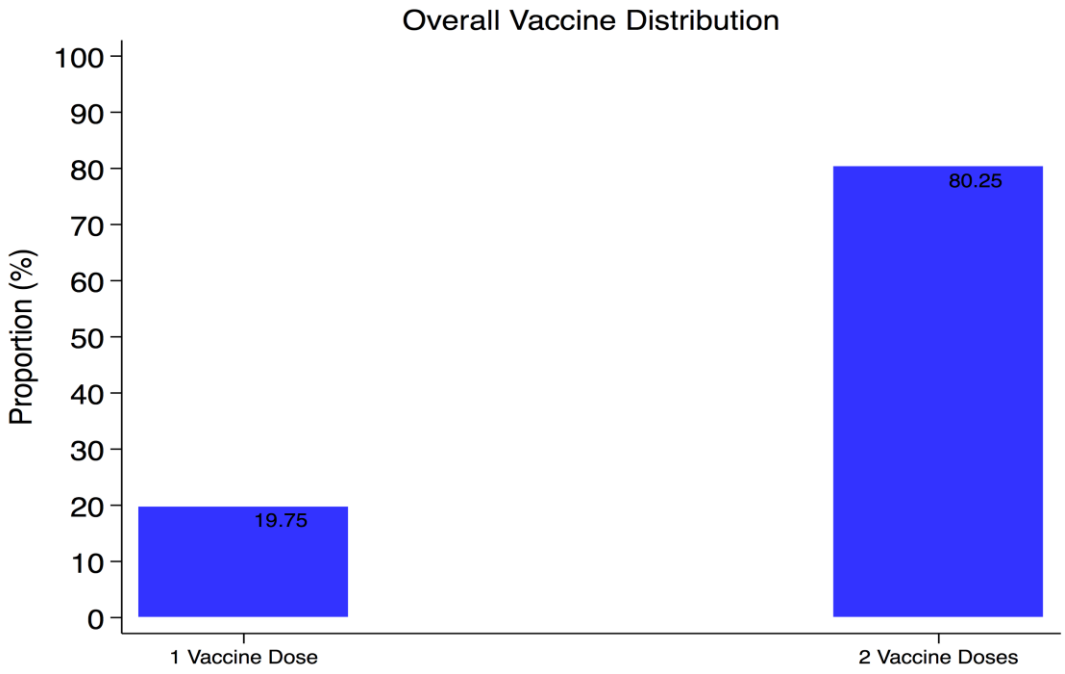

Figure 4.6: Proportion of children below 1year vaccinated with Rotarix® vaccination in Mukuru kwa Njenga health center Nairobi county, July 2014-July 2015.

4.10 Vaccine uptake by Gender

Proportion of children below 1year vaccinated with Rotarix® vaccine by dose and gender is as shown in **(Figure 4.7).** The vaccine distribution for each of two doses was observed to be similar among both males and females.

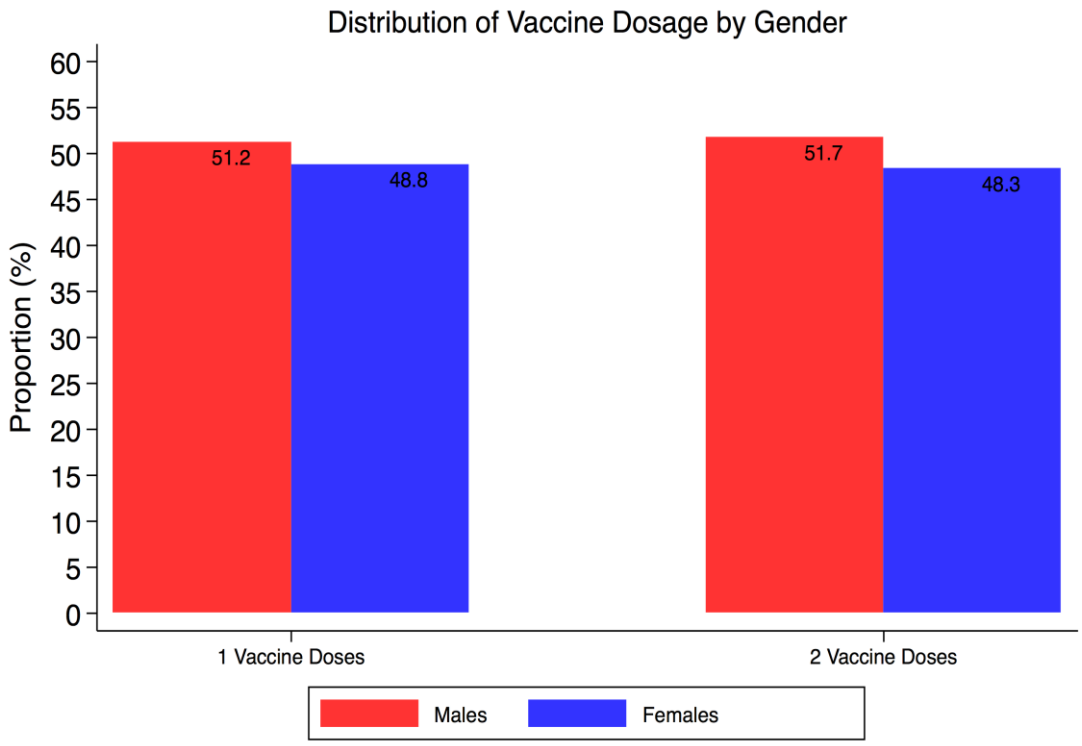

Figure 4.7: Proportion of children below 1year vaccinated with Rotarix® vaccine and vaccination by gender in Mukuru kwa Njenga health center Nairobi county, July 2014-July 2015.

4.11 Vaccine uptake trend in Mukuru for the 1st year of introduction (July 2014-July 2015)

The vaccine uptake within the 1st year of introduction was observed to be high. Figure 4.8 below shows the proportions of children who received two doses of the vaccine by each month of study period. During the first cohort between September and December 2014 the number of children who received the first dose was higher than the 2nd dose. However, it can be observed that use of health centre register records for vaccination data may be bias as compared to data from immunization cards.

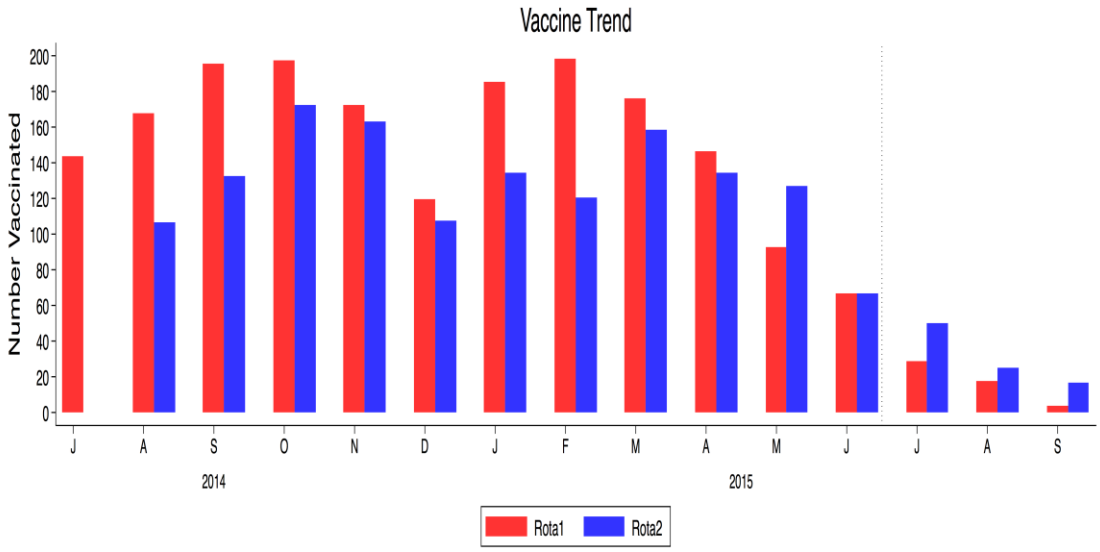

Figure 4.8: Vaccine uptake trend by months in Mukuru kwa Njenga health center Nairobi county, July 2014-July 2015. Rota1 (red) represent 1st dose at 6 weeks and Rota 2 (blue) represent 2nd dose at 10 weeks**.**

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Prevalence of rotavirus infections in children under five in Mukuru

Rotavirus infections are among the major causes of diarrhea leading to death in children in developing countries. Data on prevalence of rotavirus infections in Kenyan outpatient facilities before and after vaccine introduction is limited. The study compared the prevalence of rotavirus infections one year before and one year after rotavirus vaccine introduction in two Mukuru outpatient health facilities.

The study reports significance difference in prevalence of rotavirus during the pre-vaccine and post-vaccine periods, respectively, 10% (15/150) and 5% (6/120), (P>0.05). This shows 50% reduction of rotavirus infections after introduction of vaccine. A previous study carried out in Viwandani for the period before vaccine (2012-2013) reported prevalence in rotavirus infections of 23% in children double the prevalence that was observed in this study (Raini *et al*., 2015).

Analysis of the prevalence confirms findings from a previous study done in the same location three years ago, before vaccine introduction to be 24%, indicating that rotavirus infections was still a major pathogen causing diarrhea in children in Kenya (Gikonyo *et al*., 2010).

The high prevalence of rotavirus infections in the current and other studies in East Africa reflect the high burden of rotavirus infections reported elsewhere in sub-Sahara Africa (Sabrina *et al*., 2007, Nakawesi *et al*., 2010, Mwenda *et al*., 2010).

Rotavirus infections was more prevalent before vaccine introduction in children <12 months 6/36 (16.7%) ($P<0.05$; $\chi^2=6$; df=1) and 13-24 months 4/42 (9.5%) ($P<0.05$; $\chi^2=9.6$; df=1) as compared to the period after vaccine. This could be attributed to the introduction of the free vaccine which targeted the age below 12months. In children 25-36 months and 37- 48 months, however, there was no difference in prevalence of rotavirus pre-and post-vaccine introduction ($P>0.05$; $\chi^2=0.5$; df=1) and ($P>0.05$; $\chi^2=0.184$; df=1) respectively. Rotavirus infections was detected higher in female 8/75 (10.7%) and 7/75 (9.3%) in male for the period before vaccine. For the period after vaccine introduction cases in male were higher 4/64 (6.3%) compared to female 2/56 (3.6%). This indicated that males excreted significant high rate of rotavirus in their faeces than their female counterparts. This is in agreement with the finding of that boys have been found to be twice susceptible and likely to attend the hospitals than girls (Surajudeen *et al*., 2011). Whether this difference is due to sex susceptibility or by chance is however questionable and needs further investigation.

Reported prevalence from Mukuru study post vaccine introduction on age categories compared well to a study in Rwanda, with decrease in proportion of rotavirus infections after introduction of pentavalent rotavirus vaccine in May 2012.

The greatest effect was in children directly protected by vaccine being 13-24 months in 2014-2015 (Ngabo *et al.,* 2016). However, same study reported a reduction in admission by 61-70% including older children age-ineligible for vaccination suggesting an indirect protection through reduced transmission of rotavirus infections.

Although the effectiveness of rotavirus vaccine in Rwanda is promising, the effectiveness is still less than that in developed countries in America and Europe (Umesh *et al*., 2016). Finland, for instance, with a high vaccine coverage (92%), the effect of rotavirus vaccination was seen at 88% reduction of admittances to hospital for rotavirus gastroenteritis, with most of the remaining cases occurring in older children too old to be vaccinated in the programme (Hemming *et al*., 2013).

Indirect protection of children too old to have been vaccinated with rotavirus vaccine has been previously reported in several high-income and middle-income countries including the USA and El Salvador (Lambert *et al*., 2009, Payne *et al*., 2011). Comparative analysis of the assessment of vaccine effectiveness after the introduction of rotavirus vaccine into routine immunization programme in South Africa and Malawi showed that the vaccine was 57-64% effective against hospital admissions for rotavirus and reduced prevalence in young children (Groome *et al*., 2014, Msimang *et al.,* 2013). Partially similar data was reported in a study conducted in Brazil, where prevalence rates dropped from 11.12% in the pre-vaccine years 2002-2006 to 5.07% in the post vaccine period 2007-2011 (Andressa *et al*., 2013).

The implementation of rotavirus vaccination programme has resulted in reduction of rotavirus infection. This study could not exclude the effect of other confounding factors including and not limited to improvement of sanitation and level of education in the area. However, data from this study was in agreement with studies done after vaccine in Africa in under five years. In Ghana prevalence of rotavirus infections in children decreased from 47.7% to 27.8% after vaccine introduction (Christabel *et al*., 2014). In South Africa, results indicated that introduction of rotavirus vaccine into the national immunization program in late 2009 was associated with a one-third reduction in total diarrhea hospitalizations in children <5 years during May–December of 2010 and 2011, compared with 2009 (Veerle *et al*., 2013).

In Malawi, the prevalence of rotavirus infections was 50% before vaccine compared to 40% and 31% after one calendar year after vaccine introduction (Naor et *al*., 2015). Rwanda introduced rotateq vaccine in 2012 and reported a decrease of admissions due to rotavirus infections from 61-70% (Ngabo *et al*., 2016). The fall of number of cases positives after vaccine in Mukuru study was also similar to those reported in Brazil (2007-2010) and USA (2009-2011) after the implementation of rotavirus vaccines in these countries (Dulgheroff *et al*., 2012, Payne *et al*., 2013). This study aimed to add more recent data before vaccine and immediately after vaccine introduction as well as adding to the existing data on molecular typing of rotavirus infections in addition to finding out the vaccine coverage of children under 5years in Mukuru area in Nairobi.

5.1.2 VP4 and VP7 Genotyping in Mukuru

In this study six G strains were detected G1, G9, G8, G3, G4 and G2 and two P strains P[4] and P[8]. G1P [8] combination was the major genotype combination detected with 53.33% and 37.5% of the sequenced samples before vaccine and after vaccine respectively. Other minor genotypes reported circulating in Mukuru were G1 P[4], G2P[4], G3P[4], G4P[4], G8P[4] and G9P[8].

The phylogenetic analysis of the VP4 and VP7 partial sequence indicated G1P [8] followed by G9P [8] genotypes circulated in Mukuru before and after vaccine respectively. Within the clusters, no temporal clustering was quite evident. Combining Mukuru sequences with those from elsewhere indicated that there was no importation of viruses from other part of the globe into Mukuru. This conclusion is however limited by the fact that there were very few sequences in GenBank especially from Africa. Majority of the sequences in GenBank are from Europe and Britain. These results are consistent with those of other African studies describing various P and G common genotypes (Page *et al*., 2009; WHO, 2010).

The current vaccine introduced in Kenya immunization programme is Rotarix® (GlaxoSmithKline). This is a monovalent vaccine derived from the human rotavirus strain G1 [P8] genotype. Information on genetic diversity of rotavirus in Kenya is limited especially after vaccine introduction. This study determined genetic diversity of rotavirus in Mukuru informal settlements in Nairobi before and after vaccine introduction into national immunization.

The Mukuru study findings on prevalence and genotypes compares with previous studies reported rotavirus infections 18% in outpatient in Meru, Kenya (Kiulia *et al.*, 2006), 9% in urban slum (Kibera) and rural setting (Lwak-Siaya) in Nyanza Western Kenya (Breiman *et al*., 2014).

Data published after rotavirus vaccine introduction especially where countries had reached the coverage target of 80-90% in first years after introduction showed that the vaccine was achieving its primary goal of significantly reducing the cases of diarrhea and rotavirus infections. (Ngambo *et al.,* 2016*;* Naor *et al.,* 2015). However despite the high vaccination coverage a 4years follow-up study in Brazil reported a new scenario with disappearance of homotypic G1 P[8] strain and a coincidental rise of G2P[4] in post vaccination. (Dulgheroff *et al*., 2012).

In Africa, there is not only predominance of the G1 P[8], G2P[4], G3 P[8],G4P[8], and G9P[8] genotypes but also appearance of novel strains such as G8P[6], G10 and G12P[6] (Armah *et al.,* 2003; Mwenda *et al.,* 2010). In Kenya and Tanzania, detection and characterization of P and G genotypes has been reported with G9, G1, G3, P[8], P[6], P[4] dominating (Nyang'ao *et al.,* 2010, Sabrina *et al.,* 2007). A study previously conducted in Mukuru informal settlements in 2010 showed the main genotypes as G1, G2, G3, G4 and G9 and P[8], P[4], P[6] and P[9]. (Gikonyo *et al.*, 2010). These genotypes were similarly reported in this study and in additional, G8 for the period before vaccine.

The major circulating genotypes in the world include (G1, G2, G3, G4 and G9 and currently G12, while P include (P[8], P[4], and P[6] genotypes (Santos *et al.,* 2005, WHO 2011). Rotavirus G1P[8] is the most prevalent G/P combination reported to be responsible for 50-65% of rotavirus infections worldwide (Arora *et al*., 2011). In Africa G1P [8] still dominates with 21% followed by G2[P4] as reported by Africa Rotavirus Surveillance Network combined data from 11 countries (Mwenda *et al*., 2010). In Kenya, a more recent study in the similar informal settlement in Nairobi showed G1P8 was detected in 40% of the samples 128 samples tested from children. (Raini *et al*., 2015).

5.1.3 Proportion of the children vaccinated 2014-2015 in Mukuru kwa Njenga clinic

The proportion of children under 1year vaccinated was high at 80.3% with proportion of the children who got the two doses almost equal. This is regardless of the fact that there was no catch-up campaign prior to vaccine introduction. According to the WHO/UNICEF, rotavirus only achieved an average 38% and 50% of the national target Kenyan population in 2014. In the month of December 2014 there was a countrywide shortage of vaccine supply corresponding to the lower numbers of children vaccinated. This could have been attributed to, delayed introduction of the rotavirus vaccine, health care givers not adhering to age restrictions (children below 1year), shortage of health workers, poor logistics and frequent human migrations in health centers (GAVI., 2015).

A recent study on rotavirus vaccine coverage by sub-counties in Kenya reported similar coverage in Kiambu sub county with 82.4% coverage of two doses in 2014. This was followed by 116% in 2015 and 124.3% in 2016 (GAVI., 2015; WHO/UNICEF., 2016, Wandera *et al*., 2017).

The proportion of the children who received vaccine by gender was high in male 51% compared to 48% in female in the first year of vaccine roll out. Generally, there were more boys attending to the clinics as compared to girls. This could be attributed to more males attending the clinics compared to female. There was a marked decrease in prevalence of rotavirus infections from 9.9% to 5% one year after roll out of a vaccination programme in Mukuru informal settlement, Nairobi, Kenya. This could be attributed to the role played by the vaccine in prevention of rotavirus infections.

5.2 Conclusion

1) The overall prevalence of rotavirus infections decreased (9.9% to 5%) substantially after rotavirus vaccine implementation and majority affected children aged 12 to 24 months who were mostly eligible to be vaccinated.

2) G1 P[8] genotype was the major genotype circulating in both pre-vaccine and post-vaccine introduction period. G9 P[8] genotype was the second most prevalent genotype among pre-vaccine samples, though absent in post-vaccine samples. Other uncommon strains circulating in Mukuru informal settlement post vaccine introductions include G2

[P4], G3[P4], G4[P4]. We observed genotypes that were not observed in pre-vaccine period i.e. G2, G3 and G4.

3) Data from this study showed that the vaccine uptake in Mukuru informal settlement was 80.3% among children given two doses in the first year (July 2014 –July 2015) of vaccine roll out.

5.3 Study limitations

Missing data from hospital log books at Reuben Medical Health Center. The available data was reported cumulatively and lacked demographic data of age and gender for a period of 6 months of the study. Hence, could not be interpreted this led to excluding the clinic from the data analysis. Vaccine data used was secondary data from the health registers hence this could only provide estimates of children who were vaccinated in Mukuru. This may be biased as compared to data from the mother's immunization book which records and captures all the immunization given at different health facilities.

5.4 Recommendations

1) Data from this study support the use of rotavirus vaccine in Kenya and highlight the benefits of routine vaccination against rotavirus infections in low-income settings. However, with only one year of both pre-and post-vaccine data from Mukuru informal settlement, it is early to establish the trends of rotavirus infections in Kenya. In order to assess the impact of the vaccine on the disease occurrence, continuous monitoring of

rotavirus infections is needed. Additional studies of longer duration preferably on outpatients in other sentinel surveillance sites in Kenya from July 2014 should be carried out.

2) Working with other health sector stakeholders would provide a more comprehensive information on vaccine uptake and efficacy to determine whether it meets its primary goal of reducing diarrhea cases in under five years.

3) Simple and cost effective rapid tests that can be used to detect rotavirus infections in low health setup should be encouraged to enable early detection and management of disease. Currently the cost per sample (1790Ksh) using Prospect^{TM} ELISA which is the goal standard test is still high.

If implemented, these might greatly reduce and ensure healthy lives and promote well being for all the ages to achieve Sustainable Development Goal 3 (SDGs).

5.5 Further Studies

1) Research on other enteric viruses (Norovirus, adenovirus and sapovirus, human astrovirus) that could be causing the diarrhea in children less than five years of age is hereby recommended. This would provide evidence of indirect protection from rotavirus vaccination in a high-burden, low income settings. Co-factors which may influence severity of rotavirus infections disease e.g. H.I.V, malaria, poor sanitation and malnutrition should also be investigated, especially in informal settlement setups.

2) Understanding the dynamics of herd immunity and the length of protection of rotavirus vaccine would be important to the policy makers in Kenya.

3) Continued rotavirus genotype surveillance should provide a clearer understanding of rotavirus evolution.

4) In future there might be need for introduction of a multivalent vaccine that would target different genotypes to prevent against minor variant genotypes.

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APPENDICES

Appendix 1: Elisa Template OD and the Excel Work Sheet

plate1	1	2	3	4	5	6	7	8	9	10	11	12	
A	2.768	0.042	0.037	0.05	0.044	0.071	0.053	0.048	0.054	0.544	0.053	0.064	450
B	0.07	0.038	0.05	0.05	0.076	0.287	0.43	0.049	0.05	0.05	0.06	0.049	450
C	0.76	0.046	0.475	0.07	0.046	0.112	0.05	0.053	0.086	0.437	0.053	1.61	450
D	0.051	0.04	0.239	0.07	0.049	0.05	0.058	0.043	0.056	0.339	0.044	0.001	450
E	0.049	0.038	0.135	0.04	0.04	0.052	0.072	0.051	0.047	0.057	0.042	0.001	450
F	0.052	0.039	0.043	0.04	0.663	0.046	0.042	0.038	0.048	0.041	0.041	0.001	450
G	0.042	0.039	0.038	0.06	0.046	0.043	0.128	0.047	0.063	0.04	0.059	0.001	450
H	0.045	0.038	0.042	0.04	0.034	0.051	0.047	0.045	0.047	0.046	0.296	0.001	450

IDS		Cut off-0.27										
	PC	2.768		36	M485	0.663	pos	73	M1158	0.437	pos	
	NC	0.07		37	M500	0.046	Neg	74	M1165	0.339	pos	
1	M007	0.76	pos	38	MR515	0.034	Neg	75	M1175	0.057	Neg	
2	M0016	0.051	Neg	39	M578	0.071	Neg	76	M1179	0.041	Neg	
3	M020	0.049	Neg	40	M600	0.287	pos	77	M1194	0.04	Neg	
4	M-031	0.052	Neg	41	M611	0.112	Neg	78	M1206	0.046	Neg	
5	M885	0.042	Neg	42	M623	0.05	Neg	79	M1216	0.053	Neg	
6	M038	0.045	Neg	43	M643	0.052	Neg	80	M1217	0.06	Neg	
7	M039	0.042	Neg	44	M669	0.046	Neg	81	M1235	0.053	Neg	
8	M046	0.038	Neg	45	M670	0.043	Neg	82	M1237	0.044	Neg	
9	M065	0.046	Neg	46	M683	0.051	Neg	83	M1248	0.042	Neg	
10	M073	0.04	Neg	47	M771	0.053	Neg	84	M1254	0.041	Neg	
11	M085	0.038	Neg	48	M798	0.43	pos	85	M1239	0.059	Neg	
12	M086	0.039	Neg	49	M786	0.05	Neg	86	M1267	0.296	pos	
13	M100	0.039	Neg	50	M805	0.058	Neg	87	M1263	0.064	Neg	
14	M115	0.038	Neg	51	M861	0.072	Neg	88	M1270	0.049	Neg	
15	M117	0.037	Neg	52	M873	0.042	Neg		PC	1.61	Pos	
16	M163	0.05	Neg	53	M905	0.128	Neg					
17	M186	0.475	pos	54	M913	0.047	Neg					
18	M188	0.239	pos	55	M924	0.048	Neg					
19	M199	0.135	Neg	56	M925	0.049	Neg					
20	M124	0.043	Neg	57	M968	0.053	Neg					
21	M225	0.038	Neg	58	M1041	0.043	Neg					
22	M229	0.042	Neg	59	M1057	0.051	Neg					
23	M237	0.046	Neg	60	M1058	0.038	Neg					
24	M246	0.048	Neg	61	M1097	0.047	Neg					
25	M249	0.071	Neg	62	M1098	0.045	Neg					
26	MR251	0.066	Neg	63	M1114	0.054	Neg					
27	M288	0.043	Neg	64	M1135	0.05	Neg					
28	M304	0.041	Neg	65	M1145	0.086	Neg					
29	M326	0.064	Neg	66	M1146	0.056	Neg					

Appendix 2: Pwani Study Approval

NACOSTI ACCREDITED

ERC/MSc/043/2015

Pwani UNIVERSITY

ETHICS REVIEW COMMITTEE

ACCREDITTED BY THE NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY AND INNOVATION (NACOSTI, KENYA)

CERTIFICATE OF ETHICAL APPROVAL

THIS IS TO CERTIFY THAT THE PROPOSAL SUBMITTED BY:
REGINA WAMUYU NJERU

REFERENCE NO:
ERC/MSc/043/2015

ENTITLED:
Prevalence and molecular characterization of group A Rotavirus in children under five years, before and after vaccine introduction at Mukuru informal settlements, Nairobi County

TO BE UNDERTAKEN AT:
NAIROBI COUNTY, KENYA

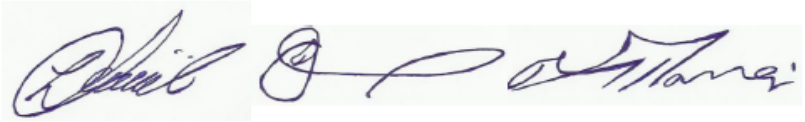
FOR THE PROPOSED PERIOD OF RESEARCH

HAS BEEN **APPROVED** BY THE ETHICS REVIEW COMMITTEE

AT ITS SITTING HELD AT PWANI UNIVERSITY, KENYA

ON THE 30th DAY OF OCTOBER 2015

CHAIRMAN SECRETARY LAY MEMBER



PTO

Pwani UNIVERSITY
Ethics Review Committee,
Pwani University, www.pu.ac.ke, email: r.thomas@pwaniuniversity.ac.ke, tell: 0719 182218.
The ERC, Giving Integrity to Research for Sustainable Development

Appendix 3: KEMRI Study Approval Letter

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200 NAIROBI - Kenya
Tel: (254) (020) 2722541, 254 (020) 2713349, 0722-205901, 0733-400003 Fax (254) (020) 2720030
Email: director@kemri.org info@kemri.org Website: www.kemri.org

KEMRI/RES/7/3/1 **December 09, 2015**

TO: **REGINA NJERU,**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CMR,**
NAIROBI

Forwarded JS 4/1/2016

Dear Madam,

RE: **PROTOCOL No. KEMRI/SERU/CMR/P0026/3144 (*RESUBMISSION OF INITIAL SUBMISSION*): PREVALENCE AND MOLECULAR CHARACTERIZATION OF GROUP A ROTAVIRUS IN CHILDREN UNDER FIVE YEARS BEFORE AND AFTER VACCINE INTRODUCTION AT MUKURU INFORMAL SETTLEMENT IN NAIROBI COUNTY-*(VERSION 2.0 DATED 8TH NOVEMBER, 2015)***

Reference is made to your letter dated 8th November, 2015. KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on 10th November, 2015.

This is to inform you that the Committee notes that the issues raised during the 244th A meeting of the KEMRI/SERU held on 13th October, 2015 have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **9th December, 2015** for a period of one year. Please note that authorization to conduct this study will automatically expire on **December 08, 2016.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **October 27, 2016**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

EAB

PROF. ELIZABETH BUKUSI,
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT

Appendix 4: Publication

Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.6, No.14. 2016

Prevalence of Group a Rotavirus before and after Vaccine Introduction in Mukuru Informal Settlement in Kenya

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Abstract

Background: Rotavirus vaccines have been shown to be a lifesaving and cost-effective public health intervention in Africa and have resulted in reduced rotavirus mortality. In Kenya, rotavirus diarrhea causes 19% of hospitalizations and 16% of clinic visits among children <5 years of age and causes 4471 deaths and 8,781 hospitalizations per year. Nationally, rotavirus disease costs the health care system $10.8 million annually. It is estimated that routine vaccination with a 2-dose rotavirus vaccination series would avert approximately 2,467 deaths (55%), 5,724 hospitalizations (65%), 852, 589 clinic visits (59%) and would save 58 disability-adjusted life-years (DALYs) per 1000 children annually. In July 2014, Kenya introduced rotavirus vaccine into its routine expanded programme immunisation, with two doses given at 6 and 10ths week of age.WHO recommend having surveillance studies before and after vaccine as baseline data and monitoring the possible effect after vaccine introductions. The aim of this study was to determine the prevalence of rotavirus in pre- and post-vaccine stool samples collected from children under five years, attending two selected clinics in Mukuru informal settlement in Nairobi, Kenya.

Methods: Archived samples collected during a *Salmonella* surveillance study (SSC No. 2074) conducted between July 2013 and July 2015 were used for this study. A total of 270 samples (150 pre-vaccine and 120 post-vaccine) were tested for rotavirus using ELISA Prospect kit (Oxoid Ltd UK) and data analyzed using SPSS version 20.

Results: Rotavirus prevalence was 10% (15/150) and 5% (6/120) in pre-vaccine and post-vaccine samples respectively. There was significant difference in prevalence pre and post vaccine samples for children less than 12 months (P=0.014), 13-24 months (P=0.002) and over 49 months (P=0.01). However, there was no difference in prevalence for age categories 25-36 and 37- 48 months.

Conclusion: This study showed a reduction in prevalence of Group A rotavirus in Mukuru selected clinics one year after vaccine introduction into National immunization program in Kenya. Rotavirus prevalence differed significantly for cases less than 12 months, 13-24 months and over 49 months pre and post vaccine introduction. However, there was no difference in prevalence for age category 25-36 and 37- 48 months thus the vaccine proved to have a significant protection in the most vulnerable group of children.

Keywords: Rotavirus, Kenya, vaccine, pre-vaccine, post-vaccine, prevalence, Kenya.

Introduction

Background information

Rotavirus is a leading cause of diarrhea in children under five years (WHO 2009). It is estimated to cause 527,000 deaths globally with approximately 232,000 occurring in South Asia and Sub-Saharan Africa (Mwenda *et al.*, 2010).

Previous studies in Kenya (Nyanza and Western Kenya) found that rotavirus infections caused 19% of hospitalizations and 16% of clinic visits for diarrhea among children <5 years. Between 2005-2007 the annual mortality burden associated with rotavirus was estimated to be 68 deaths per 100,000 children (Tate *et al.*, 2009). A prospective surveillance for children <13 years at Kilifi County Hospital reported admissions with diarrhea as

3,296 (22%) of which 2,039 were tested for rotavirus with 588 (29%) positive cases reported (Nokes *et al.,* 2008).

Rotavirus is contracted through the fecal oral route. It is highly contagious and spreads easily from person to person through contaminated hands and objects following contact with fecal matter (WHO, 2012). Children with severe rotavirus infections have frequent diarrhea and vomiting leading to dehydration and often need to be rehydrated with intravenous fluids, Oral rehydration solution (ORS) and zinc tablets (WHO, 2009). In developing countries, this type of emergency care is largely inaccessible, making the rotavirus prevention through vaccination critical to saving children's lives (Albert *et al*., 2012). Other measures that would be combined in preventing diarrhea include improvement of water quality, hygiene and prevention of bacterial and parasitic infections (Navaneethan and Giannella, 2008).

Following clinical trials in America and Europe that showed high efficacy of over 85-98% against severe rotavirus infection, there was recommendation of introduction of rotavirus vaccine trials in countries in Africa and Asia (WHO 2007). Rotavirus vaccine studies in South Africa and Malawi reported efficacy estimates ranging between 57-64% against a hospital admissions end point for rotavirus associated diarrhea and similarly reduced the prevalence of rotavirus in young children (Madhi *et al.,* 2010, Msimang *et al*., 2013). Similar studies in Mali and Ghana have reported an efficacy of 83% in the first year of life (Armah *et al.,* 2010, Christabel *et al*., 2014). In Kenya, a rotavirus vaccine trial in the Western part of the country reported efficacy of 63.9% through two years of follow up in 1308 infants (Felkin *et al.,* 2012).

WHO extended the recommendation for introduction of rotavirus vaccine into national immunization programs in African countries (WHO 2009, Jiang *et al*., 2010, Armah *et al*., 2010) following review of clinical trial data from Africa. Through Global Alliance for Vaccines and Immunization (GAVI), 19 countries in Africa have introduced the vaccine in their immunization programs (www.gavi.org). Rotarix (GSK) was included in Kenya immunization program in July 2014 in all government health facilities. Children aged below one year were administered two doses vaccine at 6 and 10 weeks of age (www.gavi.org/country/kenya).

WHO, however, also recommends local disease surveillance studies prior to the introduction of vaccines. Part of this should be monitoring of rotavirus diarrhea cases and as well as circulating strains. Having baseline surveillance for prevalence of rotavirus positive cases and trends pre and post vaccine introduction is important in monitoring the effects of the vaccine and for the country to get an understanding of epidemiology of rotavirus (WHO /IVB/08/16). This study set out to determine the possible influence of vaccination on number of rotavirus positive after vaccine introduction in Kenya.

Methodology

Ethical statement

Ethical approval for the study was obtained from the Scientific Ethics & Review Unit (SERU) KEMRI/SERU/CMR/P0026/3144. This study was part of the ongoing *Salmonella* surveillance study (SSC 2074). Consent from parents/guardians for their children had already been sought by personnel involved in study SSC 2074. All samples were anonymized and no personally identifiable information was present on the same labels.

Study population and sample collection

This study was conducted in two outpatient health facilities, Medical Missionaries of Mary and Reuben Medical center in Mukuru urban informal settlement situated 15 km East of Nairobi city. This is an overcrowded area with closely clustered houses, garbage dumps, open drains and inadequate sanitation.

A single time point fecal sample was collected in sterile specimen collecting tubes (poly pots). Upon receipt, the samples were transported in cool boxes for other bacterial pathogens testing at the Centre for Microbiology Research, Kenya Medical Research Institute in Nairobi. An aliquot of sufficient sample was stored at -20^{0}C until use in this study.

Inclusion criteria were children below 5years of age, with diarrhea 3 or more watery, non-bloody stool within 24 hour period.

Exclusion criteria was, <5years, bloody stool, no stool available or stored sample insufficient.

A total of 270 stool samples were purposively selected from 3995 children that had been recruited between July 2013-July 2015. Additional demographic data including age, sex and clinical characteristics were also gathered from case report form.

Laboratory Procedures

ELISA(Enzyme Linked Immunoassay) using Prospect™ rotavirus kit

Diagnosis of rotavirus was based on the detection of virus antigen in human stool using a commercial kit (PROSPECT™ Oxoid LMD, UK). This is a qualitative technique which utilizes a polyclonal antibody in a solid phase sandwich enzyme immunoassay to detect group specific antigen present in Group A rotaviruses.

Briefly, 1ml of sample diluent (Tris Buffered Saline solution) was added to approximately 0.1g of solid fecal material (small pea-sized portion) or approximately 100ul of liquid stool and mixed thoroughly on a vortex mixer and left to settle for 10 minutes prior to testing.

To conduct the ELISA test, 100ul of prepared sample was added in each well of the microtitre plates supplied with the kit that is pre coated with antibody. A 100µl horse peroxidase-conjugated anti-human antibody was added to each well and mixed by rotating the plate manually 3 times. The plates were then incubated at 20-30°C for 60 minutes and subsequently washed 4 times using 350-450 µl PBS in each well. Substrate 100 µl was then added to each well and incubated at 20-30°C for 10 minutes, followed by addition of 100µl of the 0.46 mol/L sulphuric stop solution. The plates were mixed thoroughly and read at 450nm within 30 minutes of addition of the stop solution using BioTek Synergy4-Gen5 microplate reader. The negative control value was set at less than 0.150 absorbance units while positive control was a value greater than 0.500 absorbance units. The cut-off value was calculated by adding 0.200 absorbance units to the negative control value. Any specimen with absorbance value less than the cut-off was negative while those above the cut off were considered positive.

Statistical Analysis

Data analysis was carried out using the SPSS version 20. Chi square tests were used to compare proportions of rotavirus cases before and after vaccine and to determine significance at 95% CI and $P<0.05$. To assess the possible influence of vaccination, the data was divided into two: pre vaccine (July 2013-July 2014) and post vaccine (July 2013-July 2015).

Results

In the study period from July 2013-July 2015, 270 samples (150 before vaccine and 120 after vaccine) were used and demographic data regarding age and gender was recorded. Out of the 150 children for the period before vaccine, there was equal distribution of sample size 75 (50%) of female and male. For the period after vaccine introduction 56 (46.7%) were female while 64 (53.3%) were male. There was no significant ($P>0.05$) difference between males and females in the selected sample set (Table 1).

Table 1: Sample size distribution by gender in Mukuru Informal settlement pre and post vaccine introduction.

Gender	Female		Male		Total				
	N	%	N	%	N	%	Chisquare	df	P-value
Pre-vaccine	75	50	75	50	150	55.6	0	1	1
Post-vaccine	56	46.7	64	53.3	120	44.4	0.533	1	0.465
Total	131	100	139	100	270	100			

Age wise distribution of patients showed significant difference ($P=0.001$) in sample distribution among the age categories for pre vaccine period (Table 2).With the highest age category being 13-24 months 24/150 (28%). However, the sample sizes for post vaccine were equally distributed ($P>0.05$) in the different age categories.

Table 2: Age stratification of sample size distribution in Mukuru Informal settlement pre and post vaccine introduction

Age range (months)	<12		13-24		25-36		37-48		>49		Total			
	N	%	N	%	N	%	N	%	N	%	N	Chi square	df	P-value
Pre-vaccine	36	24	42	28	39	26	23	15.3	10	6.7	150	23.667	4	0.001
Post-vaccine	18	15	18	15	33	27.5	26	21.7	25	20.8	120	6.583	4	0.160
Total	54	20	60	22.2	72	26.7	49	18.1	35	13	270			

Rotavirus was more prevalent before vaccine introduction in children <12 months 6/36 (16.7%) and 13-24 months 4/42 (9.5%) (Table 3). In children 25-36 months and 37- 48 months, however, there was no difference in rotavirus prevalence pre and post vaccine introduction.

Table 3: Age stratification of rotavirus positives in Mukuru Informal settlement pre and post vaccine introduction

Vaccine	Pre-vaccine			Post vaccine					
	Negative	Positive	Total	Negative	Positive	Total			
	N %	N %	N %	N %	N %	N %	chi-square	df	P-value
Age(Months) <12	30(83.3)	6 (16.7)	36(100)	18(100)	0(0)	18(100)	6	1	0.014
13-24	38(90.5)	4(9.5)	42(100)	16(88.9)	2(11.1)	18(100)	9.6	1	0.002
25-36	35(89.7)	4(10.3)	39(100)	31(93.9)	2(6.1)	33(100)	0.5	1	0.485
37-48	22(95.7)	1(4.3)	23(100)	25(96.2)	1(3.8)	26(100)	0.184	1	0.668
>49	10(100)	0.0(100)	10(100)	24(96)	1(4)	25(100)	6.429	1	0.011

Rotavirus was detected slightly higher in female 8/75 (10.7%) and 7/75 (9.3%) in male for the period before vaccine. For the period after vaccine introduction cases in male were slightly higher 4/64 (6.3%) compared to female 2/56 (3.6%).

Table 4: Rotavirus positives distribution by gender in Mukuru Informal settlement pre and post vaccine introduction

Vaccine	Pre-vaccine			Post vaccine					
	Negative	Positive	Total	Negative	Positive	Total			
	N %	N %	N %	N %	N %	N %	chi-square	df	P-value
Female	67(89.3)	8 (10.7)	75(100)	54(96.4)	2(3.6)	56(100)	2.756	1	0.097
Male	68(90.7)	7(9.3)	75(100)	60(93.8)	4(6.3)	64(100)	0.871	1	0.351

A total of 15/150 (15%) and 6 /120 (5%) cases tested positive for rotavirus pre and post vaccine introduction periods respectively. The figure below shows the overall prevalence between pre and post vaccine. Pre vaccine was ($p<0.05$) higher prevalence compared to post vaccine.

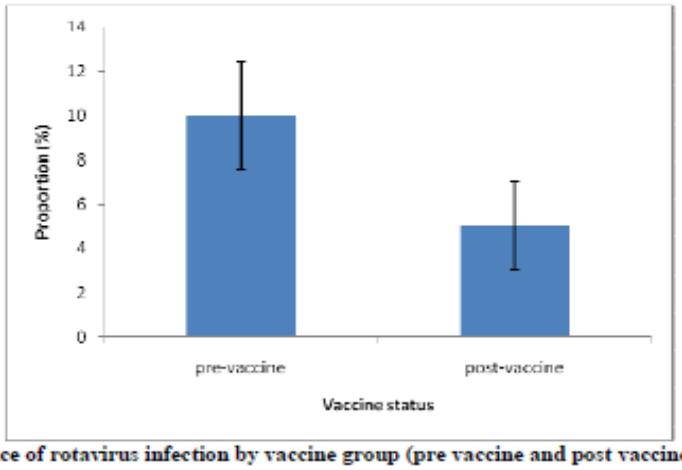

Figure1: Prevalence of rotavirus infection by vaccine group (pre vaccine and post vaccine)

Discussion

Rotavirus is among the major causes of diarrhea leading to death in children in developing countries. Data on prevalence of rotavirus in Kenyan informal settlements before and after vaccine introduction is limited. In this study, we set out to compare prevalence of rotavirus infections one year before and one year after rotavirus vaccine introduction in outpatient facilities.

We found rotavirus prevalence of 10% (15/150) and 5% (6/120) during the pre-vaccine and post-vaccine periods, respectively, which was statistically confirmed (P=0.05) leading to a 50% reduction of rotavirus cases. A previous study carried out in Mukuru for the period before vaccine (2012-2013) reported a rotavirus prevalence of 23% in children (Raini *et al*., 2015) that is almost double the prevalence that we observed in our study. Analysis of the prevalence confirms findings from a previous study done in the same location three years ago before vaccine introduction to be 24%, indicating that rotavirus was still a major pathogen causing diarrhea in children in Kenya (Gikonyo *et al*., 2010). The high prevalence of rotavirus in the current and other studies in East Africa reflect the high burden of rotavirus reported elsewhere in sub-Sahara Africa (Nakawesi *et al*., 2010, Mwenda *et al*., 2010, Sabrina *et al*., 2007).

Rotavirus prevalence differed significantly for cases less than 12 months, 13-24 months (p value-and over 49 months pre and post vaccine introduction. However, there was no difference in prevalence for age category 25-36 and 37- 48 months. There was no significant difference in prevalence between male and female study participants over the two periods. Reported prevalence from Mukuru study post vaccine introduction on age categories compared well to a study in Rwanda, with decrease in proportion of rotavirus positive after introduction of pentavalent rotavirus vaccine in May 2012, with the greatest effect in children directly protected by vaccine 13-24 months in 2014-2015 (Ngabo *et al.,* 2016). However, same study reported a reduction in admission by 61-70% including older children age-ineligible for vaccination suggesting an indirect protection through reduced transmission of rotavirus.

Although the effectiveness of rotavirus vaccine in Rwanda is promising, the effectiveness is still less than that in developed countries. Finland, for instance, with a high vaccine coverage, the effect of rotavirus vaccination has seen at 88% reduction of admittances to hospital for rotavirus gastroenteritis, with most of the remaining cases occurring in older children too old to be vaccinated in the programme (Hemming *et al*., 2013).

Generally, information on rotavirus vaccine effectiveness from Rwanda and other African countries is non-existent and future studies should address the effectiveness of vaccines after complete and uncomplete series of vaccinations. Indirect protection of children too old to have been vaccinated with rotavirus vaccine has been previously reported in several high-income and middle-income countries including the USA and El Salvador (Lambert *et al*., 2009, Payne *et al*., 2011). Comparative analysis of the assessment of vaccine effectiveness after the introduction of rotavirus vaccine into routine immunization programme in South Africa and Malawi showed that the vaccine was 57-64% effective against hospital admissions for rotavirus and reduced prevalence in young children (Groome *et al*., 2014, Msimang *et al.,* 2013). Partially similar data was reported in a study conducted in

Brazil, where prevalence rates dropped from 11.12% in the pre vaccine years 2002-2006 to 5.07% in the post vaccine period 2007-2011 (Andressa et al., (2013).

Conclusion

This study reported that rotavirus infection majorly affects children aged 12 to 24 months who were mostly eligible to be vaccinated. Prevalence of rotavirus decreased substantially after rotavirus vaccine implementation. The introduction of vaccine in the National immunization program in Kenya can be effective measure to decrease the rotavirus disease in young children and reduce the financial burden to the Kenyan health system. These data support the use of rotavirus vaccine in Kenya and highlight the benefits of routine vaccination against rotavirus in low-income settings. However with only one year of both pre and post vaccine data from Mukuru informal settlement, it is too early to establish the trends of rotavirus cases. In order to assess the impact of the vaccine on the disease occurrence, continuous monitoring of rotavirus cases is needed. The use of sentinel surveillance sites in Kenya from July 2014 will also provide the most comprehensive information after vaccine introduction. Research on other enteric viruses will provide evidence of indirect protection from rotavirus vaccination in a high-burden, low income settings. Routine use of rotavirus vaccine could have a large impact on diarrhea-associated mortality and morbidity in Kenya.

Acknowledgement

We would like to thank all the study participants and KEMRI-CMR Mukuru project staff for their assistance in sample collection and archiving. Special thanks go to Professor James Nokes (KEMRI-Welcome Trust Programme) for providing all the lab reagents and lab facilities used on this study.

Conflict of interest

The authors declare no conflicts of interest.

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