University of Nairobi

PREVALENCE OF ROTAVIRUS INFECTION AMONG CHILDREN WITH ACUTE DIARRHOEA AFTER ROTAVIRUS VACCINE INTRODUCTION IN KENYA

A Dissertation in Part Fulfilment of the Requirements for the Degree of Masters of Medicine in Paediatrics and Child Health, University of Nairobi

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DECLARATION

This dissertation is submitted as my original work and has neither been published elsewhere nor presented for a degree in any other university

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DEDICATION

This work is dedicated to my dear parents, Mary and Cosmas Maweu, my supportive husband Dennis Kimathi and our lovely children Jayden and Zara Kimathi. To all my siblings Mercy, Mutua, Grace and Josemaria. Thank you all for your tremendous support and constant words of encouragement.

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ABBREVIATIONS

AGE	Acute gastroenteritis
AIDS	Acquired Immune deficiency Syndrome
AVPU	Acronym for ‘alert’,’voice’,’pain’,’unresponsive’
DTap	Diphtheria, Tetanus and acellular Pertussis vaccine
DTwp	Diphtheria, Tetanus and whole cell Pertussis vaccine
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
HepB	Hepatitis B vaccine
Hib	Haemophilus Influenzae type b vaccine
HIV	Human Immunodeficiency Virus
IgA	Immunoglobulin A
IPV	Inactivated Polio Vaccine
KDHS	Kenya Demographic Health Survey
KEPI	Kenya Expanded Program of Immunization
KNH	Kenyatta National Hospital
KNH/UON/ERC	Kenyatta National Hospital-University of Nairobi Ethics Research Committee
LPA	Latex Particle Agglutination
NSP	Non Structural Proteins
OPV	Oral Polio Vaccine
ORS	Oral Rehydration Solution
PAGE	Polyacramide Gel Electrophoresis
PCV-7	Pneumococcal Conjugate Vaccine
PEU	Paediatric Emergency Unit
RAD	Rotavirus associated diarrhoea
RESOMAL	Recommended oral rehydration salts for severely malnourished children.
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription-Polymerase Chain Reaction

RV-	Rotavirus negative
RV+	Rotavirus positive
RVGE	Rotavirus gastroenteritis
RV	Rotavirus
RVV	Rotavirus vaccine
UON	University of Nairobi
VP	Viral Proteins
WHO	World Health Organization

CASE DEFINITIONS

- **Diarrhoea**- the passage of three or more loose or liquid stools per day.
- **Acute Diarrhoea**- the passage of three or more loose or liquid stools per day lasting less than 14 days.
- **Rotavirus Associated Diarrhoea**- gastroenteritis episodes meeting the above definition for acute diarrhoea and testing positive for rotavirus by ELISA.
- **Severe Rotavirus Associated Diarrhoea**- rotavirus associated diarrhoea having a score of ≥11 according to the Vesikari Clinical Severity Scoring System (Table 1 and 2)
- **Fully Vaccinated against Rotavirus**- children who have received two doses of Rotarix®, or three doses of Rotateq.
- **Vaccinated one dose of Rotavirus**-children who have received only one dose of Rotarix® or Rotateq.
- **Unvaccinated against Rotavirus**- absence of written records for Rotarix® or Rotateq vaccination in the medical record or having no recollection from guardian.

ABSTRACT

Background

Rotavirus is the commonest cause of severe and fatal diarrhoea among young children in the world with mortality occurring more in Sub-Saharan Africa and Southern Asia. Majority of these deaths are due to severe dehydration which is often not treated adequately due to the unavailability of timely and optimal medical care. The prevalence rates in Kenya are reported to be ranging from 30-40% among hospitalized children in the pre-vaccination era. Rotavirus vaccine is considered the most successful public health strategy in preventing infection and mitigating the severity of gastroenteritis. Rotavirus vaccination was introduced in Kenya in 2014. There has not been a study post introduction of the rotavirus vaccine in Kenyatta national hospital to determine whether there is any change in the profile of children being treated with acute diarrhea.

Objectives

The primary aim of the study was to determine the prevalence of rotavirus infection among children aged 3-24 months presenting with acute diarrhoea at Kenyatta National Hospital after introduction of the rotavirus vaccine. The secondary objectives was to determine the severity of the rotavirus associated diarrhoea using the Vesikari Clinical Severity Scoring System and to determine the rotavirus vaccination status among the children.

Methods

This was a short hospital-based longitudinal study at Kenyatta National Hospital among children aged 3-24 months presenting with acute diarrhea. The patients who met the inclusion criteria were enrolled sequentially. We acquired information on rotavirus vaccination status, nutritional status (z-scores), feeding practices, sociodemographic characteristics such as age, gender and caretaker characteristics such as age, level of education and relationship with the child and entered into a pre- structured questionnaire followed by a full clinical evaluation. The gastroenteritis severity was assessed using the 20 point Vesikari Clinical Severity Scoring System. The children who were admitted were followed up for 7 days using hospital ward registers. Comorbid conditions were established from patient's physical examination and medical records. Stool specimen

from study participants was tested for rotavirus using enzyme linked immunosorbent immunoassay (ELISA).

The data collected was entered and managed in Excel while data analysis was done with STATA version 13 software package and presented in figures and tables as applicable.

Results

Three hundred and sixty five children aged 3-24 months presenting with acute diarrhoea were recruited. Rotavirus was positive in 53/365 children, giving a prevalence of 14.5% (95% CI 11.1 -18.6). Of the 53 children who tested rotavirus positive, 28.3% (n=15) had severe rotavirus associated diarrhea, scored using the Vesikari Clinical Severity Scoring System, 32.0% (n=17) had moderate rotavirus associated diarrhoea and 39.6% (n=21) had mild rotavirus associated diarrhea. Three hundred and fifty six children (97.5%) were fully vaccinated against rotavirus, with only 9 (2.5%) receiving partial or no vaccination.

Conclusion

The prevalence of rotavirus infection among children with acute diarrhea at Kenyatta National Hospital is 14.5%. Twenty-eight percent of the children who tested rotavirus positive had severe rotavirus associated diarrhea as scored by the Vesikari Clinical Severity Scoring scale. The rotavirus vaccination status among the children was 97.5%, having received 2 doses of rotarix vaccine.

Recommendation

Advocacy on use of the Rotavirus vaccine should continue due to the observed reduction in the prevalence of rotavirus associated diarrhea.

CHAPTER 1: LITERATURE REVIEW

1.1 Background

Rotavirus gastroenteritis (RVGE) is the commonest cause of severe diarrhoea among children under 5 years(1).It is caused by Rotavirus (RV), a double-stranded RNA virus. The principle of management is to correct dehydration and this is dependent on the severity of the disease.

1.2 Epidemiology

Diarrhoeal diseases are one of the leading causes of mortality in the world, predominantly in developing countries with rotavirus infection being the commonest cause of severe, acute diarrhoea. It was responsible for an estimated 527,000 childhood deaths in 2008(2) to approximately 200,000 in 2015 among children who are under 5 years old according to World Health Organization (WHO)(1,3). It is estimated that about 70% of these mortalities continue to occur in South Asia and Sub-Saharan Africa(3). Furthermore, rotavirus is responsible for 25 to 50 percent of all diarrhea hospitalizations among infants and young children (2).

In Kenya, rotavirus infection causes approximately 19% (~9,000) of diarrhea hospitalizations and more than 4,000 deaths among children under 5 years of age annually (4).The peak infection age range with rotavirus is 3-24 months, the highest rate being between the ages of 6-11 months. Approximately 80% of rotavirus hospitalizations are in the 1st year of life(5). Jacqueline Tate et al's study on the rotavirus disease burden in Kenya, showed that every child, by 5 years of age will have visited an outpatient clinic for rotavirus diarrhoea, and that majority of rotavirus related deaths occurred in children from the poorest regions of the country, mostly in the western part of Kenya(4). This could be attributable to poor health access in the region, higher levels of malnutrition and a higher prevalence of HIV/AIDS(6). Osano et al's study in 2008 showed a 38.2% prevalence of rotavirus gastroenteritis among children less than 36 months with more than 80% of children affected below 12 months at Kenyatta National Hospital (KNH)(7).

This is comparable to a RV surveillance study done by Mwenda et al in 2006 at KNH, which reported a rotavirus positivity rate of 40% amongst hospitalized children below 5 years(8). In a study done at Gertrude's Children's Hospital and its satellite clinics, Kenya's largest private children's hospital, by Karanja et al, reported a prevalence of 39.5% of Rotavirus Gastroenteritis among children less than 24 months(9). According to the Kenya Demographic Health Survey (KDHS) 2014, diarrhea prevalence is highest among children aged 6-11 and 12-23 months (27% and 24% respectively)(10).

However, the prevalence of RV in hospital-based studies is much higher than of that seen in outpatient studies. Mwenda et al study found the prevalence of RV infection in children treated at the outpatient department ranged from 10% to 20%, which was much lower than the inpatient prevalence(8). Waggie et al's review of rotavirus studies in Africa showed rotavirus positivity of 25% in hospital based studies compared with 16% in outpatient studies and 32% in combined studies(11).

A Rotavirus Sentinel Surveillance performance feedback report by WHO in 2016 reported a significant decline of rotavirus infection among countries in East and Southern Africa from 44% in 2010 to 25% in 2015, after introduction of rotavirus vaccine after 2013(12). Similarly, reduction in diarrheal hospitalizations has been recorded since the introduction of the rotavirus vaccine. Rotavirus infection occurs throughout the year in the tropics with seasonal peaks more common during the dry months whereas in temperate countries, it occurs in seasonal winter epidemics(5,8,13).

1.3 Clinical Features

Rotavirus illness is characterized by mild, watery diarrhoea of limited duration to severe diarrhea with fever, anorexia and vomiting that can lead to dehydration with electrolyte imbalance, shock and death. The stools are watery, pale and with a distinct milky odor. Following a short incubation period of about 1-3 days, the illness usually begins unexpectedly, and vomiting usually begins before the onset of diarrhoea. Gastrointestinal symptoms typically resolve in 3 to 7 days, but the diarrhoea can continue up to 21 days. It has been noted that severe rotavirus infection occurs predominantly among unvaccinated

children aged 3–35 months(15). A prospective community based study done in Canada, revealed that rotavirus positive (RV+) children were more likely to experience the symptoms concurrently and to be hospitalized compared with rotavirus negative children(16). Moreover, dehydration often occurred among rotavirus positive children as compared to rotavirus-negative children(16–18). Additional clinical symptoms, such as convulsions and respiratory symptoms were associated with rotavirus infection. Children who are immunocompromised such as those receiving cytotoxic agents, malnourished children, occasionally suffer from severe, protracted and even fatal rotavirus gastroenteritis(19). However, it has been demonstrated that asymptomatic rotavirus infection is not an unusual happening; with an increased incidence of asymptomatic virus shedding in carriers. Consequently, recovery of rotavirus from feces is of little diagnostic significance since it does not give a differentiation between rotavirus-induced and rotavirus-associated diarrhea(20).

Clinical severity scoring systems have been used in rotavirus vaccine efficacy and effectiveness studies as a tool for defining the primary end point, which is severe RVGE(21). It has also been used in studies to classify severity of rotavirus gastroenteritis(22). The Vesikari Clinical Severity Scoring System is a composite measure, which relies on the clinical presentation profile of patients with RVGE to identify severe RVGE episodes in combination with laboratory assays. It has been adopted and routinely used in rotavirus clinical vaccine trials globally. There are 7 scoring parameters included in the Vesikari Scoring System which include diarrhoea, vomiting, fever, dehydration, duration of diarrhoea and vomiting and lastly treatment offered. Each of the seven parameters is broken down into equal thirds depending on the severity as initially identified by Ruuska and Vesikari(23). The scores for each parameter within the clinical severity scoring system are summed up allowing for a severity score between 0 and 20 points. Severity scores above 10 points are considered severe, scores between 7 and 10 moderate, and scores less than 7 mild (see appendix 4)(13).

1.4 Diagnosis

Since the clinical features of RVGE are indistinguishable to gastroenteritis caused by other enteropathogens, diagnosis is made by detection of rotavirus antigen in stool. Antigen detection tests are qualitative tests that detect VP6 rotavirus antigens from serogroup A. They are the most practical, easy to use and cost effective methods of rotavirus diagnosis. The most common technique applied is Enzyme-Linked Immunosorbent Assay (ELISA) which has a sensitivity and specificity of 90-95%(24).

Other techniques include Latex Particle Agglutination (LPA), Polyacramide gel electrophoresis (PAGE). Rotavirus can also be identified using electronic microscopy, which is considered the standard diagnosis method. It is very specific and almost 100% sensitive, however, quite time consuming and expensive to install and mostly used for research purposes.

1.5 Prevention

Improvements in hand washimg, water quality, proper food handling and waste disposal has significantly reduced the incidence of diarrhoea but these measures do not prevent the spread of rotavirus. Placentally transferred maternal antibodies and breast-feeding provide passive immunity and play a crucial role in the protection against the occurrence of the rotaviral infection in young infants. This is why exclusive breastfeeding is recommended as early complementary feeding is associated with an increased risk of infection(25).

Studies of natural immunity indicate that initial infection with the wild type rotavirus induces immunity against subsequent severe infections. Therefore vaccination early in life, which mimics a child's first natural infection will not prevent all subsequent disease but should prevent most cases of severe rotavirus disease and their sequelae (26). Rotavirus vaccine has been recommended by WHO as the best strategy for reducing morbidity and mortality associated with severe dehydrating rotavirus infection given that factors of hygiene and sanitation do not influence the high morbidity of rotavirus diarrhoea in both developing and developed countries(26).

1.6 Rotavirus Vaccine

There are two orally administered rotavirus vaccines available on the Kenyan market today which were licensed in 2006: Rotarix®, manufactured by GlaxoSmithKline, and RotaTeq®, manufactured by Merck & Co. Inc. Both vaccines are prequalified by WHO and have been shown to be equally safe and efficacious with a comparable protective efficacy (90-100%) in preventing severe rotavirus diarrhoea among children in developed countries mainly Europe, United States, Latin America and Finland(27,28).

In a phase III clinical trial done in 6 European countries by Vesikari et al, they assessed the efficacy and immunogenicity of Rotarix® when co-administered with vaccines normally included in national immunization programmes. In this trial, the vaccine conferred 87% protection against any, and 96% protection against severe RVGE. Furthermore, protection was 100% against hospitalization due to rotavirus and 75% against hospitalization due to gastroenteritis of any cause.

On the other hand, lower efficacy rates have been noted in developing countries of Africa (61.2–64.2%) and Asia (48.3%)(30,31). In a phase III clinical trial done in South Africa and Malawi by Shabir et al, the vaccine efficacy was lower in Malawi than in South Africa (49.4 % vs 76.9%) however, the number of episodes of severe rotavirus gastroenteritis that were prevented was greater in Malawi than in South Africa (6.7 vs 4.2 cases prevented per 100 infants vaccinated per year)(30). It is postulated that the low vaccine efficacy rates in developing countries as compared to developed countries may be as a result of host characteristics, such as higher HIV prevalence, different rotavirus serotypes, poor nutritional status, increased burden of enteric coinfections; young age at immunization, interference by maternal antibody or by coadministration of the oral poliovirus vaccine, which may reduce rotavirus antibody levels(30).

In Sub-Saharan Africa, Kenya included, following a pentavalent rotavirus vaccine efficacy trial by Armah et al, the efficacy against severe RVGE was about 83% in the first year of life, and 54% in the second year of life(32). This indicates a significant protection during the first year of life; a period characterized by a sharp increase in

rotavirus infection prevalence in Kenya. Waning immunity probably explains the decline in efficacy from the first to the second year of life. Despite the decline in point estimates of efficacy between the first and second protection during the first year, the overall benefit to public health was cumulative, with a rate reduction of severe cases of rotavirus gastroenteritis(32).

Rotavirus vaccines have been included in national immunization programs for most countries in the world to date. In 2006, WHO strongly advocated for the inclusion of rotavirus vaccines into national immunization programs in American and European countries, and later in 2009 extended this recommendation to all regions of the world. It was not until 2014, that the rotavirus vaccine was incorporated into the Kenya Expanded program of immunization (KEPI).

The current rotavirus vaccines differ in immunization schedules. The Rotarix vaccine administered orally in a 2 oral doses. The first dose should be given to infants from 6 weeks of life, while the second dose after a minimum interval of 4 weeks. The schedule should be completed by age 16 weeks, and no later than 24 weeks of age(35). RotaTeq® (Merck&co) is administered orally in a 3-dose schedule in the first 6 months of life, at a time interval of at least 4 weeks between the doses; the first dose is administered between ages 6 and 12 weeks of life(29).

There are numerous studies showing the positive impact of vaccination against rotavirus among children in both high, mid and low-income countries. In a Brazil study done by Greice Madeleine et al after rotavirus vaccine introduction into the Brazilian national vaccination programme in 2006, they compared rotavirus disease trends before (2002-2005) and after rotavirus vaccine introduction (2007-2009) by analyzing national hospital data, and they reported significant declines in under 5 diarrhoea related mortality and hospital admissions(36). The rates for diarrhoea related mortality and admissions among children < 5 years were 22% and 17% lower than expected, respectively. The largest reductions in deaths and admissions were among children younger than 2 years who had the highest rates of vaccination compared to those 2 years and above. This implies that

the reduced diarrhoea burden in the < 2 years age group was associated with introduction of the rotavirus vaccine.

In a study done in South Africa by Msimang et al, looking at rotavirus vaccine's impact on childhood diarrhoeal hospitalization after rotavirus vaccine introduction into the South African national Immunization Program in 2009, they compared the proportion of enrolled children aged <5years hospitalized with acute gastroenteritis and testing RV positive from 2009 to 2011.They reported a decline in RV positivity from 46% in 2009 to 33% in 2010 and a further decline of 29% in 2011 among children < 5 years. Moreover, rotavirus hospitalizations were 54% and 58% lower in 2010 and 2011, compared with 2009(37).

In Ghana, an observational study done in two paediatric referral hospitals in Accra, by Emweronu et al in 2014 also reported significant reductions of severe diarrhoea hospitalizations following introduction of rotavirus vaccine in 2012. The annual prevalence of acute RVGE declined from an average of 50% during the pre-vaccine introduction years to 38% and 32% in 2012 and 2013 respectively. In addition, the yearly hospitalization for all-cause diarrhoea showed a 51.6% and 16.2% decline from 2011 to 2012 and 2012 to 2013 respectively. The most likely explanation for reductions in all-cause diarrhoea hospitalization was the introduction of rotavirus vaccination(38).

The prevalence of rotavirus among children under 5 years of age hospitalized in the largest referral hospital in Kenya, Kenyatta National Hospital following two years surveillance before routine vaccination was found to be 40%(8). It is anticipated that the prevalence and severity of RVGE will decline significantly following rotavirus vaccine introduction in to the Kenya Expanded Program on Immunization. According to the KDHS 2014 vaccine coverage data, it showed that 79% of children aged 12-23 months had received all basic vaccinations, and 75% were fully vaccinated. The coverage has increased slightly from 77% reported in the 2008-09 KDHS. The highest vaccine coverage rate was 98% for the first dose of DPT-HepB-Hib and first dose of polio(10).

This coincides with the first dose administration of RV vaccine thus it is anticipated that the vaccine coverage for rotavirus vaccine will be even higher.

1.7 Study Justification and Utility

Rotavirus is the most common cause of severe and fatal diarrhoea among Kenyan children. Vaccination has been proven to be the best way to prevent severe rotavirus disease . There has not been a study post introduction of the rotavirus vaccine in Kenyatta National Hospital to see if there is any change in the profile of children treated with diarrhea. This study provides information on the prevalence of rotavirus infection and severity of rotavirus associated diarrhoea post vaccine introduction. The information obtained in this study will provide comparison data on prevalence and severity of rotavirus gastroenteritis post vaccine introduction compared to the pre vaccination era. It would also be useful in monitoring and evaluation of the outcome of rotavirus gastroenteritis patients after rotavirus vaccine initiation.

CHAPTER 2: RESEARCH QUESTIONS AND STUDY OBJECTIVES

2.1 Research Questions

1. What is the prevalence of rotavirus infection among children aged 3-24 months presenting with acute diarrhoea at Kenyatta National Hospital after rotavirus vaccine introduction in Kenya?

2.2 Study Objectives

Primary Objective

1. To determine the prevalence of Rotavirus infection among children aged 3-24 months presenting with acute diarrhoea at Kenyatta National Hospital after rotavirus vaccine introduction in Kenya.

Secondary Objectives

1. To assess the severity of the rotavirus associated diarrhoea using the Vesikari Clinical Severity Scoring System.
2. To determine rotavirus vaccination status among the children.

CHAPTER 3: RESEARCH METHODS

3.1 Study Design

Short longitudinal survey.

3.2 Study Period

From August 2016 to April 2017.

3.3 Study Location

Kenyatta National Hospital Paediatric Emergency Unit and Paediatric wards. KNH is the largest public, teaching and referral hospital in Kenya. It covers an area of approximately 45.7 hectares and has a total bed capacity of 2000 and 50 wards. The hospital serves the low and middle-income population from Nairobi and its environs as well as referrals from other hospitals in the country and the greater Eastern Africa region.

Within the KNH complex are; - the Kenya Medical Training College, University of Nairobi-College of Health Sciences and Kenya Medical Research Institute. The hospital's mission is not only to provide health care services, but to also facilitate training and research.

There are four paediatric wards with a bed capacity of 240 and has approximately 300-350 admissions per month. Most children admitted to the paediatric wards are usually referred from peripheral facilities across the country. The sick children are admitted from the PEU where the postgraduate paediatric residents' triages, stabilizes and initiates emergency care before admission to the paediatric wards. Children with acute diarrhoea with severe dehydration account for a significant proportion of the paediatric admissions. Registered clinical officers treat the non-priority cases mainly as outpatients.

3.4 Study Population

The study population included infants aged 3 to 24 months presenting with acute diarrhoea at KNH Paediatric Emergency Unit (PEU) or admitted to the paediatric wards.

3.4.1 Inclusion Criteria

To be included into the study, each of the children recruited met the following criteria;

- Children presenting with acute diarrhea.
- Children aged 3 to 24 months.
- Written informed consent for study participation obtained from their parents or primary caregivers.

3.4.2 Exclusion Criteria

Children who met any of the following exclusion criteria were excluded from the study;

- Those with bloody diarrhoea.
- Those unable to provide stool specimen within 24 hours.
- Those aged below 3 months or above 24 months.
- Those whose guardians declined to take part in the research.

3.5 Sample Size Determination

The Sample Size was determined using Fischer's Formula for Sample Size Determination in Prevalence studies:

$$n = \frac{z^2\, p(1-p)}{d^2} = \frac{1.96^2 \times 0.39 \times 0.61}{0.05^2} = \mathbf{365}$$

- n = Sample Size=365
- z = Normal Standard Deviation taken with a 95% Confidence Interval; set at 1.96.
- p = Expected Prevalence of Rotavirus Gastroenteritis, Estimated at 39.5% as per Karanja et al's Study in the Gertrude's Children's Hospital(9).

  This study was done in a private hospital where rotavirus vaccine was already being administered to children.
- d = Study Precision taken as 5%.

3.6 Study Outcomes

The study achieved the following outcomes:

- Determined the prevalence rate of rotavirus infection among children presenting with diarrhea in KNH.
- Determined the severity of rotavirus associated diarrhoea among children presenting with diarrhoea in KNH.
- Determined rotavirus vaccine status among the children presenting with rotavirus gastroenteritis.

3.7 Study Tools

A standardized questionnaire was used for collecting data from the enrolled participants (Appendix 2). The questionnaire had a unique study code for each study participant. The questionnaire was pretested in paediatric emergency unit among children with acute diarrhoea at the Kenyatta National Hospital. The questionnaire collected socio-demographic characteristics of the child and the caretaker, water and sanitation characteristics, vaccination history, clinical history and focused clinical exam to recognize dehydration status namely pulse character, capillary refill time, extremity temperature gradient, skin pinch, level of consciousness, level of thirst and eye appearance (Appendix 3). The outcome within 7 days was recorded for the admitted children using hospital ward registers.

A standardised KNH laboratory request form was used to request for the rotavirus antigen test. It documented the patients name, identification number, age, sex, date and time of stool collection, brief clinical information and clinicians name, quality of specimen on receipt at the laboratory and rotavirus results.

A confidential identification log register was used which acted as an interface containing the patient's details against the unique study code that assisted the principal investigator to extract the rotavirus results from the patient's medical record.

3.8 Study Procedures

3.8.1 Study Personnel

1. Principal investigator (myself) was the overall leader of the research team. The role was to collect data and ensure proper documentation, training of research assistants and perform standard procedures on enrolled participants. I also ensured all materials needed were available and all data collected was entered in to a computer system daily.
2. Research assistants- Data was collected with the help of seven research assistants. The research assistants were fully qualified clinical and nursing officers who had experience working in the paediatric department. They received training on the standard ways of doing procedures for the study, basic values and concepts of research ethics such as informed consent, autonomy and confidentiality.

3.8.2 Patient Recruitment Procedure

Patients were identified from either the PEU or the admitting paediatric ward by the principal investigator or the research assistants and were screened as per the case definition of acute diarrhea and those who met the inclusion criteria were recruited through sequential sampling by the investigator and research assistants. This was done every day of the week. Consent was given in written form, on a pre-designed consent form, which was availed to the caregiver at the point of recruitment. The consent form described the intentions of the study, the risks and benefits of participating in the study and also gave a brief overview of rotavirus disease. Any questions arising regarding the study were addressed before the caregiver signed the consent form. The consent obtained was voluntary and free from coercion and was countersigned by the PI or the research assistants.

Once the patient was enrolled into the study, we gave them a unique study code and documented the date of the interview. The caregiver was then issued the questionnaire which obtained both patient's and caregiver's socio-demographic information, water and sanitation characteristics, vaccination history which was verified from the mother baby booklet and/or word of mouth as reported by the parents. For the parents who did not

recall the names of the vaccines received, they described the vaccine by route of administration and the age of the child when they received the particular vaccine. Clinical history including the health seeking behavior was taken. The patient's HIV status was verified from the mother baby booklet and/or word of mouth as reported by the caregiver. We then performed a focused physical examination and recorded in the questionnaire. This included recording of weight and length, temperature recording, pulse character, temperature gradient of extremities, capillary refill time, consciousness level, skin pinch, level of thirst and eye appearance. (Refer to Appendix 3)

The severity of the RVGE was assessed using the Vesikari Clinical Severity Scoring System (refer to Appendix 4). Comorbid illnesses were extracted from the patient's medical records and/or physical examination findings based on the assumption that the primary clinicians followed standardized clinical guidelines in diagnosis and treatment. The patients who were admitted were followed up for 7 days using hospital ward registers to determine the outcome as either discharged, died or still admitted after 7 days. The duration of admission (in days) from the paediatric emergency unit was recorded. This information was extracted from the hospital ward registers.

A standardized KNH laboratory request form was used to request for rotavirus antigen test.

3.8.3 Stool Sample Collection and Sample Handling

A stool sample was collected from all patients enrolled into the study in a screw-capped disposable stool container for rotavirus detection. Caregivers were given a stool container in which to put the collected stool using a spatula. They were instructed on how to collect approximately 5 mL of diarrhoeal stool by the PI or research assistant. The stool sample was only collected from the children who were able to void within 24 hours of presenting to hospital. This was intended to include the children who were to be treated as outpatients as well as minimize acquisition of nosocomial rotavirus gastroenteritis after 24 hours of admission. The guardian was requested to deliver the stool sample within five minutes of sample collection to a central collection point both in the PEU and the Paediatric wards. The central collection points had a cooler box or a refrigerator in which

the stool sample was stored. The well-marked cooler boxes were placed in the procedure rooms for the four wards with one ward and PEU having a refrigerator. The cooler boxes were maintained at a temperature range of 2-8°C by ensuring the ice packs were changed twice daily. The stool samples were collected by a well-trained research assistant and transported twice daily to the Immunology laboratory-Kenyatta National Hospital using a cool box where they were stored in a freezer at -20°C prior to testing. Rotavirus testing was done monthly according to the laboratory protocols by a well-trained and qualified laboratory technician. The PI was notified once the results were ready before they were placed in the patient's clinical record.

3.8.4 Laboratory Procedures

Stool testing for rotavirus was performed using a commercially available Enzyme-linked immunosorbent assay kit- ProSpecT Rotavirus Microplate Assay which is based on detection of group specific antigen in group A rotaviruses(39). The procedure was carried out according to the manufacturer's specifications (Refer to Appendix 5) by a qualified laboratory technician. The test has a 95% sensitivity and specificity.

3.8.5 Patient Follow Up

The enrolled children admitted to the paediatric wards were followed up using hospital ward registers for 7 days to determine the outcome of hospitalization as either discharged, died or still admitted after 7 days. The number of admission days was recorded. The study flow chart is outlined in figure 1 below.

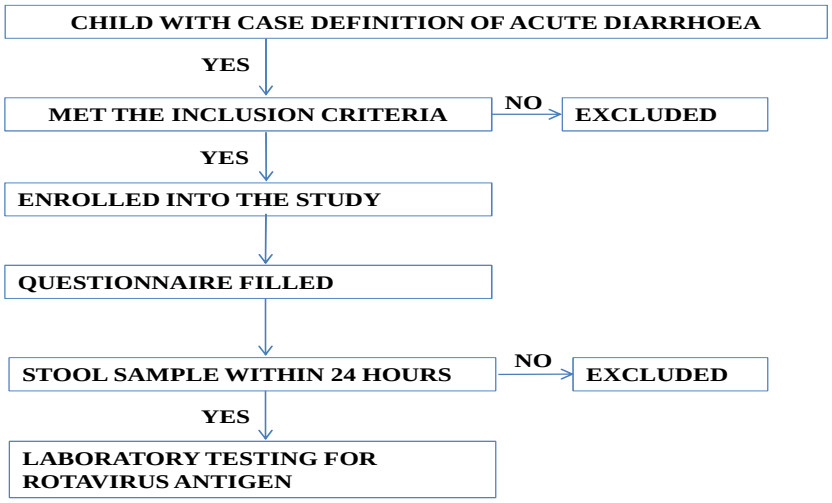


Figure 1: Study Flow Chart

3.9 Data Management and Analysis

Data was collected using a well-structured questionnaire. (Refer to Appendix 2).

The filled questionnaires were kept safely under lock and key ready for data entry for purposes of confidentiality of the patient's details. A database was designed in MS Excel. The data was cleaned and verified to ensure that quality was maintained. Statistical analysis was executed using STATA version 13 software.

The variables analyzed were rotavirus status, vaccination, age, gender, hand washing, education level of the care giver, duration of exclusive breastfeeding and Vesikari Clinical Severity score. All the variables except age and duration of exclusive breastfeeding were categorical variables. If the distribution was not normal, we used medians and interquartile ranges. Proportions amongst different categories were graphically expressed as pie charts. The dataset had no missing variables. All the data was used to conduct the analysis.

Data is presented in tables, graphs and figures as applicable.

3.9.1 Control of Bias and Errors

1. **Measurement bias**- the questionnaire was pretested to reduce bias, ensuring the questions are sensitive enough to detect what might be important difference in the variable of interest. Training of the research assistants on the data collection procedure reduced bias.
2. **Sampling bias**- only those who met the eligibility criteria were included.
3. **Instrument error**- digital thermometers, digital infant scale and balance beam were inspected daily to ensure correct data measurements.
4. **Information bias**- each research assistant was familiarized with the study and the questionnaire. They received a copy of study definition of terminologies and procedure guide to ensure uniform interpretation of terms. The principal investigator assessed the responses given to the questionnaire on daily basis to oversee data entry to ensure validity of collected data.
5. **Recall bias**- A short time frame of 14 days was used as the duration for which the frequency of symptoms was assessed.

3.10 Ethical Considerations

Ethical consent was granted by the KNH/UON Ethical and Research Committee before conducting the study. Informed consent was granted by the primary caretaker for each child recruited. No experimental investigations or products were employed in this study. Non-invasive procedures were used in sample collection therefore inflicting no pain to the children. Emergency treatment took precedence over the interview and no treatment was delayed due to the interview. Patients suffered no loss if they declined to participate in the study.

Strict confidentiality was safeguarded throughout the entire study period, held in trust by participating investigators, research staff and the study institutions. The filled questionnaires and the patient log register were kept in a safe place under lock and key for confidentiality of the patient's details.

Dissemination of the research findings will be availed to the primary health care team in the paediatric emergency unit and paediatric wards thereby, contributing to the improvement of care delivered to this children.

CHAPTER 4: RESULTS

The study was carried out between August 2016 and April 2017. Figure 2 below illustrates the flow of patients recruited in the analysis.

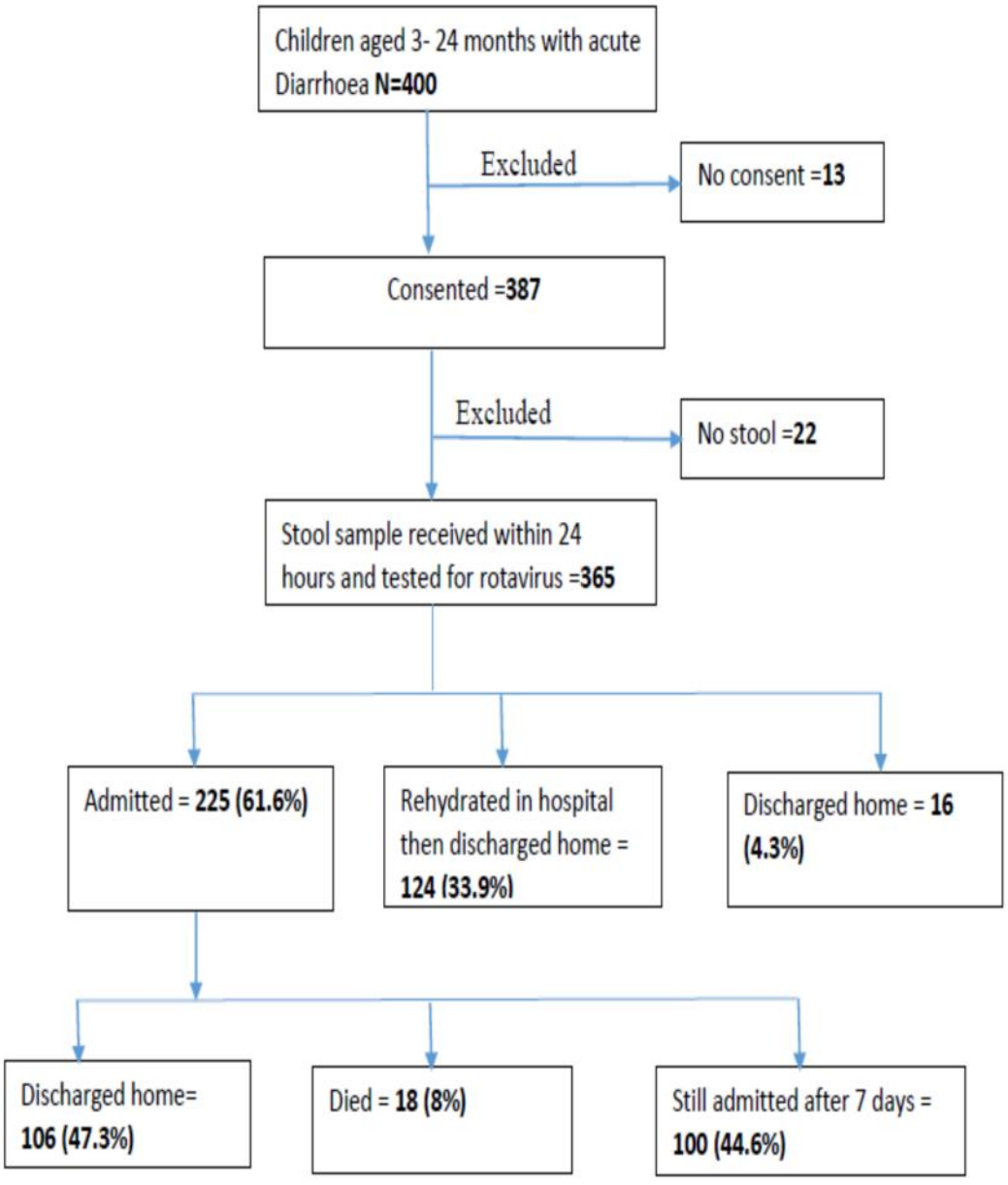


Figure 2: Flow of patients

Sociodemographic Characteristics of the Study Participants

Table 1: Sociodemographic Characteristics of the children (n=365)

Variable	Characteristics	Frequency (%)
Age group (months)	3-12 months	135(36.9)
	13-24 months	230(63.1)
Gender	Male	206(56.4)
	Female	159(43.6)
Nutritional status (W/L) Z scores	>-2	250(68.5)
	-3 to -2	50(13.7)
	<-3	65(17.8)
Duration of exclusive breastfeeding Age categories (months)	0-3	40(11)
	4-5	57(15.6)
	≥6	268(73.4)
Residential area	Within Nairobi	329(90)
	Outside Nairobi	36(10)
Residence within Nairobi	Informal settlements	241(73.3)
	Formal settlements	88(26.7)

The median age of the population studied was 11 months (IQR: 7- 16 months) with a mean age of 11.73 months (SD =6.07) and ranging from 3 to 24 months. The 3-9 month old children formed the bulk at 43.8%. The age distribution is shown in Figure 3.

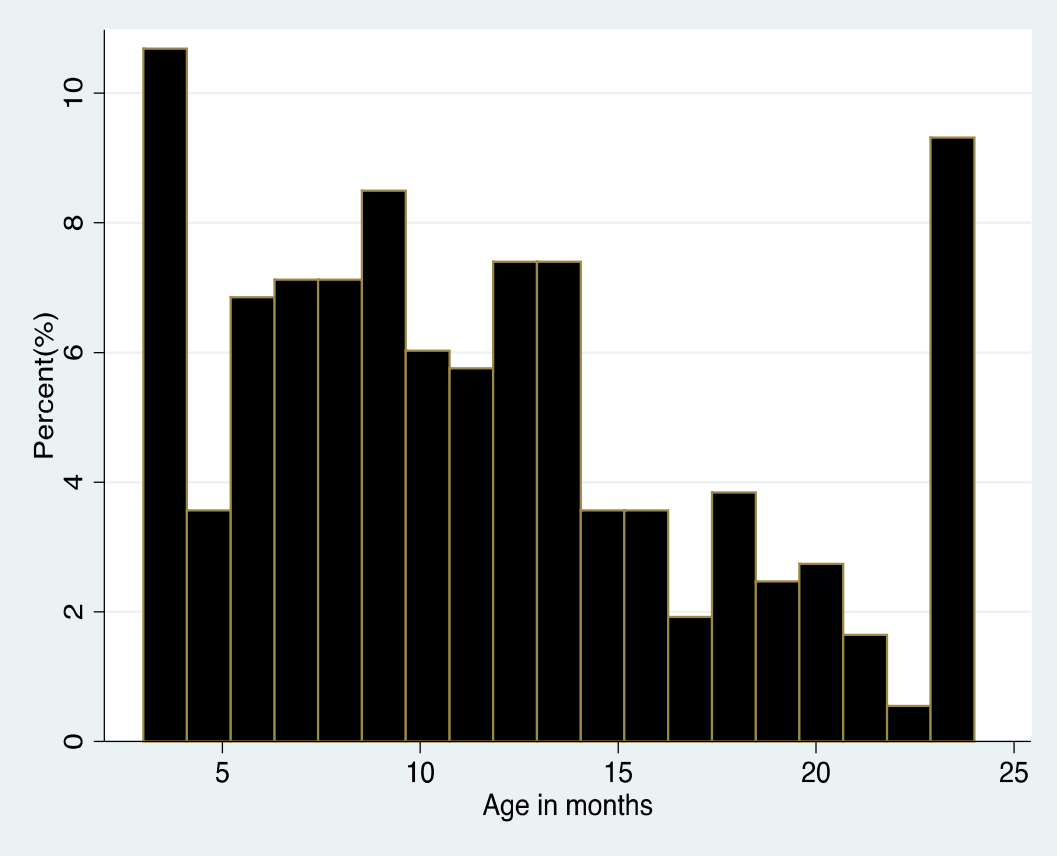


Figure 3: Age distribution of study participants

Of the 365 patients, 206 (56.4%) were males and 159(43.5%) were females, with a male to female ratio of 1.2:1. More than 60% of the children were infants. The mean weight of the recruited children was 7.9 kilograms (SD 2.45) with a range of 2.8 to 16 kilograms. The mean length was 71 centimeters (SD 9.50) with a range of 48 to 100 centimeters. Using the WHO reference ranges, the weight for length z-scores of the study population was determined. Weight for length z-scores greater than -2SD were 68.5% while 13.7% had z-scores between -3 to -2SD, and 17.8% had z-scores <-3SD. Majority of the children 268 (73.4%) were exclusively breastfed for 6 months. The mean duration of exclusive breastfeeding was 5.4 months (SD 1.19). Three hundred and fifteen mothers (86.5%) were still breastfeeding with a mean of 10.7 months (SD 5.38). The mean age when breastfeeding was completely stopped was 14 months (SD 5.83) with a range of 3 to 24 months. The patients came from within Nairobi environs as well as outside Nairobi with most children coming from informal settlements.

Caregiver Sociodemographic characteristics

Table 2: Caregivers Sociodemographic characteristics

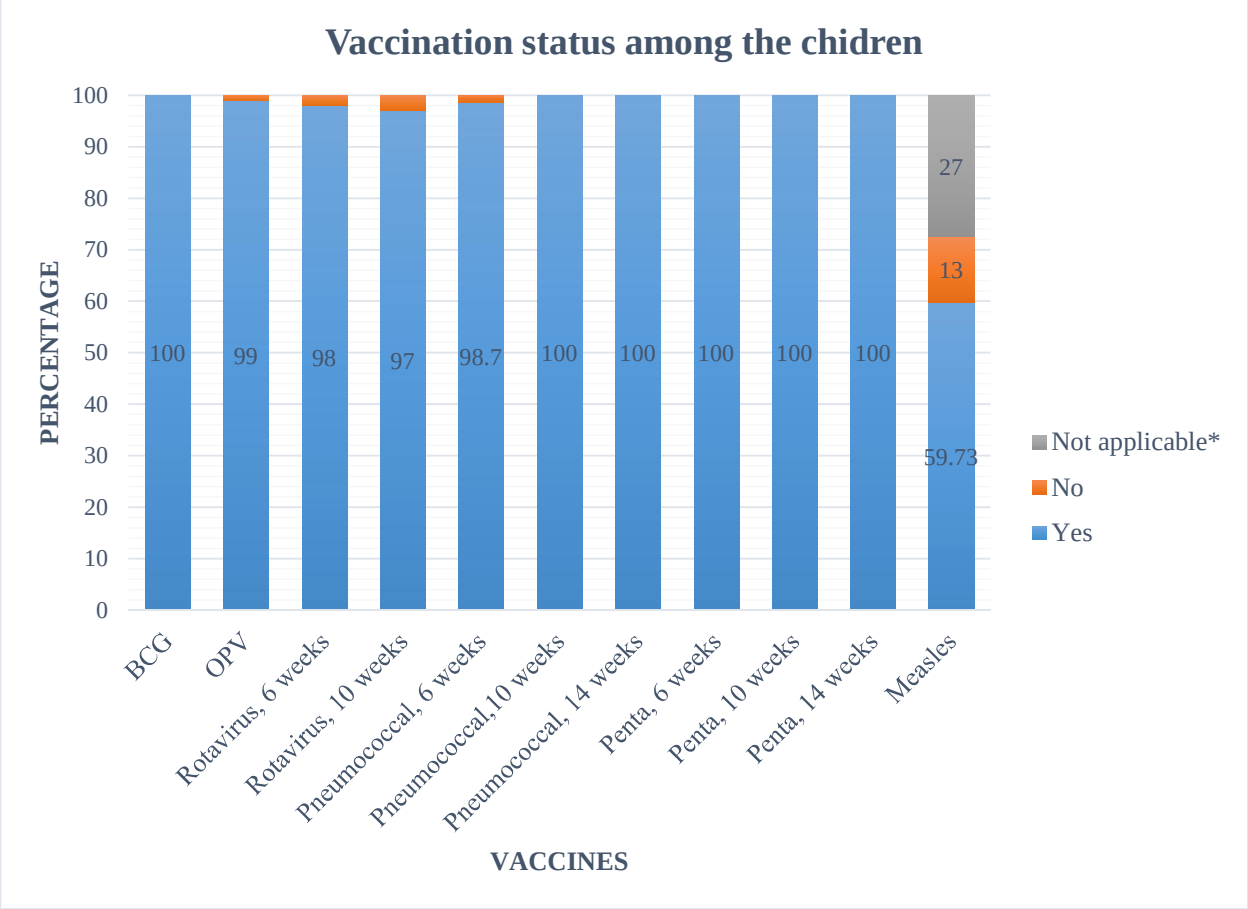
Variable	Characteristic	Frequency (%)
Relationship with guardian	Mother	357(97.8)
	Others (Father, Grandmother, Aunt)	8(2.2)
Age of guardian	≤20	16 (4.4)
	21-30	263(72)
	31- ≥40	86(23.6)
Level of education	Post-secondary	54(14.8)
	Secondary	208(57)
	Primary	99(27.1)
	None	4(1.1)
Occupation	Salaried employment	38(10.6)
	Self employed	87(24.4)
	Unemployed	232(65)
Caregivers monthly income	≤5000/-	176 (48.2)
	5001-10000/-	110(30.1)
	10001- ≥20000/-	79(21.7)
Number of children in the household	1	195(53.4)
	2	87(23.8)
	>2	27(7.4)

Most children (97.8%) were under the care of their mothers whose age ranged from 17 to 44 years with mean of 27.30 (SD 4.74). Mothers aged 21-30 years formed the bulk at 72%. Majority of the mothers had some form of education with 54 (14.8%) being educated post-secondary level, 208 (57%) secondary, 99 (27.1%) up to primary. Four mothers (1.1%) did not attend formal school. Two hundred and thirty two mothers (65%) who formed the bulk were unemployed while 176 mothers (48.2%) were earning less than 2 dollars a day.

One hundred and ninety five mothers (53.4%) had only one child, 87 (23.8%) had two children and 27(7.4%) had more than two children.

Rotavirus vaccination Status

Most children 353 (96.7%) had received 2 doses of rotavirus vaccine, 3(0.8%) children had received only one dose while 9(2.4%) children had not been vaccinated for rotavirus. For the children who did not receive the rotavirus vaccine, lack of knowledge about vaccination and poor communication by health workers were the commonest reasons provided. The status of vaccination among the children is illustrated in figure 4.



Not applicable implies not attained age for receiving measles vaccination.

Figure 4: Status of vaccination among the children

Clinical Presentation

Table 3 below summarizes the presenting complains.

Table 3: Presenting Complains

CLINICAL PARAMETER	CHARACTERISTICS	DISTRIBUTION OF CASES (%)
Diarrhoea Duration (Days)	1-4	303(83)
	5	35(9.6)
	≥6	27(7.4)
Frequency of diarrhea per day	3	241(66)
	4-5	98(26.9)
	≥6	26(7.2)
Duration of vomiting (days)	0	57(15.6)
	1	146(40)
	2	85(23.3)
	≥3	77(21.1)
Frequency of vomiting per day	0	57(15.6)
	1	125(34.2)
	2-4	149(40.8)
	≥5	34(9.3)
Temperature (°C)	37.1-38.4	151(41.4)
	38.5-38.9	162(44.4)
	≥39	52(14.3)

All the children had diarrhea, which was an inclusion criteria. Vomiting and diarrhea were the most frequent symptoms in 308 (84.3%) children. The duration of vomiting was reported to be for 1 day with 2-4 vomiting episodes per day in most children. The combination of fever, vomiting and diarrhea was in 276 (75.6%) children.

Clinical Evaluation of study participants

Table 4: Clinical Evaluation of Study participants

Clinical Parameter	**Characteristic**	**Frequency (%)**
Sought health services elsewhere prior to KNH		229(62.7)
Sent to Kenyatta Hospital		207(56.7)
Facilities where prior care was sought.	Public	96(26.3)
	Private	110(30.1)
	Over the counter	158(43.3)
	Herbalist	1(0.3%)
Level of dehydration	Hypovolaemic shock	36(9.9)
	Severe dehydration	50(13.7)
	Some dehydration	11(54.5)
	No dehydration	80(21.9)
Nutrition status	Normal	313(85.8)
	Visible severe wasting	52(14.2)
Associated problems	Convulsions	66(18.1)
	Respiratory distress	136(37.3)
	Abdominal distention	8(2.2)
Any Comorbidities		216(59.18)
Comorbidities present	Pneumonia	124(57.4)
	Meningitis	62(28.7)
	Severe acute malnutrition	48(22.2)
	Rickets	31(14.4)
	Others	45(20.8)
HIV status	Exposed	29(7.4)
	Not exposed	330(90.4)
	Positive	3(0.8)

One hundred and thirty six children (37.3%) sought care at KNH as the first contact with a health provider while 229 (62.7%) children had sought care in other public or private clinics. One patient acknowledged having taken herbal or traditional medications. Most children 279 (76.4%) had no to some dehydration while only 36 children (9.8 %) had hypovolemic shock. Two hundred and twenty five children (61.6%) were admitted to the

paediatric wards. Of those children admitted to the wards, 216(96.4%) children had associated comorbidities with the commonest being pneumonia. The outcome of the clinical evaluation is outlined in figure 5 below.

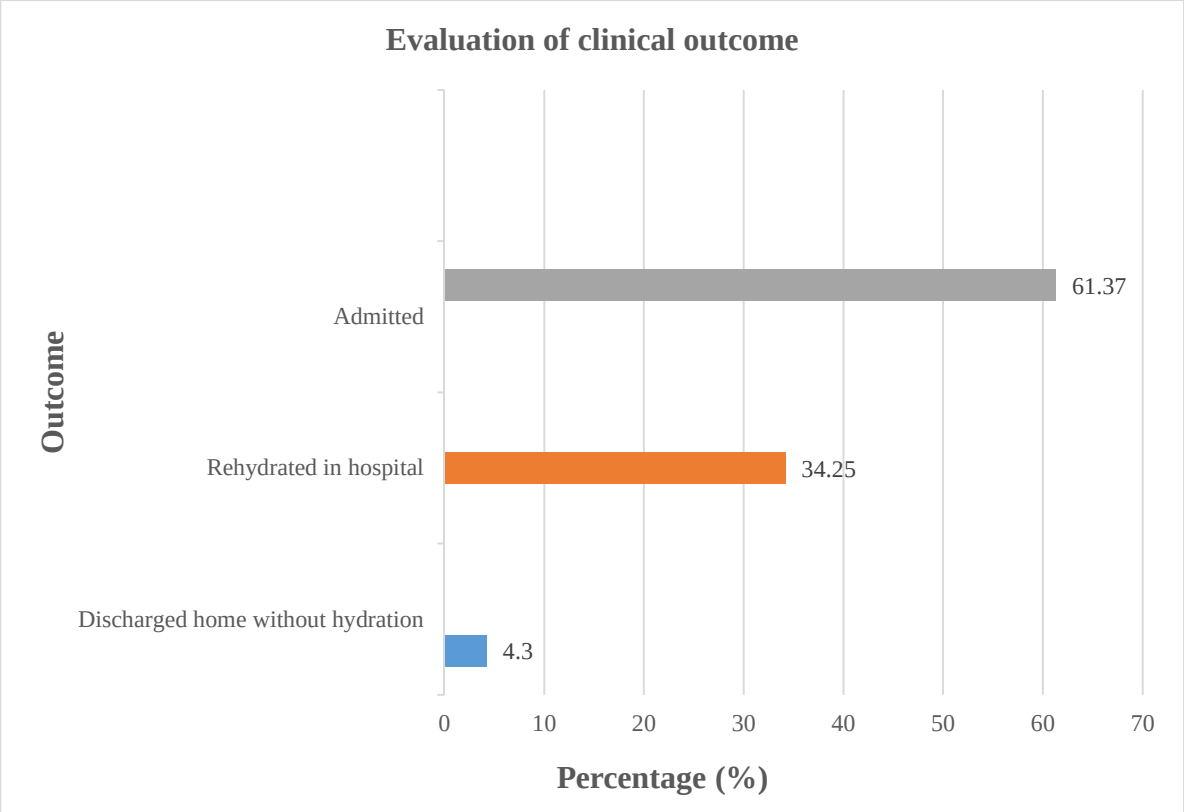


Figure 5: Outcome of clinical evaluation

Table 5: Duration of admission (days) in hospital

Duration of stay	N= 224	Any Comorbidity	No comorbidity	P-Value
1-2 days	12 (5.4%)	10	2	**0.007**
3-4 days	40 (17.9%)	31	9	
5-6 days	62 (27.7%)	54	8	
≥7 days	110 (49.1%)	105	5	

One hundred and ten children (49.1%) were admitted for >7 days. More than 90% of the children admitted for > 7 days had associated comorbidities. One hundred and six children (47.3%) were discharged while 18 (8.0%) died. This is outlined in figure 6.

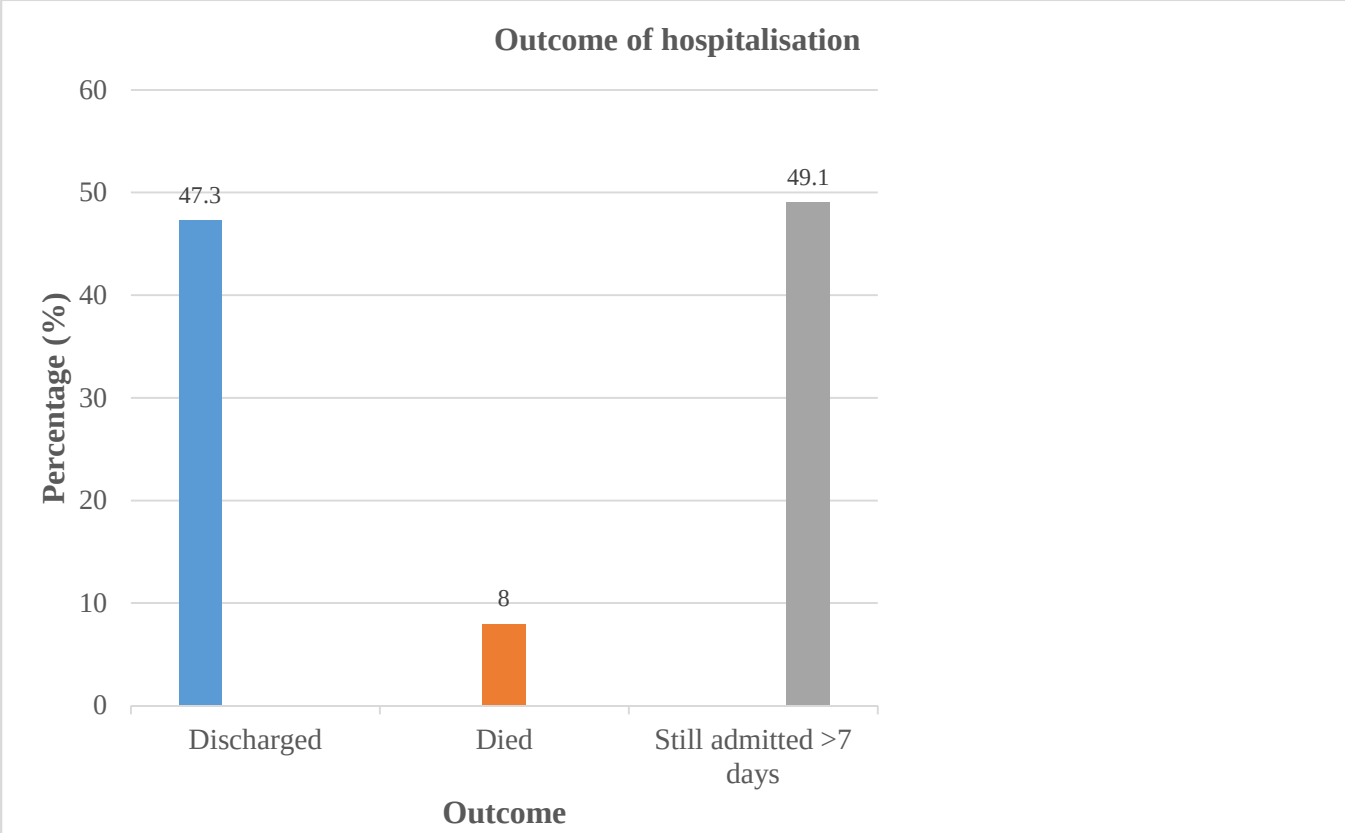


Figure 6: Outcome of hospitalization

Of the 365 children, 53(14.5%) stool samples tested positive for rotavirus while 312(85.5%) were negative. The prevalence of rotavirus infection was 14.5% (95% CI 11.1 -18.6) among children aged 3-24 months presenting with diarrhea at KNH.

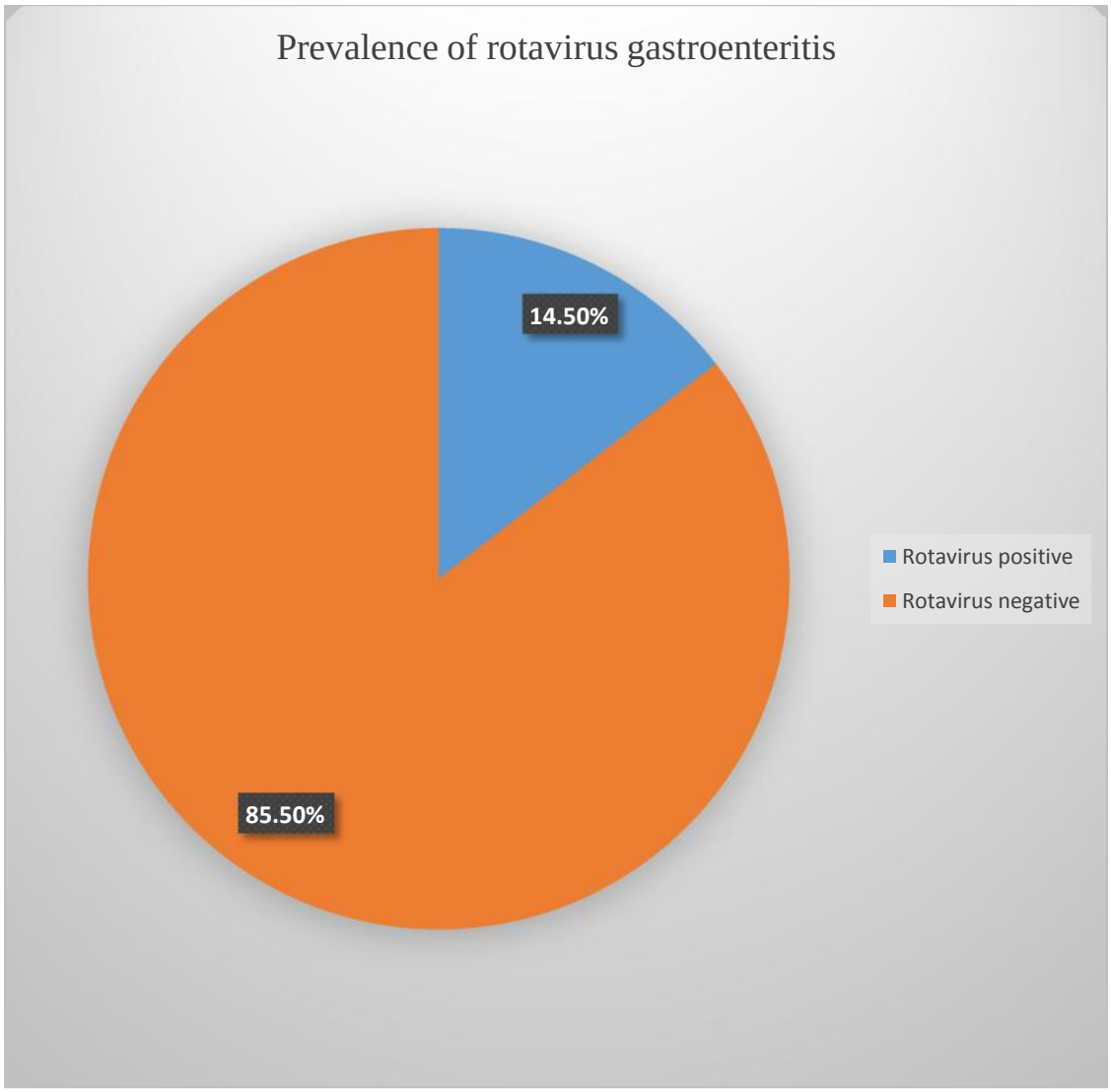


Figure 7: Prevalence of rotavirus in stool

As illustrated in table 9, using the Vesikari Clinical Severity score; 21 children (39.6%) were rated mild, 17 (32.1%) were rated moderate while 15 (28.3%) were rated severe.

Table 6: Distribution of cases within the clinical parameters of the vesikari scoring for severe gastroenteritis n=53

CLINICAL PARAMETER	CHARACTERISTICS	DISTRIBUTION OF CASES (%)
Diarrhoea Duration (Days)	1-4	42(79.3)
	5	5(9.4)
	≥6	6(11.3)
Frequency of diarrhea per day	3	29(54.7)
	4-5	19(35.9)
	≥6	5(9.4)
Duration of vomiting (days)	0	5(9.4)
	1	22(41.5)
	2	15(28.3)
	≥3	11(20.8)
Frequency of vomiting per day	0	5(9.4%)
	1	17(32.1)
	2-4	24(45.3)
	≥5	7(13.2)
Temperature (°C)	37.1-38.4	18(33.9)
	38.5-38.9	25(47.2)
	≥39	10(18.9)
Dehydration status	None	9(17)
	Some dehydration	6(11.3)
	Severe dehydration/shock	38(71.7)
Treatment	Rehydrated in outpatient	21(39.6)
	Admitted and rehydrated	32(60.4)
Severity Category	Mild	21(39.6)
	Moderate	17(32.1)
	Severe	15(28.3)

Three hundred and fifty three children (96.7%) had received 2 doses of rotavirus vaccine, 3(0.8%) children had received only one dose while 9(2.4%) children had not been vaccinated for rotavirus.

CHAPTER 5: DISCUSSION

The findings in this study show that the prevalence of rotavirus infection is 14.5% (95% CI 11.1 -18.6) among children aged 3-24 months presenting with acute diarrhoea at Kenyatta National Hospital. This is much lower than that found in previous studies carried out in a similar setting with a similar sample population before the introduction of the rotavirus vaccine. A study done in 2008 by Osano et al at KNH showed a rotavirus prevalence of 38.2 % among children less than 36 months(7). A similar study done at Gertrude's Children's Hospital and its satellite clinics, by Karanja et al in 2009, reported a prevalence of 39.5% among children less than 24 months(9). The above referenced studies were conducted prior to the rotavirus vaccine introduction into the national immunization program. The difference in the study findings could be attributed to the rotavirus vaccine introduction. A Rotavirus Sentinel Surveillance performance feedback report by WHO in 2016 reported a decline of rotavirus among countries in East and Southern Africa from 40% in 2014 to 25% in 2015, after introduction of rotavirus vaccine after 2013(12). This hospital-based study finding however, may not be a true reflection of rotavirus burden in the community since the study was conducted in the hospital paediatric casualty and wards. Interestingly, some studies have observed a reduction of rotavirus prevalence in the community. A community study done in Nicaragua detected a 40% lower incidence rate of diarrheal episodes suggestive of rotavirus infection in the vaccine period as compared with the pre-vaccine period. This reduction may be attributable to an overall protective effect of the immunization program on both immunized and unimmunized children(40). Similarly, this study noted that the children who presented with acute diarrhoea and were not vaccinated did not have rotavirus positive stools; it is postulated that it may be as a result of the herd immunity phenomenon(40). In addition, due to the presence of other diarrhea aetiologies, the use of rotavirus vaccines should be part of a comprehensive strategy to control diarrhoeal diseases with the scaling up of both prevention and treatment services.

The duration of diarrhea in majority of the children was 1-4 days. This is much lower than is described by Gatinu et al at 4.9 days(41). In addition, the frequency of diarrheal episodes was reported by most children as 3 episodes per day in this study compared to

5.6 episodes in Osano et al study(7). The Vesikari Clinical Severity scoring system for severe gastroenteritis was used in this study. The prevalence of severe rotavirus associated diarrhoea in this study was 28.3% while 71.4% had mild to moderate rotavirus associated diarrhoea. Some studies have described severity of rotavirus associated diarrhoea using the vesikari clinical scoring system, while some studies have described severity using the hydration status(9,41) or the need for hospitalisation as a marker for severity of illness(7). Gatinu's study in 2007 reported a 47.9% prevalence of severe dehydration as a marker of severe rotavirus disease, this is much higher compared to 8% in Karanja et al's study in 2010 and 28.3% in this study(9,41). The lower prevalence of severe rotavirus associated diarrhoea in this study could be explained by introduction of the rotavirus vaccine. Despite having few cases of severe rotavirus associated diarrhoea, the hospitalisation rate was found to be above 60%. Majority of the children admitted had associated comorbidities necessitating hospitalisation with the commonest being pneumonia. Severe dehydration commonly presents as fast and deep acidotic breathing due to electrolyte imbalances and metabolic acidosis as a result of fluid loss and may be misdiagnosed as pneumonia due to similar presentation(42). However, there have been studies that have shown concurrent pneumonia infection in children presenting with diarrhoea(43). Severe rotavirus associated diarrhoea in this study was found to be increased twofold in children aged 6-12 months compared to those aged 13-24 months. This is similar to a study done in India that showed a similar increase in the prevalence of severe gastroenteritis in children aged between 7-12 months(44). According to the WHO scientific working group, the peak incidence of of rotavirus infections occurs at 9 to 12 months with most cases occuring in children between 6 and 24(45). Younger children tend to be at an increased risk of developing severe gastroenteritis due to their small body size, as they appear to lose a greater portion of their total fluid volume during the illness.

The vaccination status among the children in this study was found to be at 96.7% against rotavirus. This is relatively high as compared to what was reported in the KDHS 2014/2015 where 79% had received all the basic vaccinations. Vaccination of the child was verified from the maternal and child booklet and/or word of mouth from the parent. For the parents who did not recall the names of the vaccines received, they described the vaccine by route of administration and the age of the child when they received the

particular vaccine. The latter method of vaccine verification is unlikely to be as accurate as the written and dated records with a high likelihood of over reporting. There was no specific rotavirus vaccine coverage in the KDHS by 2014 as the vaccine had just been rolled out for use in the country in mid-2014 however, it is estimated that the vaccine coverage would approximate 87-90% in relation with the other vaccines co-administered together with rotavirus in the KEPI schedule. The high vaccination status in this study can explain the reduced prevalence of rotavirus infection in this study. This finding is similar to what was reported by Gurgel et al in brazil following commencement of rotavirus vaccine into the Brazil immunization program in 2006, by 2008 the vaccine coverage was found to be 90.3% and the prevalence of rotavirus gastroenteritis was 11%(46). In a similar study done in South Africa by Msimang et al, following commencement of rotavirus vaccine into the national immunization program in 2009, they reported a decline in RV positivity from 46% in 2009 to 33% in 2010 and a further decline of 29% in 2011 among children < 5 years with a rotavirus vaccine coverage in the range of 40-78%(37). Majority of the children enrolled in this study, attended various public hospitals for immunization. The high vaccination status reported could be attributed to increased awareness by sensitization of the mothers by health workers in the maternal and child health clinics and also mass campaigns in the media.

STRENGTHS

One of the strengths of this study was the large sample size which enabled us to give good estimates of the prevalence of rotavirus associated diarrhea. We were able to use the Vesikari Clinical Severity Scoring Scale which is a uniform and objective tool of determining the severity of rotavirus gastroenteritis.

LIMITATIONS

Interpretation of our study results should be with caution given the following limitations;

1. Sample population in one location hence not reflective of the general population.
2. Vaccination of the child was verified from the maternal and child booklet as well as word of mouth from the parent with a high likelihood of over reporting.

3. Those children who were unable to provide a stool sample for rotavirus antigen testing within 24 hours were excluded automatically from the study even though they may have had rotavirus associated diarrhoea.
4. The study assumes that all the study patients received standardized care in terms of clerkship, accurate diagnosis and timely management.
5. The study did not look at the timing of rotavirus vaccine dose in relation to diarrhea to differentiate those with vaccine induced diarrhea.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The findings from this study indicate that the prevalence of rotavirus infection among children with acute diarrhoea is 14.5%. Twenty eight percent of children had severe rotavirus associated diarrhoea as scored by the Vesikari Clinical Severity Score scale. The rotavirus vaccination status was 96.7% among the children.

6.2 Recommendations

Advocacy on use of the Rotavirus vaccine should continue due to the observed reduction in the prevalence of rotavirus associated diarrhoea.

More studies need to be carried out in the region to determine what serotypes are causing diarrhea following vaccine introduction in Kenya.

REFERENCES

1. Tate JE, Burton AH, Boschi-Pinto C, Parashar UD. Global, Regional, and National Estimates of Rotavirus Mortality in Children under 5 Years of Age, 2000–2013. Clin Infect Dis. 2016;62(suppl 2).
2. Tate JE, Burton AH, Boschi-Pinto, Cynthia. et al. 2008 Estimate of Worldwide Rotavirus-Associated Mortality in Children Younger Than 5 Years Before the Introduction of Universal Rotavirus Vaccination Programmes: a Systematic Review and Meta-Analysis. Lancet Infect Dis. 2012;12(2):136–41.
3. Kovacs SD, Mullholland K, Bosch J, Campbell H, Forouzanfar MH, Khalil I, et al. Deconstructing the differences : a comparison of GBD 2010 and CHERG ' s approach to estimating the mortality burden of diarrhea , pneumonia , and their etiologies. 2015;15:1–15.
4. Tate JE, Rheingans RD, O'Reilly, Ciara E. et al. Rotavirus Disease Burden and Impact and Cost-Effectiveness of a Rotavirus Vaccination Program in Kenya. J Infect Dis. 2009;200(s1):S76–84.
5. Cunliffe NA, Kilgore PE, Bresee, J S. et al. Epidemiology of rotavirus diarrhoea in Africa: A review to assess the need for rotavirus immunization. Bull World Health Organ. 1998;76(5):525–37.
6. Parashar UD, Hummelman EG, Bresee, Joseph S. et al. Global illness and deaths caused by rotavirus disease in children. Emerg Infect Dis. 2003;9(5):565–72.
7. Osano BO, Kamenwa RW, Wamalwa D. Short Term Clinical Outcome of Children with Rotavirus infections at Kenyeatta National Hospital, Nairobi. East Afr Med J. 2010;87(6):7–9.
8. Mwenda JM, Ntoto KM, Abebe, Almaz. et al. Burden and epidemiology of rotavirus diarrhea in selected African countries: preliminary results from the African Rotavirus Surveillance Network. J Infect Dis. 2010;202 Suppl(Suppl 1):S5–11.
9. Karanja, C., Kamenwa, R., Nduati, R. et al. Prevalence of rotavirus in children presenting with acute gastroenteritis at Gertrude's Children's Hospital and clinics. 2010.

10. Statistics KNB of, Nairobi K, Health M of, Nairobi K, Council NAC, Nairobi K, et al. Kenya. 2014.
11. Waggie Z, Hawkridge A, Hussey, Gregory D. et al. Review of rotavirus studies in Africa: 1976-2006. J Infect Dis. 2010;202 Suppl:S23–33.
12. Africa S. NEW VACCINES SURVEILLANCE FEEDBACK WHO Inter-Country Support Team : East and Southern Africa WHO Inter-Country Support Team : East and Southern Africa. 2016;1–9.
13. Lewis K. Vesikari Clinical Severity Scoring System Manual. 2011;1.3(May):7–11.
14. Gatheru Z, Kobayashi N, Adachi, N. et al. Characterization of human rotavirus strains causing gastroenteritis in Kenya. Epidemiol Infect. 1993;110(2):419–23.
15. Agocs M. Rotavirus Surveillance-Worldwide , 2009. Mmwr Cent Dis Control Prev. 2011;60(16):514–6.
16. Sénécal M, Brisson M, Lebel, Marc H. et al. Measuring the Impact of Rotavirus Acute Gastroenteritis Episodes (MIRAGE): A prospective community-based study. Can J Infect Dis Med Microbiol. 2008;19(6):397–404.
17. Rivest P, Proulx M, Lonergan, Guy. et al. Hospitalisations for gastroenteritis: the role of rotavirus. Vaccine. 2004 May 7;22(15–16):2013–7.
18. Parashar UD, Burton A, Lanata, Claudio. et al. Global Mortality Associated with Rotavirus Disease among Children in 2004. J Infect Dis. 2009;200(s1):S9–15.
19. Liakopoulou E, Mutton K, Carrington, D. et al. Rotavirus as a significant cause of prolonged diarrhoeal illness and morbidity following allogeneic bone marrow transplantation. Bone Marrow Transplant. 2005;36(8):691–4.
20. Champsaur H, Questiaux E, Prevot J, Goldszmidt D, Bourjouane M, Bach C. Rotavirus Carriage, As)!mptomatic Infection, and Disease in the First Two Years of Life. I. Virus Shedding. J Infect Dis. 1984;149(5):667–74.
21. Lewis KDC, Dallas MJ, Victor, John C. et al. Comparison of two clinical severity scoring systems in two multi-center, developing country rotavirus vaccine trials in Africa and Asia. Vaccine. 2012 Apr 27;30 Suppl 1:A159-66.

22. Omore R, Tate JE, Reilly CEO, Ayers T, Williamson J, Moke F, et al. Epidemiology , Seasonality and Factors Associated with Rotavirus Infection among Children with Moderate-to-Severe Diarrhea in Rural Western Kenya , 2008 – 2012 : The Global Enteric Multicenter Study (GEMS). 2016;2008–12.
23. Ruuska T, Vesikari T. Rotavirus disease in Finnish children: use of numerical scores for clinical severity of diarrhoeal episodes. Scand J Infect Dis. 1990 Jan;22(3):259–67.
24. Alonso L, Domínguez G. Tests for the rapid diagnostic of rotavirus English translation. 2007;1–6.
25. GLASS RI, STOLL BJ. The Protective Effect of Human Milk against Diarrhea: A Review of Studies from Bangladesh. Acta Paediatr [Internet]. 1989 Mar [cited 2017 May 1];78(s351):131–6. Available from: http://doi.wiley.com/10.1111/j.1651-2227.1989.tb11225.x
26. Prevention of Rotavirus Gastroenteritis Among Infants and Children: Recommendations of the Advisory Committee on Immunization Practices (ACIP).
27. Vesikari T, Karvonen A, Prymula, R. et al. Efficacy of human rotavirus vaccine against rotavirus gastroenteritis during the first 2 years of life in European infants: randomised, double-blind controlled study. Lancet. 2007;370(9601):1757–63.
28. Linhares AC, Velázquez FR, Pérez-Schael, Irene. et al. Efficacy and safety of an oral live attenuated human rotavirus vaccine against rotavirus gastroenteritis during the first 2 years of life in Latin American infants: a randomised, double-blind, placebo-controlled phase III study. Lancet. 2008;371(9619):1181–9.
29. WHO. Rotavirus Vaccines: WHO Position Paper. Wkly Epidemiol Rec. 2007;82(32):285–96.
30. Madhi SA. Effect of Human Rotavirus Vaccine on Severe Diarrhea in African Infants — NEJM. NEJM. 2010. p. vol 362.
31. Zaman K, Anh DD, Victor, John C. et al. Efficacy of pentavalent rotavirus vaccine against severe rotavirus gastroenteritis in infants in developing countries in Asia: a randomised, double-blind, placebo-controlled trial. Lancet. 2010;376(9741):615–23.

32. Armah GE, Sow SO, Breiman, Robert F. et al. Efficacy of pentavalent rotavirus vaccine against severe rotavirus gastroenteritis in infants in developing countries in sub-Saharan Africa: A randomised, double-blind, placebo-controlled trial. Lancet. 2010;376(9741):606–14.
33. Vesikari T, Matson DO, Dennehy, Penelope. et al. Safety and efficacy of a pentavalent human-bovine (WC3) reassortant rotavirus vaccine. N Engl J Med. 2006;354(1):23–33.
34. Ruiz-Palacios GM, Pérez-Schael I, Velázquez, F Raúl. et al. Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis. N Engl J Med. 2006 Jan 5;354(1):11–22.
35. Rotarix - Summary of Product Characteristics (SPC) - (eMC).
36. Carmo G. M, Yen, C., Cortes, J. et al. Decline in diarrhea mortality and admissions after routine childhood rotavirus immunization in Brazil: A time-series analysis. PLoS Med. 2011;8(4):11.
37. Msimang VMY, Page N, Groome, Michelle J. et al. Impact of rotavirus vaccine on childhood diarrheal hospitalization after introduction into the South African public immunization program. Pediatr Infect Dis J. 2013;32(12):1359–64.
38. Enweronu-Laryea CC, Boamah I, Sifah, Eric. et al. Decline in severe diarrhea hospitalizations after the introduction of rotavirus vaccination in Ghana: a prevalence study. BMC Infect Dis. 2014;14(1):431.
39. Gautam R, Lyde F, Esona MD, Quaye O, Michael D, Viruses R. Detection of Rotavirus Antigen in Stool Specimens. 2015;58(1):292–4.
40. Liu L, Zambrana LE, Paniagua M, Weber DJ, Becker-dreps S, Mele M, et al. Community Diarrhea Incidence Before and After Rotavirus Vaccine Introduction in Nicaragua. 2013;89(2):246–50.
41. Gatinu BW. Prevalence of Group A Rotavirus and Electrolyte profiles in children presenting with Acute Diarrhoea at Kenyatta National Hospital. 2007;
42. Center for Disease Control and Prevention (CDC). Rotavirus Rotavirus. Pink B. 2014;263–74.

43. Das SK, Faruque ASG, Malek MA, Chisti MJ, Leung DT, Qadri F, et al. Concurrent Pneumonia in Children Under 5 Years of Age Presenting to a Diarrheal Hospital in Dhaka, Bangladesh. Am J Trop Med Hyg [Internet]. 2015 Oct 7 [cited 2017 Jun 12];93(4):831–5. Available from: http://www.ajtmh.org/content/journals/10.4269/ajtmh.15-0074

44. Jain V, Parashar UD, Glass RI, Bhan MK. Epidemiology of rotavirus in India. Indian J Pediatr [Internet]. 2001 Sep [cited 2017 May 1];68(9):855–62. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11669034

45. Group WSW. Rotavirus and other viral diarrhoeas: WHO scientific working group. Bull World Health Organ [Internet]. 1980 [cited 2017 May 1];58(2):183–98. Available from: http://www.ncbi.nlm.nih.gov/pubmed/6249509

46. Gurgel RG, Bohland AK, Vieira SCF, Oliveira DMP, Fontes PB, Barros VF, et al. Incidence of Rotavirus and All-Cause Diarrhea in Northeast Brazil Following the Introduction of a National Vaccination Program. Gastroenterology [Internet]. 2009;137(6):1970–5. Available from: http://dx.doi.org/10.1053/j.gastro. 2009.07.046

APPENDICES

Appendix 1: Consent Form

ROTAVIRUS DIARRRHOEA STUDY GUARDIANS CONSENT FORM:

Date: ……..

Study Title: PREVALENCE OF ROTAVIRUS GASTROENTERITIS AMONG CHILDREN WITH DIARRHOEA IN KENYATTA NATIONAL HOSPITAL AFTER INTRODUCTION OF ROTAVIRUS VACCINE.

Investigator: Dr Catherine Muendo

Paediatric Resident, University of Nairobi

Tel Number: - 0723-657149

Investigator's Statement:

We are requesting you and your child to kindly participate in this research study. The purpose of this consent form is to provide you with the information you will need to help you decide whether to participate in the study. This process is called 'Informed Consent'. Please read this consent information carefully and ask any questions or seek clarification on any matter concerning the study with which you are uncertain.

Introduction:

Rotavirus gastroenteritis is the most common cause of severe diarrhoea among infants and young children. It is caused by a virus called Rotavirus. It is highly communicable. Other symptoms may include vomiting which precedes the diarrhoea and fever.

The main transmission mode is the faecal-oral route. Since the virus is stable in the environment, the transmission can occur through close person to person contact, ingestion of contaminated water or food, through contact with contaminated surfaces such as toys or food preparation counters. There is no specific treatment for Rotavirus. The principle of management is to correct dehydration. Antidiarrhoeal medicines as well as antibiotics are discouraged.

Vaccination has been recommended to be the most effective method in reducing morbidity and mortality associated with rotavirus infection. This is offered in Kenya by KEPI.

This study seeks to establish the prevalence of rotavirus gastroenteritis among children 3-24 months presenting with diarrhoea and to identify factors associated with positive rotavirus disease.

Benefits:

You will receive education regarding various components that help prevent diarrhoea such as proper food handling, hand washing practices, exclusive breastfeeding and vaccination against rotavirus.

Risks:

There will be no risks to you or your child during the study. There will be no invasive procedures carried out in the study that may harm your child.

Refusal to participate will not jeopardize the treatment of your child in any way.

Voluntariness:

The study will be fully voluntary. One is free to participate or withdraw from the study at any point. We expect you to answer the questions in the questionnaire truthfully and provide us with your child's stool specimen for testing within 24 hours. Refusal to participate will not compromise your child's care in any way.

Compensation:

There will be no financial rewards to you for participating in the study due to limited resources.

Confidentiality:

The information obtained about you, your child and your family will be kept in strict confidence. All results will be given to you and your primary clinician. No specific information regarding you, your child or your family will be released to any person without your written permission. We will, however, discuss general overall findings regarding all children assessed but nothing specific will be discussed regarding your child. We will also, not reveal the identity of you or your child in these discussions.

Problems or Questions:

If you ever have any questions about the study or about the use of the results you can contact the principal investigator, **Dr Muendo Catherine** by calling **0723-657149.**

If you have any questions on your rights as a research participant you can contact the **Kenyatta National Hospital-University of Nairobi Ethics and Research Committee (KNH- UON ERC)** by calling **2726300 Ext. 44355.**

Consent Form: Participant's Statement:

I……having received adequate information regarding the study research, risks, benefits hereby AGREE / DISAGREE (Cross out as appropriate) to participate in the study with my child. I understand that our participation is fully voluntary and that I am free to withdraw at any time. I have been given adequate opportunity to ask questions and seek clarification on the study and these have been addressed satisfactorily.

Parents Signature……..

Date……..

I .....declare that I have adequately explained to the above participant, the study procedure, risks and benefits and given him /her time to ask questions and seek clarification regarding the study. I have answered all the questions raised to the best of my ability.

Interviewers Signature……. Date……

Appendix 2: Questionnaire

Date..... Unique Study number.....

SECTION A: SOCIODEMOGRAPHIC INFORMATION OF THE CHILD

1. Age (months).....

2. Sex (check one)

……. (1) Male

……. (2) Female

3. Weight (grams).....

4. Length/Height (cms).....

5. Residence.....

6. Breastfeeding status of child (check where appropriate.)

…… (1) Still breastfeeding

……..... (2) Stopped breastfeeding if so when..... (Age in months)

……... (3) Never breastfed

7. Duration of exclusive breastfeeding..... (Months)

8. If not breastfed, what type of feed (Check one)

……... (1) Formula

…… (2) Cow's milk

…… (3) Others (specify)…….

SECTION B: CAREGIVER SOCIODEMOGRAPHIC INFORMATION.

9. Age of primary caregiver..... (years)

10. Relationship of caregiver to the child (check one)

…… (1) Mother

…… (2) Father

…… (3) Other(s) (Specify).....

11. Level of education of caregiver (check one)

……. (1) Tertiary

..... (2) Secondary

..... (3) Primary

..... (4) None

12. Occupation (check one)

……. (1) Employed

..... (2) Self-employed

..... (3) Unemployed

13. Caregiver's monthly income(Kshs) (check where appropriate)

…… (1) <3000

…… (2) 3000-5000

……. (3) 5001-7000

……. (4) 7001-10000

……. (5) 10000-20000

……. (6) >20000

14. Age of other children within the household ..... …..... ….....

15. What is the source of water in the homestead?(check where appropriate)

……... (1) Individual tap water

……. (2) Community tap water

……. (3) Borehole

……. (4) Well

……. (5) River

……. (6) Other(s) specify……..

16. How is water stored?(check where appropriate)

…… (1) Water tanks

……. (2) Water drums

……. (3) Water bucket

……. (4) Sufuria

……. (5) Other(s) specify…….

17.How often do you wash your hands?(check one)

Before preparing a meal? …… (1) Yes ……(2) No

After visiting a toilet? ……... (1) Yes …… (2) No

Before eating a meal? ……... (1) Yes …….(2) No

When hands are dirty? ……...(1)Yes …….(2) No

After cleaning the house?.....(1) Yes …….(2) No

After cleaning the baby?.....(1) Yes ……(2) No

18. On average, how many times do you wash your hands in a day?(check one)

…….. (1) 1-3 times

…….. (2) 4-6 times

…….. (3) >6 times

19. What kind of toilet do you use at home?(check where appropriate)

……... (1) Personal toilet

……... (2) Communal toilet

……... (3) None

……... (4) Other(s) specify…….

SECTION C: VACCINATION HISTORY (From mother baby booklet and/or word of mouth by parents.)

20. Has the child been vaccinated for rotavirus? (check one)

……. (1) Yes

..... (2) No (If no, skip and proceed to number 24)

..... (3) Unknown

21. If vaccinated, has the child completed vaccination against rotavirus? (check one)

……. (1) Yes

..... (2) No

..... (3) Unknown

22. Number of doses of rotavirus vaccine received? (check where appropriate)

……. (1) one

……. (2) two

……. (3) three

……. (4) unknown

23. If not vaccinated, what are the barriers to vaccination? (check where appropriate)

……. (1) Lack of access to a health facility

…….. (2) Poor communication by providers

……. (3) Vaccine not useful

……. (4) Vaccine not safe

……. (5) Lack of knowledge about vaccination

…….(6) Other(s) specify……

24. Are other KEPI vaccines up to date? (check where appropriate)

BCG …….. (1) Yes …… (2) No

Oral polio …… Dose 1 ……. Dose 2 ……. Dose 3

Pentavalent …….. Dose 1 ..... Dose 2 ……. Dose 3

Pneumoccocal …….. Dose 1 ……. Dose 2 ..……Dose 3

Measles …….. (1) Yes ……. (2) No

SECTION D: CLINICAL HISTORY

25. Duration of diarrhoea? (Days) (check one)

……..(1) 1-4

……..(2) 5

……..(3) ≥6

26. Maximum number of stools per day (check one)

……..(1) 1-3

……..(2) 4-5

……..(3) ≥6

27. Duration of vomiting? (Days) (check one)

……..(1) 1

……..(2) 2

……..(3) ≥3

28. Maximum number of vomiting episodes per day (check one)

……..(1) 1

……..(2) 2-4

……..(3) ≥5

29. Presence of fever? (check one)

……. (1) Yes

..... (2) No

30. Temperature recorded during triage (°C) (check one)

…….. (1) 37.1-38.4

…….. (2) 38.5-38.9

…….. (3) ≥39.0

31. Prior care sought before child brought to KNH? (check one)

……. (1) Yes

……. (2) No (if no, proceed to question 35)

32.If yes, check where appropriate. If no, move to question 35

…… (1) Public facility

…… (2) Private facility

…… (3) Over the counter/chemist

…… (4) Herbalist/traditional healer

…… (5) Others (specify)……

33. Referring facility? (check where appropriate)

…… (1) Private

…… (2) Public

…… (3) Self-referral

34. Assessment for dehydration (check one)

A) Level of consciousness

……..(1) Alert

……..(2) Verbal

……..(3) Pain

……..(4) Unconscious

B) Pulse

……..(1) Normal volume

……..(2) Weak

……..(3) Impalpable

C) Cold extremities

……..(1) Yes

……..(2) No

D) Sunken eyes

……..(1) Yes

……..(2) No

E) Skin pinch

…….(1) Immediate

…….(2) 1-2sec

…….(3) >2 sec

F) Capillary refill time

……..(1) Immediate

……..(2) 1-2sec

……..(3) 2 sec

G) Ability to drink

……..(1) Yes

……..(2) No

35. Hydration status?

…….....(1) Shock

……..(2) Severe dehydration

……..(3) Some dehydration

……..(4) No dehydration

36. Nutrition status?

……...(1) Normal

……...(2) Visible severe wasting

……...(3) Bilateral pitting oedema

37. Other associated problems (yes/no)

a) Convulsions…….(1) yes ……..(2) no

b) Respiratory distress…….(1) yes ……(2) no

c) Abdominal distension……..(1) yes ……(2) no

d) Others (specify)……, ……, ……..,

38. Comorbidities recorded? ..... (1) yes ……..(2) no

If yes, Specify……, ……., …….,

39. HIV status (check where appropriate)

……..(1) Exposed (proceed to no. 42)

……..(2) Not exposed (skip to no.44)

……..(3) Positive (skip to no. 44)

……..(4) Not done (skip to no. 44)

……..(5) Declined (skip to no. 44)

40. If HIV exposed, PCR done? ……(1) yes ……...….(2) No

41. If PCR done, Result?.....(1) positive ……..(2) negative

42. Outcome?

…….(1) Discharged home

…….(2) Rehydrated in hospital

…….(3) Admitted

43. Duration of admission from OPD …….(days)

44. Outcome of hospitalization?

……..(1) Discharged

……..(2) Died

……..(3) Absconded

……..(4) Still admitted after 7 days

45. Stool analysis for rotavirus

……..(1) Positive

……..(2) Negative

Name of Research Assistant……

Appendix 3: Anthropometric measurements and Physical Examination Standard Operating Procedures

Weight was measured using a digital infant scale for the infants. The scale was covered with paper and activated by turning it on such that it displayed zero on the display panel. The baby was undressed and placed on the tray of the scale. The weight was recorded to the nearest 0.1kg as it appeared on the display panel. An average of 3 measurements taken was recorded. For the toddlers who are able to stand, the weight was measured using a well calibrated balance beam. The scale was placed on a hard-floor surface. Calibration was done at the beginning and end of each examining day. The toddler was undressed and put to stand in the centre of the platform. The weights were moved until the beam balanced. The weight was recorded to the nearest 0.1 kg. An average of 3 measurements taken was recorded.

Length was taken using an infantometer. The child's socks and shoes were removed where applicable and child was placed in recumbent position on top of the horizontal board with the feet towards the foot piece and the head against the fixed head piece. Both legs were straightened with toes pointing directly up and the foot-board was moved into position against the child's feet. The length was measured to the nearest 0.1cm. An average of 3 measurements taken was recorded.

Temperature recording was taken by use of a digital thermometer that was placed in the axilla. The digital thermometer was cleaned with alcohol swabs after each use. All non-rectal temperatures were converted to the rectal equivalent*

*Rectal Equivalent Conversion:

1. Convert the temperature to Fahrenheit

• Tfahrenheit = (9/5*Tcelsius) +32,

2. Add 2 degrees for Axillary (1 degree for oral or otic)

3. Convert back to Celsius

• Tcelsius = (5/9)*(Tfahrenheit -32)

This formula raised the axillary temperature by 1.1^{0}C.

Hydration Status Assessment

1. **Pulse character**- was felt at the brachial artery for those under 1 year, and the carotid artery for those above 1 year and defined as weak/thready, normal or impalpable.

2. **Limb extremities**-upper and lower limb was examined from distal to proximal to identify for any temperature gradient.

3. **Capillary refill time**- was performed according to the Emergency Triage and Treatment (ETAT) Kenya protocols 2015. The palmar aspect of the thumb/toe was pressed for 5 seconds and released and the time taken for refill was recorded in seconds.

4. **Skin Pinch**-Landmarks were between the umbilicus and the lateral flanks. The skin was grasped and tented up between the thumb and the forefinger and held up for a few seconds then released and the time taken for return to its original state recorded in seconds. This maneuver was excluded in the malnourished children because of high false positive results due to laxity of the skin. However, they were assessed using the other maneuvers mentioned in bullet 3, 4,5,7,8 and 9.

5. **Consciousness level**-was determined using the 'AVPU' scale which is an acronym that represents whether the child is Alert, responding to Voice, responding to Pain or Unconscious.

6. **Eyes**- were examined with the help of the guardian's response and classified as either sunken or normal.

7. **Level of thirst**- was assessed by offering the child clean water or ORS and assessed whether the child drinks poorly/not able to drink, thirsty/drinks eagerly, or drinks normally/not thirsty.

Appendix 4: Vesikari Clinical Severity Scoring System

		SCORE	
PARAMETER	1	2	3
Diarrhoea			
Maximum Number Stools per Day	3	4-5	≥6
Diarrhoea Duration (Days)	1-4	5	≥6
Vomiting			
Maximum Number Vomiting Episodes per Day	1	2-4	≥5
Vomiting Duration (Days)	1	2	≥3
Temperature (rectal °C)	37.1-38.4	38.5-38.9	≥39.0
Dehydration	None	Some	Severe
Treatment	Rehydration	Hospitalization	N/A

SEVERITY CATEGORY			
MILD	**MODERATE**	**SEVERE**	**MAXIMUM SCORE**
<7	7-10	>11	20

Appendix 5: Laboratory Procedures

Approximately 0.1g of solid faeces or approximately 100 µl of liquid faeces was placed in a suitable labelled container and 1ml of sample diluent was added to prepare a 10% suspension which was mixed thoroughly.

Two drops of each diluted specimen, negative control or positive control was added to the separate microwells. At least one Negative Control and one Positive Control was included in each batch of tests. After addition of all specimens and controls, 2 drops of Conjugate was added to each microwell. The plate was covered and the microwells were incubated at 20-30°C for 60 ± 5 minutes. The contents of the wells were aspirated. Each well was washed by completely filling each well with diluted Wash Buffer (~350-400 µl per well). The contents of the wells were aspirated after each wash. The wells were washed a total of 5 times. After the last wash, the contents were removed and the plate was inverted and tapped on absorbent paper to remove the last traces of wash buffer. Two drops of Substrate was added to each microwell. The plate was covered and the microwells incubated at 20-30°C for 10 minutes. The Substrate reaction was stopped by adding 2 drops of Stop Solution to each microwell. The microwells were thoroughly mixed before reading the results. The result was read spectrophotometrically at 450 nm. A positive result is when the clinical sample absorbance value > the cutoff value. A negative result is when the clinical sample absorbance value < the cutoff value. An equivocal result is when the clinical sample absorbance value within 0.010 absorbance units of the cut-off value. The Negative Control value, or mean of the Negative Control values, should be less than 0.150 absorbance units. The Positive Control value must be greater than 0.500 absorbance units.

Quality Control Measures

At least one Positive and one Negative Control was included each time the test is performed.