AETIOLOGICAL AGENTS OF DIARRHOEA AMONG UNDERFIVES IN DAR ES SALAAM, TANZANIA

By

Sabrina J Moyo M.D (UDSM)

A dissertation submitted in partial fulfilment of the requirements for the degree of Master of Medicine in Microbiology and Immunology of the Muhimbili University of Health and Allied Sciences.

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CERTIFICATION

The undersigned certify that they have read and here by recommend for acceptance by the Muhimbili University of Health and Allied Sciences a dissertation entitled ***Aetiological agents of diarrhoea among under fives in Dar es Salaam Tanzania,*** in fulfilment of the requirements for the degree of Master of Medicine (Microbiology/Immunology) of the Muhimbili University of Health and Allied Sciences

.....

Prof . S. Y Maselle

Muhimbili University of Health and Allied Sciences

(Supervisor)

Date 5th November 2007

.....

Dr. Haima Mylvaganam

University of Bergen

(Supervisor)

Date 5th November 2007.

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I Sabrina J Moyo, declare that this dissertation is my own original work and that it has not been presented and will not be presented to any other University for a similar or any other degree award.

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DEDICATION

I dedicate this dissertation to my beloved husband Joshua and my parents.

ABSTRACT

Background

Diarrhoea in children is caused by viruses, bacteria and parasites that show geographical differences in distribution.

Objective: To determine the etiologic agents of diarrhoea among under fives in Dar es Salaam Tanzania.

Study design, population and setting: A cross sectional study involving 280 children aged <5 years admitted with diarrhoea in four hospitals in Dar es Salaam.

Methods: Bacterial pathogens were identified using conventional methods. ELISA and rapid assay were used to detect viruses and intestinal protozoa, respectively. Virulence genes for diarrhoeagenic *E.coli* (DEC) and typing of group A rotavirus were done using multiplex PCR. Antimicrobial susceptibility of bacteria was done using Kirby-Bauer method.

Results: At least one enteric-pathogen was detected in 68.5% of the cases, mixed infection detected in 20.0% cases. DEC was the commonest enteropathogen isolated 22.9%, followed by *C.parvum* 18.9%, rotavirus 18.5% and norovirus 13.3%.Typical EAEC and EPEC were the most prevalent DEC strains and serotype G9 of group A rotavirus predominated. Bacteria isolates were highly resistant to erythromycin (76.5%) and co-trimoxazole (73.5%).

Conclusion: Most of diarrhoea was of acute watery type and affected mainly children < 2 years. DEC, *Cryptosporidium,* rotavirus and norovirus were the most prevalent pathogens. Typical EAEC and EPEC were the commonest DEC strains and G9 is the predominating group A rotavirus serotype.

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LIST OF ABBREVIATIONS

AA	Aggregative adherence
AIDS	Acquired immunodeficiency syndrome
DAEC	Diffusely adherent *E. coli*
DA	Diffusely adherence
DEC	Diarrheagenic *E.coli*
DNA	Deoxyribonucleic acid
ETEC	Enterotoxigenic *E. coli*
EPEC	Enteropathogenic *E.coli*
EIEC	Enteroinvasive *E. coli*
EHEC	Enterohemorhagic *E.coli*
EAEC	Enteroaggregative *E.coli*
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunoassay
Hep-2	Human epithelial cell-2
HIV	Human immunodeficiency virus
KIA	Kligler iron agar
LA	localized adherence
LT	labile toxin
MCA	Mac Conkey agar
MIC	Minimum Inhibitory Concentration

MNH	Muhimbili National Hospital
MUHAS	Muhimbili University of Health and Allied Sciences
MOH	Ministry of Health
NA	Nutrient agar
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase-PCR
SF	Selenite F
SSA	Salmonella Shigella agar
ST	Stable toxin
STEC	Shiga toxin producing *E.coli*
TCBS	Thiosulphate Citrate Bile-salt Sucrose
VP	Viral Proteins
WHO	World health Organisation
XLD	Xylose lysine deoxycholate
ZN	Ziel Nelsen

1. INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

Diarrhoea is one of the leading causes of morbidity and mortality among children under five years in the developing world [1]. During the period from 1950 to 1970s it was estimated that 4.6 million children died annually from diarrhoea in developing world [2, 3]. Mortality due to diarrhoea declined to approximately 3.3 million annually in the 1980s [1, 3]. Currently diarrhoea has been reported to account for 1.6–2.5 million deaths annually [3, 4]. Despite the decline in mortality, diarrhoea still remains one of the principal causes of morbidity in the developing world, with each child experiencing an average of three episodes of diarrhoea per year [4]. In these countries, diarrhoeal diseases are the second most common illness of children after acute respiratory infections [5]. About 80% of deaths due to diarrhoea occur in the first 2 years of life and are mostly due to dehydration, which results from loss of fluid and electrolytes and reduced ability to absorb nutrients [6]. Diarrhoeal diseases also present an economic burden for the developing countries, with more than a third of the hospital beds for children being occupied by patients with diarrhoea, often involving the use of expensive intravenous fluids and drugs [6]. Diarrhoea is more common in non-breast-fed infants than in exclusively breast- fed infants for the first four to six months [6]. Prolonged breast- feeding reduces the incidence and severity of certain types of diarrhoea diseases such as shigellosis and cholera [6]. Other factors that may contribute to transmission of diarrhoea include, prolonged storage of cooked food at room temperature, drinking contaminated water, poor hand hygiene and poor sanitary conditions. Host factors include malnutrition, intercurrent illness

and infection with human immunodeffiency virus (HIV) [6]. In addition, age has also been cited as another factor, with most diarrhoea episodes occuring during the first two years of life, especially in the 6 -11 months age group when weaning and supplementary feeding starts [6]. This age specific pattern reflects the combined effects of declining levels of maternally-acquired antibodies and the introduction of food that may be contaminated with faecal bacteria [6]. Most enteric pathogens stimulate partial immunity against repeated infection, which may explain the declining incidence of disease in older children and adults [6].

1.2 Types of diarrhoea

According to WHO guidelines diarrhoea is defined as the passage of three or more loose or watery stools in a 24-hour period [6]. However, a variety of terms have been used to describe diarrhoea, depending, for example, upon whether the stool is loose, watery, bloody or mucoid. The guidelines stipulate three forms of diarrhoea namely:
i) acute watery diarrhoea defined as diarrhoea that begun acutely and lasted less than or equal to 13 days,

ii) Dysentery defined as mucoid bloody stool associated with anorexia, abdominal cramps, and tenesmus and

iii) Persistent diarrhoea defined as diarrhoea with duration of 14 or more days.

1.3 Aetiology of diarrhoea

Until few years ago pathogenic organisms could be identified in the faeces of only about 25% of patients with diarrhoea.

Today, using new techniques, experienced laboratories can identify pathogens in about 75% of cases seen at a treatment facility [6]. Diarrhoea can be caused by a wide range of bacteria, viruses and parasites [7, 8].

1.3.1 Enteric Bacterial-pathogens

A number of bacteria have been associated with diarrhoea including; diarrhoeagenic *Escherichia coli* (DEC), *Campylobacter jejuni*, *Salmonella* species, *Shigella* species, *Yersinia enterocolitica* and *Vibrio cholerae* [6, 7]. The pathogenic mechanisms include adhesion, toxin production and invasiveness.

1.3.1.1 DEC

DEC have been classified into at least six categories according to their pathogenicity traits and they include; enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), shiga toxin producing or enterohaemorrhagic *E. coli* (STEC/ EHEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E.coli* (DAEC) [9]. The epidemiological significance of the different categories varies with the geographical area [9].

DEC categories have been differentiated by serotyping. Currently, characterisation of DEC categories invloves detection of different chromosomal and plasmid-encoded virulence genes as well as detection of their patterns of interaction with cultured cells (Hep-2 cells or He La cells) and the identification of specific bacterial surface antigens [9, 10]

ETEC; strains cause diarrhoea through the action of the enterotoxins labile toxin (LT) and stable toxin. (ST) These strains may express an LT only, an ST only, or both an LT and an ST.

The LTs of ETEC are oligomeric toxins that are closely related in structure and function to the cholera enterotoxin (CT) expressed by *Vibrio cholerae* [9, 11]. There are two major serogroups of LT, LT-I and LT-II, which do not cross-react immunologically [9]. LT-I is expressed by *E. coli* strains that are pathogenic for both humans and animals. LT-II is found primarily in animal *E. coli* isolates and rarely in human isolates, but in neither animals nor humans has it been associated with disease. The genes encoding LT (*elt* or *etx*) reside on plasmids that may also contain genes encoding ST. STs are small, monomeric toxins that contain multiple cysteine residues, whose disulfide bonds account for the heat stability of these toxins [9]. There are two unrelated classes of STs that differ in structure and mechanism of action namely ST I and ST II. ST I is methanol soluble and is active in the infant mouse model, and ST11 is methanol insoluble and it is not active in the infant mouse model [11].

Genes for both classes are found predominantly on plasmids, and some ST-encoding genes have been found on transposons [9]. There are two variants of ST1 designated ST1a and ST1b. Both variants can be found in human ETEC strains [9, 11]. The genes encoding for ST (*st1a*\ *st1b*) also reside on plasmid [12]. ST 1 toxins are produced by ETEC and several other gram-negative bacteria including *Yersinia enterocolitica* and *V. cholerae* non-O1[9]. ST 11 has been found only in ETEC and it is associated primarily with ETEC strains isolated from pigs, although some human ETEC isolates expressing ST11 have been reported [9].

EPEC; causes infantile diarrhoea in developing countries and sporadic diarrhoea in developed countries [13].The organism produces localized adherence (LA) pattern on Hep-2 cells and HeLa cells [9, 14].

The central mechanism of pathogenesis is a lesion called 'attaching and effacing' (A/E), which is characterized by intimate localized adherence of the bacteria to the intestinal epithelium [9, 15]. For the identification of EPEC and classification of this group of bacteria into typical and atypical strains, two genes have been used the *eae* which is a chromosomal gene coding for adhesion intimin located in the pathogenicity island 'locus of enterocyte effacement' (LEE) and the *bfpA* which is a plasmid gene responsible for the formation of bundle-forming pilus [9, 15]. *E.coli* strains with both (eae^+) and the ($bfpA^+$) genes are classified as typical EPEC. Strains of *E.coli* with (eae^+) gene which do not posses ($bfpA^-$) gene are classified as atypical EPEC. Typical EPEC is well recognized as a cause of gastroenteritis in infants [9, 16]. The role of atypical EPEC in childhood diarrhoea is still controversial [15, 17].

EHEC; the term enterohaemorrhagic *Escherichia coli* is used to designate a subset of Shiga toxin –producing *E. coli* STEC, also known as verotoxin-producing *E. coli* VTEC that causes severe diseases in humans, including bloody diarrhoea and the haemolytic uraemic syndrome (HUS) [18, 19]. More than 100 serotypes of *E. coli* were shown to produce shiga toxin 1 (Stx1) or shiga toxin 2 (Stx2) coded by the genes stx_1 and stx_2, respectively [18, 19]. However, the most-common serotype of this category of DEC is O157:H7 [20]. The Stx1 group consists of Stx1, Stx1c, and Stx1d [18]. Five subtypes of Stx2 have been identified, and these include Stx2, Stx2c, Stx2d, Stx2e, and Stx2f [18]. *Eae* positive *E. coli* strains harbouring Shiga

toxin genes (*stx*$_1$ and/or *stx*$_2$) are classified as enterohaemorrhagic *E. coli*. EHEC strains also carry other putative genes which may be associated with virulence, these include genes encoding adhesion molecule intimin (*eae)* [12], enterohaemolysin gene (*ehx*),and an immunomodulator gene (*lif*) [21]. Consequently, the pathogenesis of EHEC strains is likely to be complex and multifactorial [21].

EAEC; is rapidly gaining recognition as an important cause of persistent diarrhoea among children in developing countries, outbreaks of diarrhoea in developed world, diarrhoea of travelers and HIV-infected patients [22]. EAEC strains are characterized by the ability to adhere to HEp-2 cells in an aggregative adherence (AA) pattern, described as a stacked brick formation [23, 24].The pathogenesis of EAEC infection is thought to involve initial adherence to the host intestinal mucosal epithelium via aggregative adherence fimbriae and subsequent destruction of the mucosa, possibly by toxins [23-25]. However, the precise mechanisms by which EAEC causes diarrhoea, and the role of the various pathogenicity factors, are poorly understood. Most strains carry a high molecular- weight plasmid (pAA) associated with AA [25], on which a number of virulence genes are located. These include the AA fimbrial genes, AAFI (*aagA*), AAFII (*aafA*), and AAFIII (*agg3A*). Also located on the plasmid are the transcriptional activator gene (*aggR*), antiaggregation protein (dispersin) gene (*aap*), previously called *asp*U, and an antiaggregation protein transporter gene (*aat*) previously called CVD432 or the AA probe [24, 25]. Other plasmid encoded genes include the plasmid-encoded toxin (*pet*) and enteroaggregative heat-stable toxin (EAST) genes (*astA*) [25]. EAEC also carry chromosomal encoded genes responsible for virulence. These genes include the

*pic*gene which is responsible for expression of a protein involved in colonization, The irp2 gene responsible for expression of yersiniabactin, the *aai* gene, which is located on a pathogenicity island and is thought to be under control of *aggR*, and the putative invasion determinant gene *tia* [25].

EIEC; causes a watery diarrhoea and dysentery in humans [26]. The precise pathogenetic scheme of EIEC has yet to be elucidated. However, pathogenesis studies of EIEC suggest that its pathogenetic features are virtually identical to those of *Shigella* spp. [9, 12]. Both organisms have been shown to invade the colonic epithelium, a phenotype mediated by both plasmid and chromosomal loci. In addition, both EIEC and *Shigella* elaborate one or more secretory enterotoxins that may play roles in diarrhoeal pathogenesis. The site of EIEC infection is the colonic mucosa [9]. All genes required for invasiveness are carried on a 140-MDs plasmid in EIEC. The plasmid gene *ipa* (invasion plasmid antigen) plays essential role in bacterial entry, but the *ial* gene is effective in a multiplex PCR system to identify EIEC strains simultaneously with other *E. coli* categories [9].

DAEC; the term "diffusely adherent *E. coli*" was initially used to refer to any HEp-2-adherent *E. coli* strain that did not form EPEC-like adherence pattern [9]. With the discovery of EAEC, most authors now recognize DAEC as an independent category of potentially diarrhoeagenic *E. coli.* Little is known about the pathogenetic features of DAEC-induced diarrhoea [9]. Bilge et al have described the cloning and characterization of a surface fimbria in this strain, which mediates the diffusely adherent (DA) phenotype pattern [9]. The genes encoding the fimbria can be found

on either the bacterial chromosome or a plasmid. Several recent studies have implicated DAEC strains as agents of diarrhoea, while other studies have not recovered DAEC strains more frequently from diarrhoeal patients than from asymptomatic controls [9]. DAEC strains are defined by the presence of the DA pattern in the HEp-2 adherence assay [9].

A 700-bp polynucleotide fragment derived from the *daaC* gene has been used as a DAEC DNA probe[9]. The *daaC* gene encodes a molecular usher necessary for expression of the F1845 fimbriae. Approximately 75% of DAEC strains from around the world are positive with this F1845 gene probe [9].

1.3.1.2 *Shigella*

Shigella species are the most important cause of dysentery, accounting for about 60% of all episodes [6] and are the major cause of acute dysentery in children [27]. It has been estimated that each year more than 163 million episodes of endemic shigellosis occur in developing countries accounting for 1.1 million deaths including 580 000 children [28, 29]. Person-to-person transmission accounts for most infections, which occur most commonly in children between the ages of 6 months and 10 years [28]. *Shigella* species cause bloody diarrhoea by invading and destroying mucosal epithelial cells. This occurs mostly in the colon and the distal part of the ileum. Invasion is followed by the formation of micro-abscesses and superficial ulcers, and hence the presence of red and white blood cells, or frank blood, in the stool. The organisms can also produce toxins that cause tissue damage and possibly mucosal secretion of water and electrolytes [6]. Four species of *Shigella* (*S. dysenteriae*, *S.*

flexneri, *S. boydii* and *S. sonnei*) are responsible for the disease. Each species is subdivided into serotypes with *S. dysenteriae* comprising 12 serotypes, *S. flexneri* 6, *S. boydii* 18 and *S. sonnei* 1. Of these four species *S. flexneri* is the most common in developing countries, but *S. dysenteriae* type 1, which occurs in regional epidemics, causes the most severe disease [6, 28].

1.3.1.3 *Salmonella*

Salmonella infections (other than typhoid which is not a cause of diarrhoea) follow much the same pattern as shigella infections, but with the important difference that the infective dose is high (10^5). Diarrhoea is usually watery, but dysentery may occur [6]. In developing countries *S. Typhimurium* and *S.Enteritidis* are common [6].

1.3.1.4 *Vibrio cholerae* 01

Vibrio cholerae 01 has two biotypes (classical and El Tor) and three serotypes (Ogawa, Inaba and hikojima) [6]. *V. cholerae* 01 is non-invasive and uses pili or fimbriae to bind to receptors on the intestinal surface. Mucosal adherence may sometimes cause changes in the gut epithelium that may reduce its absorptive capacity or cause fluid secretion. *V. cholerae* elaborates enterotoxins which result in massive secretion of water and electrolytes in the small bowel through activation of cyclic AMP [6]. Diarrhoea is watery, may be severe, leading to dehydration and collapse within a few hours if the lost fluids and salts are not replaced. In non-endemic areas, epidemics of the disease have equal frequency in adults and children. In endemic areas, cholera occurs mostly in children, adults having substantial immunity from previous infections [6].

1.3.1.5 *Campylobacter jejuni*

In developing countries *Campylobacter jejuni* causes disease mostly in infants. It is spread by contact with faeces or consumption of contaminated food, milk, or water. *C. jejuni* can cause either watery diarrhoea (two-thirds of cases) or dysentery (one third of cases) [6]. The organism may cause bloody diarrhoea by the same mechanisms described under *Shigella* species. Fever may be present and the disease is usually not severe and lasts 2-5 days [6].

1.3.1.6 *Yersinia enterocolitica*

This organism has only recently been identified as a cause of acute diarrhoea in children. Some strains of *Yersinia enterocolitica* are invasive and toxigenic. In infants and young children infection usually produces watery diarrhoea without blood. *Y.enterocolitica* exists in more than 50 serotypes and most isolates from human disease belong to serotypes 04, 08, and 09 [6].

1.3.2 Enteric viral-pathogens

Several different groups of viruses have been shown to be responsible for the high incidence of acute viral diarrhoea among children during their first few years of life [30, 31]. Viruses cause diarrhoea by replicating within the villous epithelium of the small bowel, causing patchy epithelial cell destruction and villous shortening [6]. The loss of normally absorptive villous cells and their temporary replacement by immature, secretory, crypt-like cells causes the intestine to secrete water and electrolytes. Villous damage may also be associated with the loss of disaccharidase

enzymes, leading to reduced absorption of dietary disaccharides, especially lactose. Recovery occurs when the villi regenerate and the villous epithelium matures [6].There are four major genera of viruses that are now recognized as clinically important including rotaviruses, caliciviruses, adenoviruses and astroviruses [31].

1.3.2.1 Rotavirus

Rotavirus is the single most important aetiological agent in severe dehydrating diarrhoea among children. Each year, rotavirus causes approximately 111 million episodes of gastroenteritis requiring only home care, 25 million clinic visits, 2 million hospitalizations and 440,000 deaths in children under five years of age [32]. The high frequency of infection with the rotavirus is reflected by the fact that approximately 80% of the populations have antibodies against this virus by the age of 3 years [6]. Structurally rotaviruses are icosahedral particles composed of multiple protein layers enclosing the genome that consists of 11 segments of double-stranded (ds) RNA. Each segment codes for one protein, with the exception of segment 11 which codes for two proteins. Out of a total of 12 proteins, six proteins are structural proteins and six are non-structural proteins [33]. The outer capsid layer is made of VP7 and VP4, these two proteins are associated with attachment and entry of virus into the cells. The intermediate layer is made of VP6, which distinguishes group specific antigens A to G and most human rotaviruses belong to group A [31, 33, 34]. Group C rotaviruses have been shown to cause sporadic diarrhoea outbreaks [33]. The two proteins VP7 and VP4 carry distinct epitopes, which specifically are determined by neutralising antibody responses, and thus determine serotypes. VP7-specific serotypes are termed G types (G stands for glycoprotein) and VP4-specific

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serotypes are termed P types (P stands for protease-sensitive protein). The genes coding for VP4 and VP7 have also been used for genotypic classification of strains. Among group A rotaviruses, 14 G serotypes and 20 P serotypes have been identified [33, 34]. There is co-circulation of different G and P serotypes/genotypes of rotaviruses. Initially almost all episodes of rotavirus diarrhoea were found to be caused by G serotypes 1, 2, 3 and 4, however recently G 9, G5 and G8 serotypes which were previously known only as animal rotaviruses have been found in humans [33].

1.3.2.2 Caliciviruses

These viruses belong to the family caliciviridae with four genera in which two genera, norovirus (previously known as Norwalk-like virus) and sapovirus (previously known as Sapporo-like virus) infect humans [33] . Sapovirus infection occurs less frequently [35]. Noroviruses are among the leading causes of non-bacterial gastroenteritis outbreaks in children and adults worldwide [30, 33]. Norovirus appears in sporadic cases but also in outbreaks in children and adults with no seasonality reported [35]. The viruses probably represent the second most common aetiological agent of sporadic diarrhoea in young children after rotavirus [36], and sapovirus infection occurs less frequently [35]. Both norovirus and sapovirus have been divided into three major genetic groups, genogroup I, II and III (GI, GII, GIII)based on the sequence analysis [35]. Each of these genetic groups comprises genomically and antigenically diverse strains.

1.3.2.3 Enteric adenoviruses

The genus Adenovirus has six subgroups (A–F) and 49 distinct serotypes, which are differentiated by type-specific neutralisation tests [31]. Enteric adenoviruses of subgroup F belonging to serotypes 40 and 41 are a major cause of acute gastroenteritis, responsible for up to 7.9% cases of diarrhoea in children worldwide[30]. Other adenoviruses, serotypes 12, 18 and 31 of subgroup A and serotypes 1, 2, 5 and 6 of subgroup C have been found in the diarrhoeal specimens [33]. Early surveillance had shown that the incidence of occurrence of serotypes 40 and 41 was approximately equal, however after 1986 the serotype 41 is more predominant worldwide [33].

1.3.2.4 Astrovirus

Human astrovirus belongs to the family Astroviridae, which contains a single genus, astrovirus. Astroviruses are classified into eight serotypes based on the reactivity of the antibodies [31, 37]. Previous studies in various countries have shown that human astrovirus type-1 is the most common type causing disease, followed by type 2,3,4 and 5 whereas types 6, 7 and 8 have been detected only rarely [31, 33]. Astrovirus has only recently been recognized as a common cause of diarrhoea in children[38].The epidemiological studies carried out in different locations in the world have reported astrovirus prevalence rates of 2 to 16% among children hospitalized with diarrhoea and 5 to 17% among children with diarrhoea in community-based studies [38, 39]. Though in limited number of studies, the prevalence of human astrovirus in children with no diarrhoeic symptoms has also been determined and viruses have been found in 2.0% of the children analyzed [39].

1.3.3 Enteric parasites

Diarrhoea is neither a usual nor a frequent feature of parasitic infection. In tropical regions virtually all underprivileged children are carriers of intestinal parasites, but these parasites are only responsible for 5–10% of acute diarrhoea in infancy [40] and slightly more in cases of chronic diarrhoea. In developed countries, in temperate zones, parasitic infections are much less frequent, but parasitic diarrhoea is not uncommon. Only some of the parasites can cause diarrhoea, the protozoa (*Entamoeba histolytica*, *G. lamblia*, *Cryptosporidium*) do so more often than the helminths (*Strongyloides* and *Trichuris trichiura*) [40]. Occasionally *Ascaris lumbricoides*, *Ancylostoma duodenale* and *Schistosoma* are involved in endemic areas [40]. Enteric parasites cause diarrhoea by adhering to the small bowel epithelium and cause shortening of the villi which results into impaired absorption, with the exception of *E.histolytica* which causes micro abscesses and ulcers, in much the same way as *Shigella.* However, about 90% of human *E. histolytica* infections involve strains that are non-virulent, with no mucosal invasion and no symptoms occur, although amoebic cysts are present in the faeces [6].

1.3.3.1 *Cryptosporidium*

Since the recognition of *Cryptosporidium* as human enteric- pathogen in 1976, *Cryptosporidium parvum* has been described all over the world [41, 42]. In tropical countries it has been associated with 4–27%of all incident diarrhoeal illnesses in children [41]. *Cryptosporidium* has also been recognized as a cause of persistent diarrhoea particularly among children in developing countries [41]. Several studies

have suggested that cryptosporidiosis is most common in children younger than 1 year and is associated with malnutrition [43]. Most of these studies are largely incidence studies therefore it has not been clear if malnutrition predisposed children to cryptosporidiosis or cryptosporidiosis led to malnutrition. In some countries cryptosporidium infection in early childhood has been reported to be associated with subsequent impairment in growth, physical fitness and cognitive function [44-46]. Infection with this parasite is asymptomatic or mild and self-limiting in immunocompetent hosts, but can be severe and chronic in immunocompromised individuals, such as patients with acquired immunodeficiency syndrome or in severely malnourished children [47].

1.3.3.2 *Giardia lamblia*

Giardia lamblia is one of the most common protozoa worldwide and can be found in the diarrhoeic stools of humans, provided it is properly sought [40]. The prevalence of infection varies considerably with the level of hygiene, ranging from 1 to 5% in developed countries and from 20 to 50% in developing countries [40]. Infection can occur in the first days of life, but breast milk is at least partially protective [40, 48]. On first contact with the parasite, infants usually develop diarrhoea (of short duration if they are breast-fed), whereas infected older children and adults are most often asymptomatic[48]. When first contact with the parasite occurs later in life, diarrhoea is common, but asymptomatic carriage may occur just after initial contact. Diarrhoea follows 3–40 days after the ingestion of cysts.

1.3.4 Mixed enteric infection

This occurs when two or more enteric pathogens are isolated from a single patient [49]. Mixed enteric infections can occur in up to one third of cases of diarrhoea in developing countries [6]. Individuals harbouring such infections may suffer exacerbated morbidity, making children even more vulnerable. Thus, these infections pose a serious threat to the health and development of children in low-income countries [49].

There is increasing evidence that mixed infections in which microorganisms present specific pathologies may act in a synergistic or inhibitory fashion, impacting on either tissue, host cell destruction or the maintenance of health. Among these mixed infections, bacteria–bacteria-, virus–virus-, parasite–parasite- or virus–bacteria interactions have been well documented [49]. For example, enteroaggregative *Escherichia coli* (EAEC) or enteropathogenic *E. coli* (EPEC) may provide a second pathogen with better conditions to invade the intestine and cause diarrhoea.

1.4 Laboratory diagnosis of diarrhoea diseases

Definitive diagnosis of diarrhoea depends on laboratory results. Requests may be made for chemical, bacteriological, viral and parasitological analysis. Specimen include stool but if not possible rectal swabs can be used instead [50].

1.4.1 Specimen collection, transportation and storage

Stool specimen should be collected in a clean, dry container of appropriate size with a tight-fitting, leak-proof cap. Ideally stool specimen should be processed as soon as

possible within 2 hours of collection for examination or detection of bacteria, viruses and parasites [50]. Stool is first examined visually for consistency (e.g. formed, liquid), colour (white, yellow, brown, black) and atypical components such as mucus, blood and segments of parasites. For detection of bacterial pathogens, longer storage of stool would require use of appropriate transport media, for example, Carry-Blair transport medium is appropriate for *Salmonella*, *Shigella, Vibrio*, *Campylobacter* and *Yersinia* species. These organisms survive well in Carry-Blair medium for up to 48 hours, and *Campylobacter* for up to 6 hours [50]. Alkaline peptone water is suitable for *Vibrio* species. For rectal swabs, a sterile cotton swab is moistened with sterile non bacteriostatic fluid or transport media. If the swab is to be processed within 2 hours, it should be placed in a sterile empty tube with a cotton plug or screw cap. If delay of more than two hours is anticipated rectal swab should be inoculated in transport medium [50]. Stool specimen for detection of enteric viruses should also be processed as soon as possible and if there is likely to be a delay of more than a few hours 1 gram of fecal specimen can be suspended in 9mls of phosphate buffered saline.For longer storage days, months or even years stool specimen can also be preserved at -20^0C or -70^0C for later analysis.

1.4.2 Isolation of bacterial pathogens

To isolate bacterial pathogens stool should be cultured onto selective and differential media for enteric bacteria [50]. These media include; Mac Conkey Agar (MCA), Xylose lysine Deoxycholate (XLD), Deoxycholate Citrate Agar (DCA), Salmonella Shigella Agar (SSA), Thiosulphate Citrate Bile-salt Sucrose Agar (TCBS) and Butzler medium. XLD is a selective and differential medium for the isolation of

Salmonella and *Shigella*; it contains an indicator phenol red which is red at an alkaline pH (7.4) and yellow at an acid pH. The medium is incubated aerobically at 35-37^{0}C for overnight. SSA is also a selective and differential medium for *Salmonella* and *Shigella*. To isolate these organisms the plate is incubated aerobically at 35-37°C. TCBS is used to isolate *Vibrio* species; it is a selective and differential medium with an indicator bromothymol blue. It differentiates sucrose fermenting colonies of *V.cholerae* from non-sucrose fermenting colonies of *V.parahaemolyticus*. The medium is incubated aerobically at 37^{0}C overnight. MCA medium contains neutral red which differentiates lactose fermenting colonies of *E.coli* (pink) from non lactose fermenting colonies of *Salmonella*, *Shigella*, *Y.enterocolitica* (colourless). The medium is incubated aerobically at 37^{0}C overnight. To isolate *Y.enterocolitica* SSA and MCA media are used. After inoculation on MCA/ SSA the plate is incubated aerobically at room temperature 20-28^{0}C for up to 48hours, but growth can be examined after overnight. Butzler medium is used to isolate *Campylobacter* species and the plate is incubated at 42^{0}C with 5-10% CO_2 [50].

1.4.3 Identification of the bacterial isolates

Identification of the bacterial isolates is based on colony morphology and gram staining reaction which gives the presumptive identification. T o make definitive identification, biochemical tests, serological test and molecular techniques can be done [50]. The biochemical tests that can be done include oxidase test, urease test, Indole, Methylred, Voges-Proskeur and Citrate utilization. There are also commercially available test kits that give rapid identification of enterobacterociae based on conventional and chromogenic substrates. These tests include Rapid API

20E (OXOID) and RapiD ONE system (Remel, Lenexa, USA). Serological tests are rapid agglutination tests that use specific polyvalent O and monovalent antisera to identify and or speciate bacterial pathogens such as *Vibrio cholerae* serogroups and serotypes and *Salmonella* and *Shigella* species and serotypes [50].

1.4.4 Antimicrobial susceptibility testing of bacterial enteric-pathogens

Since antibiotic resistance is high among enteric pathogens, a continuous surveillance of antimicrobial susceptibility testing should be done routinely to select suitable antibiotic for patient treatment. It also helps to monitor resistance pattern and identify presence of resistance. In addition to that antimicrobial susceptibility results can provide information for empiric therapy and epidemiological marker of strain relatedness in outbreaks. Sensitivity tests measure antimicrobial activity against bacteria under laboratory conditions (in vitro activity). This is done by either diffusion technique or dilution technique [50]. Disc diffusion techniques are used by most laboratories to test routinely for antimicrobial sensitivity and the methods commonly used are Stoke's disc diffusion and Kirby-Bauer methods. E-test is used to estimate minimum inhibitory concentration (MIC) [50]. When using disc diffusion techniques the sizes of the zones of inhibition of the organisms are interpreted as sensitive, intermediate, or resistant to the agents that have been tested.

1.4.5 Detection of enteric viruses

Detection of gastroenteritis producing viruses has traditionally been based on techniques of direct visualization using electron microscopy, of which its use is limited to reference laboratories [31]. Cell culture of these viruses is not considered

to be useful for diagnostic purposes as it is technically cumbersome and slow. Propagation of caliciviruses in cell culture media has not yet been achieved [31]. The progressive development of more sensitive techniques for detection of enteric viruses in faeces based on immunoassay techniques, as well as the application of molecular biology techniques has improved the diagnosis of these viruses.

Techniques for the detection of viral antigen in faecal samples have been developed [31]. These are based on enzyme immunoassay (EIA), agglutination with latex particles (LA) and more recently immunochromatography (ICG). These tests use monoclonal and polyclonal antibodies for the detection of viruses. There are comercially available EIA for group A rotavirus, calicivirus, enteric adenovirus and astrovirus. EIA has proven to be more sensitive than direct visualization by electron microscopy and has also a high specificity in the detection of group A rotavirus especially when monoclonal antibodies are used. The LA techniques have lower sensitivity than EIA[31]. ICG showed high sensitivity and the results are comparable to those achieved with EIA. The technique is rapid and technically very simple [51].

1.4.6 Detection of enteric parasites

Microscopy is the 'gold standard' for detecting faecal parasites. Wet mount preparation, saline and eosin preparation can be done to observe protozoan trophozoites and cysts such as *E. histolytica* cysts, *Giardia*, and helminths eggs of intestinal parasites [50]. Modified ZN staining is usually done to observe cysts of *Cryptosporidium* species and *Entamoeba histolytica*. There are several permanent

safranin and trichrome staining procedures. The permanently stained smears provide laboratories with a permanent record that can be reexamined as needed. Modified Ziehl-Neelsen staining is useful for the identification of oocysts of the coccidian species *Cryptosporidium*, *Isospora*, and *Cyclospora*. Unlike the Ziehl-Neelsen, Modified Acid-Fast Stain does not require heating.

Since faecal examination using microscopy is very labor-intensive and requires a skilled microscopist, progr ess has been made in developing and validating non-morphologically-based diagnostic tests for detection of intestinal parasite antigens e.g. immunofluorescence microscopy (IF), enzyme-linked immunosorbent assay (ELISA) and rapid dipstick-like tests. Rapid dipsick-like test methods can be performed quickly and do not require an experienced and skilled morphologist. Currently there are commercially available rapid test kit reagents for the intestinal parasites such as *Cryptosporidium parvum, Entamoeba histolytica*, and *Giardia lamblia* [40]. Fresh or preserved stool samples are the appropriate specimens for antigen detection testing with most kits.

1.4.7 Molecular techniques in the diagnosis of diarrhoeal diseases

Hybridization methods and Polymerase Chain Reaction (PCR) can also be done to detect enteric pathogens which are viruses, bacteria and parasites direct from the stool specimen as well as typing/speciation of these parasites. PCR allows invitro amplification of specific target deoxyribo nucleic acid (DNA) sequence by a factor of 10^6. It is based on an enzymatic reaction involving use of synthetic oligonucleotides which act as primers. The method involves; extraction of nucleic acid RNA/DNA

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from isolated pathogens or stool sample by either chemical or physical methods. Amplification of the target DNA starts with denaturation at 94°C, cooling to 52°C for 20 seconds for annealing to occur, temperature is raised again to 72°C for extension (polymerization). Detection of the amplified targeted DNA can be done by gel electrophoresis, ELISA or Real time PCR. PCR techniques have been developed for many viruses that produce gastroenteritis, although they are not used routinely [31]. These techniques have been shown to be more sensitive than immunoassay methods. They are useful for confirmation of results of other techniques. In the case of human caliciviruses reverse transcriptase polymerase chain reactions (RT-PCR) is the most sensitive and widely used technique at present for the detection of these viruses. Molecular techniques such as RT-PCR are also used to genotype the viruses such as genotyping of group A rotavirus using specific primers for G and P serotypes[31]. PCR can offer increased sensitivity for detection of intestinal parasites. However these tests remain too costly for diagnostic laboratory use, given the requirement to screen stools for a variety of pathogens [40]. Nevertheless PCR might be useful as several targets can be combined in one multiplex RT-PCR assay, allowing the simultaneous detection of multiple infections such as *Entamoeba histolytica*, *Giardia* and *Cryptosporidium* species and genotypes infectious to humans. With sufficient quality assurance and the judicious choice of controls, RT-PCR can be more specific and more sensitive than microscopy in detection of parasites.

1.5 Treatment of diarrhoea

The main principle of treatment of watery diarrhoea is fluid and electrolyte replacement irrespective of its etiology [6]. Feeding should be continued during all

types of diarrhoea to the greatest extent possible, and should be increased during convalescence so as to avoid any adverse effect on nutritional status.

According to Intergrated Management of Childhood Illnesses (IMCI) guidelines antimicrobials and anti-parasitic agents should not be used routinely, but the exceptions are dysentery, suspected cases of cholera and persistent diarrhoea [6]. Dysentery cases should be treated with antibiotic effective for *Shigella* which are ampicillin, chloramphenicol, co-trimoxazole, nalidixic acid and flouroquinolones [6]. Cases not responding to this treatment should be studied for possible amoebiasis where metronidazole is the drug of choice [6]. Suspected cases of cholera should also receive antibiotics which will shorten the duration of symptoms and reduce the time of excretion of the organisms. The antibiotics of choice are tetracyclines, erythromycin, co-trimoxazole, and flouroqionolones depending on the sensitivity pattern in the area [6]. In persistent diarrhoea, patients are normally treated with co-trimoxazole, but sensitivity testing should be done [6].

1.6 Anti microbial resistance of bacterial enteric-pathogens

Resistance to antimicrobial agents has resulted in morbidity and mortality from treatment failures and increased health care costs. The resistance of enteric-pathogenic bacteria to commonly prescribed antibiotics is increasing both in developing as well as in developed countries and it has emerged even to newer, more potent antimicrobial agents [52]. This is due to extensive unchecked use of antibiotics and inadequate laboratory surveillance of antimicrobial resistance [52, 53].

Inappropriate use results from physicians prescribing antimicrobial drugs to treat viral infections, using inadequate criteria for diagnosis of infections that potentially have a bacterial aetiology, unnecessarily prescribing expensive, broad-spectrum agents, and not following established recommendations for using chemoprophylaxis. The availability of antibiotics over the counter, despite regulations to the contrary, also fuels inappropriate usage of antimicrobial drugs [52]. The association between increased rates of antimicrobial use and resistance has been documented for nosocomial infections as well as for community acquired infections [52]. Over the past several decades resistance has been reported among DEC, *Salmonella*, *Shigella*, and *Vibrio cholerae* [52, 53]. For bacteria, such as *Shigella* species or DEC susceptibility to antibiotics varies from place to place, making it difficult to know which antibiotic might be effective [6]. The development of antimicrobial resistance limits treatment options available for potentially fatal epidemics of *Shigella* and *Vibrio cholerae*.

As resistance develops to "first-line" antibiotics therapy with new, broader spectrum and more expensive antibiotics also increases, but this is followed by development of resistance to the new class of drugs.

The following four basic mechanisms of resistance have been documented; development of mechanisms that prevent antimicrobial access to the site of action by increasing efflux or decreasing influx through the cell membrane; development of enzymes that degrade or alter the antimicrobial agent; alteration of the site of antimicrobial action, rendering the drug ineffective and development of site-of-action bypass mechanisms [52, 53].

Resistance (R) factor plasmids are extrachromosomal DNA elements of bacteria that confer drug resistance on their host bacteria. They can transfer themselves to other bacteria by conjugation and by phage-mediated transduction [52, 53]. They can also integrate into the host chromosome. These factors can be transferred not only to enterobacteriaceae, but also to a variety of gram-negative bacilli such as *Pseudomonas*, *Vibrio cholerae*, and others. An important point with R factors is that many of them carry multiple drug resistance genes [52, 53].

1.7 Prevention and control of diarrhoea

Although a wide variety of infectious agents cause diarrhoea, they are all transmitted by common faecal-oral pathways, such as contaminated water, food, and hands. Measures to interrupt transmission of the causative agents should focus on these pathways which include; giving exclusive breast feeding for the first 4 -6 months of life, avoiding the use of infant feeding bottles, improving practices related to the preparation and storage of weaning foods (to minimize microbial contamination and growth) [6]. Other preventive measures are use of clean drinking water, washing hands (before and after preparing food or eating), and safely disposing of feces, including infant feces [6]. Measures that can be taken to improve host defenses and thus diminish the risk of diarrhoea include, immunizing against measles, continuing to breast-feed for at least the first year of life, and improving nutritional status by improving the nutritional value of weaning foods and giving children more food [6].

Among the major approaches to preventing diarrhoeal disease in developing countries, vaccination offers the most feasible remedy for the short and medium

term. While safe water and access to treatment are for the most part inaccessible to the most impoverished people, mass vaccination campaigns generally seek to protect these groups. Since the early 1990s the WHO has focused on a priority list of five vaccines against: Rotavirus, ETEC, *Shigella*, cholera, and typhoid fever. The main target group for all these pathogens is children. The incidence of particular serotypes remains unknown for most critical populations in the key endemic areas. There is available oral vaccine for cholera and oral vaccine for *S.Typhi*. Vaccines against shigella and ETEC are still under development. Currently there are two licensed rotavirus vaccine in use RotaTek® which is a live, oral human-bovine reassortment rotavirus vaccine (Merk and Company, Whitehouse Station, New Jersey) and RotaRix® which is a monovalent vaccine based on attenuated human rotavirus strain (GSK Biologicals Belgium). These two vaccines are designed for genotypes G1 to G4 [54].

1.8 Diarrhoeal diseases in Tanzania

In Tanzania diarrhoea disease is the second most common illness in children aged less than five years after acute respiratory infections [5]. According to the WHO /MOH figures, diarrhoea accounted for 408,862 cases and 616 deaths among under-fives, while dysentery accounted for 80,569 cases with 100 deaths in the year 2004. Despite this magnitude there are relatively few studies that have been conducted to search for aetiologic agents of diarrhoea among children in Tanzania. Most were conducted in late eighties and early nineties and focused on relatively few aetiologic agents of diarrhoea mainly rotavirus, enteric adenovirus and intestinal parasites.

In two studies that were conducted at Muhimbili National Hospital in 1988 and 1992 among children with diarrhoea showed the prevalence of *Rotavirus* infection to be 19.5% and 43.4%, respectively, while that of enteric *Adenovirus* was 2.3% [55, 56]. It is important to note that these two studies searched for enteric viruses only and used either latex agglutination only or latex agglutination and electron microscopy.

In 1993 Cegielski JP et al found chronic diarrhoea to be predominant among HIV infected children hospitalized in Muhimbili National Hospital in Dar es Salaam, and that *Blastocystis_hominis* was detected in HIV –infected patients only [57].

Cegielski JP et al 1994 using electron microscopy reported small round structured viruses (SRSV) currently known as norovirus to be more frequent in HIV-infected (19%) than in HIV- uninfected (3%) children with chronic diarrohea and that rotavirus and coronavirus-like particles were not associated with HIV infection [58].

Another study that was done in Mwanza Tanzania in 1995 showed that 18% of children with diarrhoea were infected with *Campylobacter* and 20% were infected with ETEC [59].

Cegielski et al in 1996 using Human epithelial cells-2 (HEp-2) cell adherence and DNA probes reported a higher prevalence of DEC among children with chronic diarrhoea than among children without diarrhoea (45.7% versus 20%) at the Muhimbili National Hospital [60]. Among children with chronic diarrhoea the prevalence of different virulence factors of DEC were 25.4%, 22%, 16.9% and 11.9% for DAEC, EAEC, EPEC and ETEC respectively [60]. There was no EIEC or EHEC [60]. In another study conducted in 1999 by Cegielski et al in Dar es Salaam *Cryptosporidium* was identified in 8.5% of children with chronic diarrhoea of whom

60% were co-infected with HIV [61]. A recent study conducted in Ifakara Tanzania in 2004 reported that during the dry season DEC (37%), *Shigella* spp (24.14%) and rotavirus (23.56%) were most commonly isolated pathogens [5]. DEC were also common during the rainy season (30%) followed by *Giardia lamblia* (14.5%) and *Shigella* spp (12.6%) [5].

Regarding antmicrobial susceptibility patterns of bacterial pathogens in children, Jordi Villa et al conducted a study in Ifakara Tanzania and showed that 38% of the cases of childhood diarrhoea were due to multresistant ETEC, EAEC, or EPEC, with high levels resistance to ampicillin, tetracycline, co-trimaxazole, chloramphenicol [62]. In that same year Margarita M. et al reported resistance of *Shigella* species in children of underfive years in Ifakara Tanzania [63]. Overall the antimicrobial susceptibility testing showed a high level of resistance to ampicillin (81.8%), chloramphenicol (72.7%), tetracycline (96.9%), and co-trimoxazole (87.9%). However, all strains were susceptible to nalidixic acid, ciprofloxacin, ceftazidime, cefotaxime, and cefoxitin [63].

In the year 2000 Urassa WK et al reported a significant increase in the proportion of *V.cholerae* 01 isolates resistant to tetracycline, ampicillin, nalidixic acid and erythromycin but there was no change for susceptibility to ciprofloxacin and trimethoprim/sulphamethoxazole between 1997 and 1999 [64]. Among the 94 *V.cholerae* 01 strains isolated in 1997; 98.6%, 93.6%, 83%, 81.9%, 36.2%, 35.5%, 3.2% were sensitive to ciprofloxacin, tetracycline, ampicillin, erythromycin, nalidixic acid, chloramphenicol and trimethoprim/sulphamethoxazole, respectively. Among the 87 *V. cholerae* 01 isolates collected in 1999, 100%, 58.6%, 46.0%, 46%, 47.1%,

60% were co-infected with HIV [61]. A recent study conducted in Ifakara Tanzania in 2004 reported that during the dry season DEC (37%), *Shigella* spp (24.14%) and rotavirus (23.56%) were most commonly isolated pathogens [5]. DEC were also common during the rainy season (30%) followed by *Giardia lamblia* (14.5%) and *Shigella* spp (12.6%) [5].

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19.5%, 3.4% were sensitive to ciprofloxacin, tetracycline, ampicillin, erythromycin, chloramphenicol, nalidixic acid and trimethoprim/sulphamethoxazole, respectively [64].

2. STATEMENT OF THE PROBLEM AND RATIONALE

2.1 STATEMENT OF THE PROBLEM

Despite the high magnitude and impact of diarrhoea among under five in Tanzania, there are relatively few studies that have been conducted in the country. Most of these studies have focused on relatively few aetiologic agents of diarrhoea. Mhalu F et al 1988 and Sam NE et al 1992 searched for rotaviruses and enteric adenovirus in children with diarrhoea [55, 56]. It is important to note that these two studies used latex agglutination test and electron microscope to detect these viruses in faecal samples of children with diarrhoea. These methods have been reported to be less sensitive compared to EIA in detection of these enteric viruses [51].

Cegielski et al 1994 also showed prevalence of enteric viruses, among HIV-infected and non HIV-infected children with chronic diarrhoea [58]. The study focused on chronic diarrhoea only, while acute diarrhoea is reported to be the most common type of diarrhoea among children of under five years [5] . A study done in Mwanza Tanzania showed prevalence of only two bacterial pathogens causing diarrhoea which are *Campylobacter* species and ETEC [59].

A recent study conducted in Ifakara in 2004 showed a prevalence of different aetiologic agents of diarrhoea among children with diarrhoea [5]. It is apparent from the above studies most of them searched for one or two enteropathogens and only one study looked for all entreropathogens causing diarrhoea among under fives. Thus, there is limited information on the spectrum of enteric-pathogens causing diarrhoea diseases in children in Tanzania. Furthermore, most of these studies were conducted in late eighties and early nineties [55, 57, 58].

For the studies on prevalence of enteric viruses the methods used are currently reported to be less sensitive and less specific [31, 51]. In addition to that there is no single study that has reported the circulating genotypes of rotavoirus in Tanzania. The information regarding antimicrobial susceptibility pattern of bacteria causing diarrhoea in children is very limited. Due to limited information regarding causative agents and their susceptibility to antimicrobial agents diarrhoea patients are treated empirically, with mainly erythromycin and co-trimoxazole as recommended by the WHO/ IMCI [6]. Due to continous change in antimicrobial sensitivity there is a need to do periodic survailance to monitor sensitivity pattern of bacterial enteric pathogens.

2.2 RATIONALE OF THE STUDY

This comprehensive study will provide:

1. An update on the spectrum of causes of diarrhoea among under-five years.

2. Information regarding molecular epidemiology of group A rotavirus, which needs to be taken into account before introduction of an effective vaccine in Tanzania.

3. Information on antimicrobial susceptibility pattern of bacterial pathogens will

i) Assist in reviewing the guidelines for the routine treatment of diarrhoea.

ii) Improve treatment thus reducing morbidity and mortality,

iii) Avoid unnecessary use of antibiotics and hence limit emergence of antimicrobial resistance.

3. OBJECTIVES OF THE STUDY

3.1 BROAD OBJECTIVE

To study the aetiological agents of diarrhoea among underfives in Dar es Salaam Tanzania.

3.2 SPECIFIC OBJECTIVES

1. To determine the prevalence of the different types of diarrhoea (acute watery, persistent and dysentery) among under fives in Dar es Salaam.
2. To identify viral, bacterial and parasitic enteric pathogens causing diarrhoea among under fives in Dar es Salaam.
3. To determine the virulence factors of diarrhoeagenic *E.coli* (ETEC, EPEC, EIEC, EHEC, EAEC) among underfives with diarrhoea in Dar es Salaam.
4. To determine molecular characteristics of group A rotavirus among underfives with diarrhoea in Dar es Salaam.
5. To determine the antimicrobial susceptibility pattern of the bacterial enteric pathogens causing diarrhoea among under fives in Dar es Salaam.

4. MATERIALS AND METHODS

4.1 Patients/study population

This was a prospective cross-sectional study that was conducted in Dar es Salaam, Tanzania between December 2005 and February 2006, which was a dry season. The study population included 280 children ≤ 5 years, who during the study period, were admitted due to diarrhoea at Muhimbili National Hospital (MNH) and Amana, Mwananyamala, and Temeke Municipal Hospitals in Dar es Salaam, Tanzania. Enrolment was subject to obtaining an informed verbal consent from parent or guardian who accompanied the child.

4.2 Case definitions of diarrhoea

Diarrhoea was defined, according to WHO guidelines, as the occurrence of three or more, loose, liquid, or watery stools within 24 hours. The guidelines stipulate three forms of diarrhoea namely: i) acute watery diarrhoea defined as diarrhoea that begun acutely and lasted less than or equal to 13 days, ii) dysentery defined as mucoid bloody stool associated with anorexia, abdominal cramps, and tenesmus and iii) persistent diarrhoea defined as diarrhoea with a duration of 14 or more days.

4.3 Sample size

This study included 280 children of underfive years with diarrhoea. The sample size was calculated using the formula $n=4p\ (100-p)/d^2$. During conception of the study, only bacterial enteric pathopgens pathogens were targeted to be searched for. There

fore p was taken as the lowest prevalent bacteria enteric pathogen *Campylobacter* in a study done in Ifakara Tanzania by Vargas et al [5].

The calculation was done as follows

$n=4p\ (100-p)/d^2$

Where

n=sample size

P=previous lowest prevalence of diarrhoea caused by *Campylobacter* spp (2.9%) in a study conducted in Ifakara, Tanzania by Martha Vargas et al (4))

D=margin of error on p

p=2.9%, and d=2%

$n=4X2.9(100-2.9)/2^2$

n=281

The minimum sample size is 281

4.4 Data/specimen collection and transportation

After obtaining consent from the mother/caretaker of under five children admitted due to diarrhoea, a special questionnaire (appendix I) was filled with social demographic information, age which was confirmed by date of birth from the Maternal and Child Health (MCH) card provided by the mother/caretaker of the child, sex, duration of diarrhoea, consistency of the stool (watery, rice stool, mucoid, or bloody). Information was also sought regarding the use of antibiotics one week prior to hospitalization. The weight of the child was measured on examination.

Infants under two years of age were weighed using a 25 kg Salter hanging scales (CMS Weighing equipment, High Holborn, London, United Kingdom). Children over two years were weighed on scales calibrated before each session.

Weight of children was recorded to the nearest kilogramme. Stool specimens from enrolled children were collected using wide mouthed sterile plastic containers, and transported immediately to Muhimbili University College of Health Sciences (MUCHS) microbiology laboratory for analysis. Portion of stool specimen was frozen and stored in 1.8ml nunc tubes at -20^{0}C until the time for analysis of parasites and viruses. Children whose parents/caretakers did not consent or children whose parents or caretaker could not ascertain their age and had no MCH card to confirm the date of birth of the child were excluded from the study.

4.5 Determination of nutritional status

Weight-for age Z-scores were calculated using EPI Info (USD, Inc., Stone Mountain, GA). Children were considered to be undernourished if the Z-scores were less than -2SD (CDC\WHO, 1978).

4.6 Laboratory Procedures

The following laboratory procedures were perfomed.

4.7.1. Isolation of bacterial pathogens

Each specimen was plated out on MCA, TCBS, SSA, XLD and selenite F and incubated aerobically at 37^{0}C for overnight for the isolation of *Salmonella, Shigella, Vibrio* species *E.coli.* Culture media were prepared according to manufactures instructions (Remel, Lenexa, USA).

4.7.2 Identification of the bacterial isolates

Isolates were identified by observing the colonial morphology, Gram staining and biochemical tests such as oxidase test and RapiD One system (Remel, Lenexa, USA) and serological tests (slide agglutination test).

4.7.3 Identification of *Vibrio* species

From TCBS media, sucrose fermenting colonies were sub cultured on nutrient agar and oxidase test was performed from colonies on NA since it is not possible to perform an oxidase test directly from a TCBS because the acid produced by the sucrose fermenting colonies will inhibit the oxidase reaction. Serological test was also done from NA colonies.

An isolate was identified as *V.cholerae* 01 if it was gram negative rod, sucrose fermenting, oxidase positive and agglutinates with *V. cholerae* 01 polyvalent antiserum. Inaba and Ogawa serotypes were also identified by slide agglutination technique using specific antisera (Remel, Lenexa, USA).

4.7.4 Identification of *Salmonella* and *Shigella* species

Non lactose fermenting colonies *Salmonella* and *Shigella* spp on XLD /SSA/MCA were identified by RapiD ONE system. Speciation of *Shigella* and *Salmonella* was done by slide agglutination test using specific anti-sera.

4.7.5 Identification of *E.coli*

Lactose fermenting colonies on MCA were provisionally identified as *E.coli* by indole test.

4.7.6 Detection of virulence factors of DEC

All species provisionaly identified as *E.coli* were confrirmed by incooperating the species specific gene for *E.coli* together with the genes coding for virulence factors of ETEC, EPEC, EHEC, EIEC and EAEC in the multiplex PCR.This multiplex PCR

has been explained previously [12, 23]. Since there is a possibility that picking one colony of *E.coli* may fail to yield the offending pathogenic *E. coli*, a sweep of colonies of *E.coli* isolates were tested per diarrhoea stool to increase sensitivity of the test. Multiplex PCR was used to detect 11 virulent genes of DEC. To extract DNA a sweep of colonies were boiled in 500 µL of sterile distilled water for 10 minutes. Then centrifugation was done at 13000 rpm for 5minutes to pellet the cell debris. One microliter (1.0 µL) of the supernatant was used as template for the PCR amplification.

Positive controls were either reference strains or strains known to posses the target genes shown in Table I. Sterile distilled water was used as negative control.

TableI: Shows Reference strains of DEC used as positive controls.

DEC category	Target genes	Refference strains
E.coli	*uidA*	ATCC 43887
EPEC	*Eae* and *bfpA*	ATCC 43887
ETEC	*Elt* and *st1a*	ATCC 35401
ETEC	*St1b*	sequenced
EAEC	*AA probe* and *aggR*	sequenced
EIEC	*Ial*	1037210-1(1059048-1)
EHEC	*Eae*, stx_1 and stx_2	ATCC 43889

For the detection of ETEC, EPEC, EIEC, EHEC and EAEC five assays, n1, n2, n3, n4 and n5 were used. Assay n1 utilized primer pairs for genes coding for Stable toxin ST (*st1a*) and labile toxin LT (*elt*) of ETEC, and (*uid*A) for the *E.coli* ß-glucuronidase. Assay n2 detects the presence of the *eae* (structural gene for intimin

found in EPEC) and *bfp*A (structural gene for the bundle-forming pilus) of EPEC, and assay n3 recognises stx_1 and stx_2 shiga-like toxin of EHEC and *ial* (invasion-associated locus of the invasion plasmid) found in EIEC. Primers of the gene *st1b* which also code for stable toxin of ETEC were included in a separate tube assay n4. To detect simultaneous two plasmid genes of EAEC namely *AA probe* and *aggR* were included in assay n5. Table II shows the primers nucleotide sequence used and the predicted length of the resulting amplicon for each primer pair. These primers have been used for detection of DEC and have been used in a number of studies including in developing countries [12, 23, 65].

PCR was carried out with 1.0 µL of the template which was added to 24 µL of primers and HotStarTaq Master Mix containing HotStarTaq DNA polymerase, PCR Buffer (with 3mM $MgCl_2$ and 400µM each dNTP (QIAGEN, Norway). Cycling conditions were initial Hotstar Taq DNA polymerase enzyme activation at 95^0C for 15 minutes, followed by 30 cycles of denaturation at 94^0C for 1 minute, annealing at 55^0C for 1 minute, extension at 72^0C for 1 minute, and final extension at 72^0C for 10 minutes.

The amplicons were checked by gel electrophoresis and their size determined by using DNA molecular weight marker XIV (100bp). The products were visualized using a UV transilluminator.

Interpretation of results:

Figure 1 and 2 show results on gell elecrophoresis and the expected amplicon size are shown in table II. Only the presence of the correct sized gene product(s) was interpreted as a positive test

Isolate was confirmed as *E.coli* if it had *uid*A gene

ETEC, *uid*A (assay n1) + *elt* or *sta* or both (assay n1)

ETEC *uidA* + *st1b* (assay n4)

EPEC, *uid*A (assayn1) + *eae* and *bfp*A or *eae* alone (assay n2)

EHEC, *uid*A (assay n1) ± *eae* (assay n2) + either stx1 or stx2 or both (assay n3)

EIEC, *uid*A (assay n1) + *ial* (assay n3)

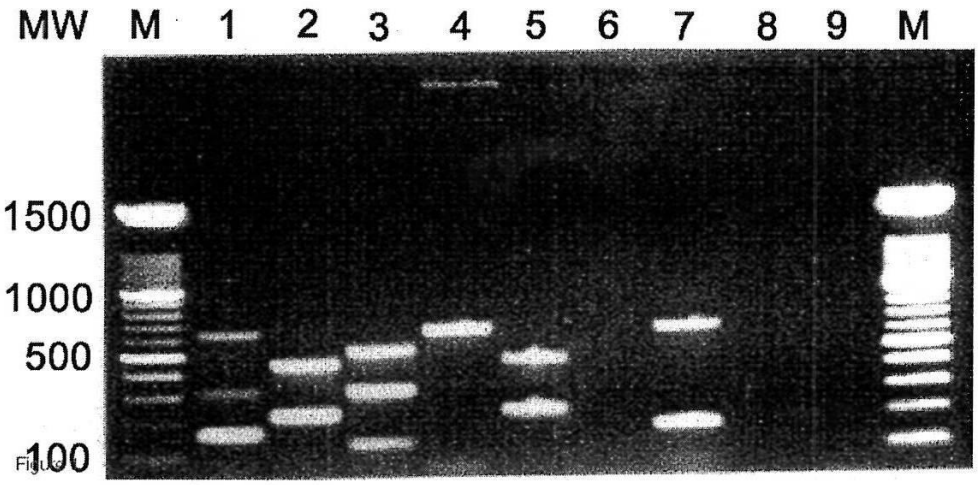
EAEC, *uidA* + *aat*/ *aggR* or both (assay n5)

Table II:. Primer sequence, primer concentration and predicted length of PCR products for multiplex PCR to identify ETEC, EPEC, EHEC, EIEC and EAEC.

	Target gene	Oligonucleotide sequence (5` to 3`)	Primer pM	Ampl Size(bp)	Reff
Assay n 1	*elt*	**F. CTCTATGTGCACACGGAGC** **R. CCATACTGATTGCCGCAAT**	10	322	[12]
	St1a	**F.TCTTTCCCCTCTTTTAGTCAGTC** **R. CCGCACAGGCAGGATTAC**	10	170	[12]
	*uid*A	**F. CCAAAAGCCAGACAGAGT** **R. GCACAGCACATCAAAGAG**	10	623	[12]
Assay n 2	*eae*	**F. TGATAAGCTGCAGTCGAATCC** **R. CTGAACCAGATCGTAACGGC**	10	229	[12]
	*bfp*A	**F. CACCGTTACCGCAGGTGTGA** **R. GTTGCCGCTTCAGCAGGAGT**	10	450	[12]
Assay n 3	stx_1	**F. GAAGAGTCCGTGGGATTACG** **R. AGCGATGCAGCTATTAATAA**	10	130	[12]
	Stx_2	**F.GGGTACTGTGTGCCTGTTACTGG** **R. GCTCTGGATGCATCTCTGGT**	10	510	[12]
	ial	**F.CTGGTAGGTATGGTGAGG** **R. CCAGGCCAACAATTATTTCC**	10	320	[12]
Assay n4	*St1b*	**F.ATTTTTCTTTCTGTATTGTCTT** **R.CACCCGGTACAAGCAGGATT**	10	190	[65]
Assay n5	*aggR*	**F. CTAATTGTACAATCGATGTA** **R. AGAGTCCATCTCTTTGATAAG**	15	457	[23]
	AA probe	**F. CTGGCGAAAGACTGTATCAT** **R. CAATGTATAGAAATCCGCTGTT**	20	629	[23]

F. forward primers, R. reverse primers.

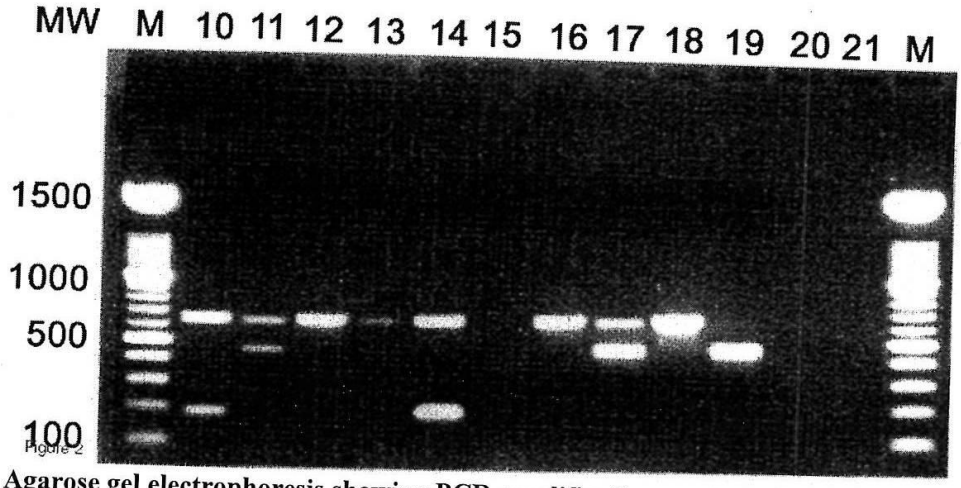
Figure 1.



Agarose gel electrophoresis showing PCR amplification products for assay n1, n2 and n3;

lane M, DNA molecular weight marker 100-bp; lane 1 positive control assay n1 *uidA*(623bp), *st1a* (322bp), *elt* (170bp)genes; lane 2 positive control assay n2 *bfpA* (450bp) and *eae* (229bp) genes; lane 3 positive control assay n3 stx_2 (510bp), *ial* (320bp) and stx_1 (130bp) genes; lane 4 and5 and positive isolate of EPEC *uidA*, *eae* and *bfpA* genes; lane 7 positive isolate of ETEC *uidA* and *st1a.*

Figure 2.



Agarose gel electrophoresis showing PCR amplification products for assay n4 and n5 :

lane M, DNA molecular weight marker 100-bp; lane 10 positive control assay n4 *uidA* (623bp) and *st1b*(190bp) genes; lane 11 positive control assay n5 *AA* probe (629bp) and *aggR* (457bp) genes; lane 12 and 13 positive isolate of EAEC *uidA* and *AA* probe genes; lane 14 positive isolate of ETEC *uidA* and *st1b*; lane 16 and 17 positive isolate of EAEC *uidA*, *AA* probe and *aggR* genes; lane 18 and 19 positive isolate of EAEC *uidA* and *aggR* genes; lane 20 and 21 negative control.

4.8 Detection of enteric viruses and molecular typing of group A rotavirus

Two hundred and seventy stool samples were available for detectetion of viruses.The presence of four enteric viruses namely rotavirus, norovirus, adenovirus and

astrovirus antigens was investigated by enzyme linked immuno assay (ELISA). Commercial immunoassays IDEIA Rotavirus, IDEIA Adenovirus, and IDEIA Astrovirus and IDEIA Norovirus (Dako Ltd., Ely, United Kingdom) were used according to the manufacturer's instructions to detect specific antigens from group A rotaviruses, adenoviruses type 40 and 41, astroviruses and noroviruses, respectively.

4.8.1 Detection of Rotavirus

The IDEIA Rotavirus test kit utilizes a polyclonal antibody to detect specific group A rotavirus proteins, particularly the internal capsid protein (VP6). This assay is able to detect rotavirus concentrations of as low as 7.8×10^5 viral particles/ml and shows good correlation, sensitivity, and specificity in comparison with electron microscopy (99.5, 100, and 99.2%, respectively) [51]. Additionally, this assay shows a good correlation with RT-PCR [66].

The samples were processed according to the manufacturer's instructions. Two positive and four negative controls were used for each kit. Briefly, 100 µl of a faecal suspension in the sample diluent buffer provided were added to each well. Then two drops (100 µl) of enzyme conjugate was added onto each well. After 1 hour incubation at room temperature, the microwells were washed five times with 500 µl of wash buffer. After five washes, specifically bound conjugate was determined by the addition of a chromogenic substrate and incubated at room temperature for ten minutes. The absorbance was measured at 450 nm. Specimens with an absorbance value equal to or greater than the cut-off value (Cut-off value was calculated on each kit by adding 0.100 absorbance units to the negative control value) were considered positive.

4.8.2 Molecular typing of rotavirus

4.8.2.1 RNA extraction

Rotavirus antigen-positive stool samples were mixed 1:10 with Eagles Minimum Essential Medium, and clarified by low-speed centrifugation. Double-stranded RNA was extracted from 140 µl supernatant using the Magna Pure LC Total Nucleic Acid Isolation Kit (Roche Applied Science).

RNA was further extracted from a subset of samples using the QIAamp Viral RNA Mini Kit (Qiagen/Westburg, Leusden, and the Netherlands). Both kits were used according to the manufacturer's instructions. 40 µl of RNA extract was used as template for RT with random primers.

4.8.2.2 Random priming reverse-transcription (RT)

A 40-µl portion of each dsRNA-extracted from the sample was used as the template for reverse transcriptase (RT) to synthesize cDNA copies from both strands. The RNA was denatured at 95°C for 5 minutes and quickly chilled on ice for 2 min. The 30 µl of the reaction mixture containing10X buffer II, 50mM Mgcl2, 10mM dNTPs, 200U/ul M-MLV, RNase-free water and random primers was then added making a total volume of 70 µl. This was then incubated at 37°C for 1 hour, then at 95°C for 5 minutes, chilled on ice again for 2 minutes. The cDNA was then used directly in the multiplex PCR.

4.8.2.3 Rotavirus genotyping by PCR

Table 3 shows primer sequences and predicted length of PCR products for multiplex PCR to identify specific P and G serotypes. Rotavirus G an P genotyping were performed using semi-nested type-specific multiplex PCR that could detect 7 G-types

(G1, G2, G3, G4, G8, G9, G10) and six 6 P-types (P[4], P[6], P[8], P[9], P[10], P[11] respectively as explained previously[67]. Each 50ul PCR mixture contained 2ul of VP7for G typing Multiplex PCR and 2ul of VP4 for P typing multiplex PCR from the amplified first round PCR products and 48ul of 10X buffer II, 50mM Mgcl2, 10mM dNTPs and 5U/ul Taq Polymerase. Conditions for the multiplex PCR were I cycle of 94°C for 4 minutes; 30 cycles of 94°C for 1 minute, 42°C for 2 minutes and 72°C for 1 minute; 1 cycle of 72°C and Hold at 15°C The PCR-products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide, and observed under ultraviolet light. The different G and P types were analyzed by comparing the size of the 2nd round PCR-products with PCR-products of known rotavirus strains. The untypeable samples were further analyzed using a single-round rotavirus VP6-specific PCR (Rotavirus Detection and Typing Protocol provided by J. Gray, HPA, UK). Negative and positive controls were used in all PCR assays.

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Table III:. Primer sequences and predicted length of PCR products for multiplex PCR to identify specific P and G serotypes.

	Target gene for genotype	Oligonucleotide sequence (5` to 3`)	Ampl Size(bp)	Reff
G genotypes	G1	**CAA GTA CTC AAA TCA ATG ATG G**	618	[67]
	G2	**CAA TGA TAT TAA CAC ATT TTC TGT G**	521	[67]
	G3	**ACG AAC TCA ACA CGA GAG G**	682	[67]
	G4	**CGT TTC TGG TGA GGA GTT G**	452	[67]
	G8	**GTC ACA CCA TTT GTA AAT TCG**	754	[67]
	G9	**CTT GAT GTG ACT AYA AAT AC**	179	[67]
	G10	**ATG TCA GAC TAC ARA TAC TGG**	266	[67]
	G12	**CCG ATG GACGTAACGTTGTA**	387	[67]
P genotypes	P[4]	**CTA TTG TTA GAG GTT AGA GTC**	483	[67]
	P[6]	**TGT TGA TTA GTT GGA TTC AA**	267	[67]
	P[8]	**TCT ACT GGR TTR ACN TGC**	345	[67]
	P[9]	**TGA GAC ATG CAA TTG GAC**	391	[67]
	P[10]	**ATC ATA GTT AGT AGT CGG**	583	[67]
	P[11]	**GTA AAC ATC CAG AAT GTG**	312	[67]

4.8.5 Detection of norovirus

The IDEIA Norovirus test kit utilises a combination of genogroup 1 and 2 specific monoclonal and polyclonal antibodies in a solid-phase sandwich enzyme immunoassay to detect genogroup 1 and genogroup 2 norovirus. The test has

sensitivity and specificity of 72.8% and 100% relative to PCR (manufactures data). The assay was run according to the manufacturer's instructions.

4.8.6 Detection of adenovirus

The IDEIA Adenovirus test kit utilises a monoclonal antibody in a solid-phase sandwich enzyme immunoassay to detect a genus-specific hexon epitope of adenovirus. The test has an overall sensitivity and specificity of 90.1% and 99.0% respectively when compared to electron microscope (manufactures data). The assay was run according to the manufacturer's instructions.

4.8.7 Detection of astrovirus

The Amplified IDEIA Astrovirus test kit combines genus specific monoclonal and polyclonal antibodies which react with and detect known strains of human astroviruses in a solid- phase immunoassay. The assay was run according to the manufacturer's instructions.

4.9 Detection of intestinal protozoa *C.parvum* and *G.lamblia*

Two hundred and seventy stool samples were available for detectetion of enteric parasites.The presence of C.*parvum* and *G.lamblia* was tested from frozen stool sample using ImmunoCard STAT! rapid assay (Meridian Bioscience, Europe). This is a qualitative immunochromatogenic assay that simultaneously detects and distinguishes between *Cryptosporidium* and *Giardia* antigens in aqueous extracts of patients stool specimens.

4.9.1 Test Procedure

The test was performed following manufactures instructions. Briefly two drops of sample buffer was added to dilution tubes using transfer pipette. Patient specimen

was added to it and mixed. Two drops of conjugate **A** (with Biotinylated rabbit anti-*Giardia* in diluent buffer with carrier protein and detergent) was further added to the tube. Finally two drops of conjugate B (a colloidal dye labelled monoclonal antibodies to *Cryptosporidium/Giardia* in diluent buffer with carrier protein and detergent) was also added to the tube and mixed the sample by manual swirling or by vortexing. The contents were then poured into the sample well of test device (which consist of a membrane coated with an avidin derivative, mouse anti-Cryptosporidium and goat anti-mouse IgG). The results were read visually after 10 minutes.

4.9.2 Interpretation of results

A result was considered positive for *Cryptosporidium* when there was a presence of grey-black bands which was visible at the crypt and cont positions, this indicated *Cryptosporidium* antigen has been detected. The intensity of the band can vary from faint to strong, visible test lines of any intensity was read as positive.Result was considered positive for *Giardia* when there was a grey-back band at the Giar and the cont positions which indicated that *Giardia* antigen has been detected. The intensity of the band can vary from faint to strong, visible test lines of any intensity was read as positive. Result was considered negative for *Cryptosporidium* when no band was visible at the cryp position and a band is present at the cont. position. That means *Cryptosporidium* antigen is absent or is below detectable levels. The Giar position can have either a band or no band.

Result was considered negative for *Giardia* when no band was visible at the Giar position and a band is present at the cont position. That indicated *Giardia* antigen is absent or below detectable levels. The cryp position can have either a band or no band.

Invalid results: result was considered invalid when no band appeared at the cont position, incomplete or beaded band appeared at the cryp or giar position, the test was repeated using another device.

4.10 Antimicrobial susceptibility testing of bacterial isolates

Antimicrobial susceptibility of the bacterial pathogens was determined by the Kirby-Bauer diffusion method according to NCCLS (see procedure in appendix II). Antibiotics tested included ampicillin10μg, augumentin 10μg, erythromycin15 μg, ciprofloxacin 100μg, gentamycin 10μg, cefotaxime 30μg, cephalothin 30 μg, co-trimoxazole 25μg chloramphenicol 30μg, and tetracycline 30μg.

4.10.1 Reading plates and interpretation of results

After 16 to 18 hours of incubation on Muller-Hinton agar, each plate was examined. Zones were measured to the nearest whole millimetre, using sliding callipers or a ruler, which is held at the back of the inverted petri plate. The petri plate was held a few inches above a black, non reflecting background and illuminated with reflected light. The zone margins were taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited growth, was ignored. However, discrete colonies growing within a clear zone of inhibition were sub cultured, re-identified, and retested.

The sizes of the zones of inhibition of the organisms were reported as sensitive, intermediate, or resistant to the agents that have been tested according to NCCLS.

4.11 Quality assurance

This ensures that the information generated from the laboratory is accurate, credible and reproducible. This was achieved by assessing the quality of the specimen, source of culture media, preparation of culture media, culture procedures, biochemical tests, and staining procedures. Dehydrated or commercially available media were used and prepared according to the directions of the manufactures. The pH of each media was checked using pH meter. For sterility check 3 plates of the batch were incubated at 37°C for 48 hrs and the ability to support growth of the suspected organisms was determined by inoculating the medium with a typical stock culture.

Negative and positive controls were used to determine the biochemical response of the reagents/test kits. Tests to detect intestinal parasites and enteric viruses were performed according to manufactures instructions.

4.12 Limitations of the study

This was a comprehensive study to detect bacteria, viruses and parasites causing diarrhoea.

1. One of the limitation of the study is that it was conducted in one season.
2. Due to unavailability of selective media and financial constrains it was not possible to look for all the agents such as *Campylobacter* and *Y. enterocolitica*.
3. Since the time between specimen collection and processing for heliminthes was more than one hour, this could have interfered with detection of *A. lumbricoides*, strongyloides and *T.trichiura*.
4. HIV infection which is one of the predisposing factors for diarrhoea diseases

4.13 Ethical consideration

Ethical and research clearance was obtained from the College Research and Publications Committee, Muhimbili University College of Health Sciences, Dar es Salaam. Permission was sought from the respective hospital authorities. Informed verbal consent was obtained from parents/caretaker of the child before enrolment into the study. Relevant treatment was offered to the children and the attending pediatrician was advised of the results of the stool examinations regarding isolation of bacterial pathogens.

4.14 Data analysis

The Statistical Package for the Social Sciences (SPSS for windows version 10.0) was used for statistical analysis.

Weight-for age z scores were calculated using EPI Info (USD, Inc., Stone Mountain, GA). Assuming the data follows a normal distribution, comparison of proportions and statistical significance were tested by using the Chi-square test. A p value < 0.05 was considered statistically significant.

5 RESULTS

During the study period from December 2005 to February 2006, a total of 280 children with diarrhoea were included in this study. Most patients were from Amana district Hospital 148 (52.9%) followed by MNH 47 (16.8%), Temeke district Hospital 47 (16.8%) and Mwananyamala Hospital 38 (13.6%) (Table IV).

Among them 172 (61.4%) were boys and 108 (38.6%) were girls. These children were aged between 0 and 60 months, with most of them (90.4%) being below 24 months. (Mean 12.96 months, 10.63SD). Children presented mainly with acute watery diarrhoea 235 (83.9%) followed persistent diarrhoea 27 (9.6%), while only few had dysentery 28 (6.4%). Of the children aged below six months 45(88.2%) were not exclusively breast-fed. Regarding antibiotic usage 128 (45.7%) patients were on antibiotic within one week of admission either because of diarrhoea, pneumonia, urinary tract infection or septicaemia. According to CDC\WHO criteria (1978), 79 (28.2%) were undernourished, with Z-scores less than -2SD). Overall the studied group had mean Z-score of -1.2453 SD.

At least one enteric-pathogen was detected in 185 (68.5%) of the cases of diarrhoea. Mixed infections were found in 54 (20%) of the cases, majority (75.9%) of which were of two pathogens, 20.4% were of three pathogens and 3.7% were of four pathogens. The mixed infections were mainly DEC with the other bacteria, viruses or parasites.

The prevalence of different pathogens is shown in tableV, DEC was the most common enteropathogen isolated 64 (22.9%), followed by *C.parvum* 51 (18.9%), rotavirus 49 (18.1%) and norovirus 37 (13.7%) while *V. cholerae* and *Shigella*

The least common pathogens isolated were astrovirus 1 (0.4%), *G.lamblia* 5 (1.8%), adenovirus 7 (2.6%) and *Salmonella* species 7 (2.6%). Regarding distribution all *V.cholerae* isolates were serogroup 0I strains of Ogawa. Of the seven *Salmonella* isolated, four were *S.Typhimurium*, two were *S.Enteritidis* and one was *S.Typhi*. Of the fifteen isolates of *Shigella*, five were *S.dysenteriae* and 10 were *S.flexineri*.

Table VI shows distribution of virulent genes of DEC. Of the 64 DEC strains, EAEC was the most prevalent strain 41 (64.1%); of these 34 (82.9%) were *aggR* positive and 38 (92.7%) were *AA* probe positive. There were13 EPEC (20.3%) strains and 92.3% were typical EPEC with both *eae* and *bfpA* genes and only one strain was atypical with *eae* gene only. ETEC were found in 10 patients (15.6%). All the ETEC strains had either *st1a* or *st1b* not both and no labile toxin. No EIEC or EHEC were detected in this study group.

Table VII summarizes genotypes of group A rotavirus. Of the 49 rotavirus-positive samples, 47 (95.9%) were typeable by RT-PCR and 2 (4.1%) were G and P nontypeable. Overall 46 were G typeable and one was G nontypeable, the dominant G type was G9 40 (85.1%) followed by G1 5 (10.6%). There was only one G3 (0.2%). The genotype G2, G4, G8, G10 and G12 were not detected in this study group. Of the P genotypes, 42 were P typeable and five were P untypeable. Genotype P [8] predominated 41 (87.2%), one strain was P [4] (10.6%). Genotypes P [6], P [9], P [10] and P [11] were not detected. The prevalent P/G combination was P [8], G9, 39 (82.9%).

Table VIII summarizes the association between enteric-pathogens and social demographic factors, in the four hospitals. DEC, *C.parvum,* rotaviruses and noroviruses were the most prevalent pathogens isolated with a range of 10.6% to 34.9%, *C.parvum* was isolated more from children admitted at MNH $p<0.05$. There was also a significant detection of norovirus in Temeke and Amana hospital $p<0.05$.

V.cholerae was isolated more in children of the age group 25-60 months (22.2%) ($p < 0.05$) while DEC were isolated more in the age group of 0-6months (39.2%) and $p < 0.05$. The four viruses, were prevalent in the age group 7-12 months while *Shigella* species, *C.parvum* and *G.lamblia* were isolated more frequently in the age group of 0-24 months but the association was not significant $p>0.05$. *Salmonella* species, adenovirus, astrovirus, *C.parvam* and *G.lamblia* were not isolated from children of 0-6 months of age who were exclusively breast-fed. Most of the pathogens (70-100%) were associated with acute watery diarrhoea, with the exception of *Shigella* species which accounted for 40% of cases of dysentery ($p < 0.05$). In children who were malnourished 26.6%, 21.8%, 16.7% and 11.5% were due to DEC, *C.parvum*, norovirus and rotavirus respectively but the association was not significant $p>0.05$.

Table IX summarises the antimicrobial susceptibility pattern of different bacterial enteropathogens. *Shigella* showed high sensitivity to erythromycin (73.3) and cephalothin (80.0%), but high resistance to tetracycline (100%), cotrimoxazole (93.3%), ampicillin (93.3%), chloramphenicol (93.3%) and augumentin (60.0%).

V.cholerae showed high sensitivity to cotrimoxazole (100%) but high resistance to erythromycin (75.0%), ampicillin (75.0%), augumentin (62.5%), chloramphenicol (68.8%), tetracycline (93.7%), and cephalothin (62.5%).*Salmonella* spp showed high sensitivity to augmentine (85.7%), chloramphenicol (85.7%), cotrimoxazole (71.4%), tetracycline (71.5%), ampicillin (57.2%) and cephalothin (57.1%) but resistant to erythromycin (57.1%). DEC strains showed high sensitivity to augumentin (92.2%) and cephalothin (68.7%) but highly resistant to ampicillin (96.9%), erythromycin (90.6%), chloramphenicol (56,3%), cotrimoxazole (90.6%)and tetracycline (71.9%).

Table X summarizes the combined sensitivity results of all the four bacterial pathogens isolated. Overall all the four bacterial pathogens showed high sensitivity to ciprofloxacin (100%), cefotaxime (97.1%), gentamycin (85.3%), augumentin (78.4%) and cephalothin (64.7%). The four bacterial pathogens showed low sensitivity to amicillin (10.8%), erthromycin (23.5%), cotrimoxazole (26.5%), tetracycline (38.2%) and chloramphenicol (39.2%).

Table IV: Social demographic data, breastfeeding, nutritional status and type of diarrhoea of the study group

	N	%
Hospital		
MNH	47	16.8
Amana	148	52.9
Temeke	47	16.8
Mwananyamala	38	13.6
Sex		
Boys	172	61.4
Girls	108	38.6
Age group		
0-6	51	18.2
7-12	135	48.2
13-24	67	23.9
25-60	27	9.6
Antibiotic use		
Yes	128	45.7
No	152	54.3
Exclusive B/F (0-6) months		
Yes	6	11.8
No	45	88.2
Nutritional status		
Malnourished	79	28.2
Normal	201	71.8
Overall mean Zscore	-1.2453	
Type of diarrhoea		
Acute watery	235	83.9
Persistent	27	9.6
Dysentery	18	6.4

Table V: Prevalence of enteric pathogens isolated from under fives with diarrhoea in Dar es Salaam

	N (% of total cases)	% within anteric pathogen group
Bacteria (n=280)		
DEC	64 (22.9)	
***V.cholerae* 01**	16 (5.7)	
***Shigella* spp**	15(5.4)	
S.flexineri	10	66.7
S.dysenteriae	5	33.3%
S.boydii	0	0.0
S.sonnei	0	0.0
Salmonella species	7 (2.5)	
S.typhimurium	4	57.1
S.enteritidis	2	28.6
S.typhi	1	14.3
S.paratyphi	0	0.0
Protozoans (n=270)		
C.parvum	51 (18.9)	
G.lamblia	5 (1.9)	
Viruses (n=270)		
Rotavirus	50 (18.5)	
Norovirus	37(13.7)	
Adenovirus	7 (2.6)	
Astrovirus	1 (0.4)	
Mixed infection (n=270)	54 (20)	

Table VI: Distribution of virulence genes among the isolated DEC (n=64)

DEC category	Number	percentage
EAEC	41	**64.1**
aggR	34	82.9
AA probe	38	92.7
EPEC	13	**20.3**
Eae	1	7.7
Eae+ bfpA	12	92.3
ETEC	10	**15.6**
elt	0	0.0
St1a	5	50.0
St1b	5	50.0
EIEC		
ial	0	0.0
EHEC		
stx1, or *stx2*	0	0.0

Table VII: Prevalence of P and G genotype of Group A rotavirus detected among underfive children with diarrhoea in Dar es Salaam Tanzania (n=49)

G or Pgenotype	N (%)
G9	40 (85.1)
G1	5 (10.6)
G3	1 (0.2)
G2,G4,G8,G10 and G12	0 (0.0)
G untypable	1
P[8]	41 (87.2)
P[4]	1 (10.6)
P[6],P[9],P[10] and P[11]	0 (0.0)
P untypable	5
P combination P[8], G 9	39 (82.9)

Table VIII: Association between social demographic factors and bacterial enteric pathogens isolated among under fives with diarrhoea in Dar es Salaam

	Salmonella n=7	*Shigella* n=15	*V.cholerae* n=16	DEC n=64	RV	NV	ADV	ASV	*C.parvum*	*G.lamblia*
Hospital[a]									*	
MNH	2(4.3%)	3(6.4%)	4(8.5%)	7(14.9%)	7(16.3%)	4(14.1%)	1(2.3%)	0	15(34.9%)	1(2.3%)
Amana	4(2.7%)	7(4.7%)	6(4.1%)	39(26.4%)	30(21.1%)	*25(17.6)	3(2.1%)	0	24(16.9%)	4(2.8%)
Temeke	1(2.1%)	2(4.3%)	3(6.4%)	10(21.3%)	4(8.5%)	*1(2.1%)	1(2.1%)	1(0.4%)	5(10.6%)	0(0.0%)
M/nyamala	0(0%)	3(7.9%)	3(7.9%)	8(21.1%)	9(23.7%)	6(15.8%)	2(5.3%)	0	7(18.4%)	0(0.0%)
Age group 0-6	0(0%)	5(9.8%)	1(2.0%)	20(39.2%) *	13(26.0%)	9(18.0%)	0(0.0%)	0(0.0%)	11(22.0%)	3(6.0%)
7-12	2(1.5%)	5(3.7%)	5(3.7%)	32(23.7%)	26(18.5%)	20(14.8%)	6(4.5%)	0(0.0%)	23(17.3%)	1(0.8%)
13-24	4(6.0%)	2(3.0%)	4(6.0%)	8(11.9%)	9(14.3%)	7(11.1%)	1(1.6%)	1(1.6%)	15(23.8%)	1(1.6%)
25-60	1(3.7%)	3(11.1%)	6(22.2%)*	4(14.8%)	2(8.3%)	1(4.5%)	0(0.0%)	0(0.0%)	2(8.3%)	0(0.0%)
Exlusive B/F[a] Yes	0(0.0%)	1(16.7%)	1(16.7%)	1(16.7%)	2(40.0%)	1(20.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)
No	0(0.0%)	4(8.9%)	0(0.0%)	19(42.2%)	11(24.4%)	8(17.8%)	0(0.0%)	0(0.0%)	11(24.4%)	3(6.7%)
Type of diarrhoea[b] Acute watery	5(71.4%)	7(46.7%)	16(100%)	52(81.3%)	44(88.0%)	32(86.5%)	6(85.7%)	1(100%)	40(78.4%)	4(80.0%)
Persistent	1(14.3%)	2(13.3%)	0(0.0%)	9(14.1%)	5(10.0%)	3(8.1%)	0(0.0%)	0(0.0%)	9(17.6%)	1(20.0%)
Dysentery	1(14.3%)	6(40.0%)*	0(0.0%)	3(4.7%)	1(2.0%)	2(5.4%)	0(0.0%)	0(0.0%)	2(3.9%)	0(0.0%)
Nutritional status[a] Malnourished	2(2.5%)	3(3.8%)	3(3.8%)	21(26.6%)	9(11.5%)	14(17.9%)	2(2.6%)	0(0.0%)	17(21.8%)	2(2.6%)
Normal	5(2.5%)	12(6.0%)	13(6.5%)	43(21.4%)	41(21.4%)	23(12.0%)	5(2.6%)	1(0.5%)	34(17.7%)	3(1.6%)
Total										

B/F =breast feeding

Hospital[a], Exclusive B/F[a], Nutritional status[a], Age group[a]; percentages calculated in rows

Type of diarrhoea[b]; percentages calculated in column; * p value < 0.05

Table IX: Antimicrobial susceptibility pattern of the different bacterial enteric pathogens isolated from under fives with diarrhoea in Dar es Salaam

Antimicrobial agent	*Salmonella*		*Shigella*		*V.cholerae*		DEC	
	S	R	S	R	S	R	S	R
Ampicillin	57.2	42.9	6.7	93.3	25.0	75.0	3.1	96.9
Augumentin	85.7	14.3	40.0	60.0	37.5	62.5	92.2	7.8
Erythromycin	42.9	57.1	73.3	26.7	25.0	75.0	9.4	90.6
Chloramphenicol	85.7	14.3	6.7	93.3	31.3	68.8	43.8	56.3
Ciprofloxacin	100	0	100	0	100	0	100	0
Cotrimoxazole	71.4	28.6	6.7	93.3	100	0	9.4	90.6
Tetracycline	71.5	28.6	0	100	6.3	93.7	28.2	71.9
Gentamycin	71.4	28.6	100	0	93.8	6.3	82.8	17.2
Cefotaxime	100	0	100	0	100	0	95.3	4.7
cephalothhin	57.1	42.9	80.0	20.0	37.5	62.5	68.7	31.3

S= sensitive, R=resistant

Table X: Combined sensitivity results of the four bacterial enteric pathogens isolated from under fives with diarrhoea in Dar es Salaam

Antimicrobial drug	% of sensitivity
Ampicillin	10.8
Erythromycin	23.5
Cotrimoxazole	26.5
Tetracycline	38.2
Chloramphenicol	39.2
Cephalothin	64.7
Augumentin	78.4
Gentamycin	85.3
Cefotaxime	97.1
Ciprofloxacin	100

6 DISCUSSION

This was a comprehensive study which investigated bacterial pathogens including five categories of DEC, *Salmonella*, *Shigella*, *Vibrio* species; four different groups of enteric viruses namely rotaviruses, noroviruses, adenoviruses, astroviruses and intestinal protozoans namely *C.parvum* and *G.lamblia*. This study was conducted during the dry season of the year. The prevalence of cases of diarrhoea with a known aetiology was 68.5%. These results concur with the findings of Martha Vargas et al in Ifakara Tanzania who detected enteric pathogens of underfive years of age with diarrhoea in 63% during the dry season and 74% in rainy season [5]. The results of this study show that 54 (20%) children had multiple infections up to four associated pathogens. In some developing countries co-infections have been detected in up to one-third of the diarrhoeal cases. Co-infections have been reported to cause more severe disease[49]. Severity of diarrheoa was not investigated in the present study however it is essential to look for all possible causative agents in children with diarrhoea because apart from its contribution in severity, multiple infections may be relevant in treatment outcome of the patient.

In this study at least one bacterial pathogen was detected in 93 patients (33.3%). The proportion of DEC (64.1%) was high, which is consistent with previous reports from Tanzania and other developing countries which showed DEC are most common pathogens in children with diarrhoea [5, 65]. This suggests that DEC remains an important cause of diarrhoea in children aged less than five years [5, 65]. The frequency of isolation of DEC was significantly higher among children of 0-6

months of age (P<0.05). Among the DEC, EAEC was the most common, accounting for 64.1% of DEC, and this is in agreement with a previous study among children in Ifakara in which, EAEC accounted for 63% of the DEC during dry season [5]. Collectively, these studies support the findings of other studies conducted in developing countries showing the predominance of EAEC among DEC [5, 68-70]. The pathogenic mechanisms of EAEC infection are only partially understood and are consistent with mucosal colonization followed by secretion of enterotoxins and cytotoxins[23]. The varying presence of the different virulence factors indicates heterogeneity of the EAEC isolates [68]. It has been hypothesized that the combination of these genes increases strain virulence. The *aggR* which is a transcriptional activator gene was originally described as a protein required for the expression of the two genes AAF/I [24] and AAF/II [71]. More recently, the *aggR* has been shown to be required for the expression of the *aap* [70]and *aatA* [72]genes. Therefore, it is likely that the *aggR* gene serves as a marker for truly virulent EAEC strains, which express a package of virulence genes. Nataro has recently suggested the term "typical EAEC" to refer to strains expressing the *aggR* regulon [72]. In the present study, the plasmid-borne genes *aggR* and *AA* probe were used to detect EAEC. The study showed high prevalence of typical EAEC than atypical among diarrhoeic children of Dar es Salaam.

In the present study EPEC was the second most common DEC isolated and most were typical EPEC with both *eae* and *bfpA* genes. Typical EPEC is well recognized as a cause of diarrhoea in children [9, 16]. In this study only one strain of atypical

EPEC was detected. The role of atypical EPEC in childhood diarrhoea is still controversial [15, 17].

ETEC in the present study accounted for 15.6% of the DEC, which is high compared with the finding of Cegielski et al 11.9% in Dare s salaam Tanzania [60], but lower than 20% and 51.6% found in Ifakara, Tanzania during dry and rainy seasons, respectively [5]. These differences could be related at least in part to seasonality and geographical differences since Ifakara is about 500km away from Dar es Salaam.
In the present study, ETEC was detected by looking for both variants of ST1 namely *st1a* or *st1b*. Using primer set for *st1a* five strains of ETEc were detected, five more strains of ETEC were detected using primer set for *st1b*. The 10 strains of ETEC detected in this study harboured either *st1a* (50%)or *st1b*(50%) gene not both. The use of primer sets for both variants of ST1 increases the sensitivity for detection of ETEC strains. This argument is supported by the results of this study where sensitivity would have decreased by 50% if only one set of primer had been used. There was a notable exception of heat-labile enterotoxin (LT)-producing ETEC strains, which was not present in this study. The results seen with ETEC strains concur with the results of other studies, in which ST-producing strains were more frequently recovered from cases of diarrhoea and showed a greater association with diarrhoea than LT-producing *E. coli* [5, 73]. EHEC and EIEC strains were not found in these children, and this is in agreement with the previous studies in Tanzania [5, 60], indicating their limited role in childhood diarrhoea in Tanzania.

Shigella species were detected in 5.7% of cases of diarrhoea in this study. *Shigella* species are frequently isolated in children with bloody diarrhoea and in this study the frequency of isolation of *Shigella* from bloody diarrhoea was 40% ($p \leq 0.05$). *S.flexineri* (66.7%) was the most frequently encountered among *Shigella* species. The predominance of *S.flexineri* is in keeping with the finding of Vargas et al in Ifakara Tanzania [5]. *S.dysenteriae* (33.3%) was the second most frequently isolated, *S.dysenteriae* type I has been reported to cause severe disease but this could not be concluded in this study due to the fact that typing of *S.dysenteriae* was not done due to unavailability of specific antisera. *S.sonnei* and *S.boydii* were not detected in this study.

With regard to *Salmonella,* serotypes isolated were *S. Enteritidis* (28.6) and *S.Typhimurium* (57.1%). The findings of 5.7% of salmonella infections fall in the range reported by WHO (1995) that *Salmonella* causes about 1±5% of gastroenteritis cases in children in most developing countries [6].

The relatively high prevalence *V.cholerae* in the present study was due to the fact that there was an outbreak of cholera during the time of study. Notably the isolation of *V.cholerae* was significantly high among older children ($P<0.05$).

The findings of this study show that all bacterial pathogens isolated are highly sensitive to ciprofloxacin, third generation cephalosporin (cefotaxime), gentamycin, augumentin and cephalothin. These findings are in agreement with the findings of previous studies [63, 64, 74, 75], and could be due to the fact that these antimicrobial drugs are not used widely. On the other hand multiple antibiotic resistance in all

pathogens was observed with commonly prescribed antibiotics such as ampicillin, chloramphenicol, tetracycline as well as cotrimoxazole and erythromycin which are currently recommended for empirical treatment of dysentery and suspected cases of cholera in Tanzania[6] . Multiple antibiotic resistance is now a common phenomen, it has been reported among *V.cholerae* [64], *Shigella* species [63], and in diarrhoeagenic *E.coli* [62]. The phenomen has also been reported in other developing countries [29, 74] and is most likely due to inappropriate/misuse of antibiotics. The observed resistance to empirically used antibiotics necessitates re-evaluation of the current guidelines for routine treatment of diarrhoea in children. The antimicrobial sensitivity pattern of *V.cholerae* observed in this study when compared with those of Urrasa *et al* [64] does show an increase in resistance to ampicillin (88.2% versus 17%), erythromycin (76.5% versus 18.1%) and tetracycline (61.8% versus 1.4%) and decrease resitance to cotrimoxazole (0.0% versus 96.8%). Although it may be tempting to speculate trends, this is difficult to substantiate due to small number of isolates in the present study.

Enteric viruses c aused nearly one-third (31.9%) of diarrhoea cases. Of the four viruses, rotavirus was by far the most frequently detected, accounting for 57.5% of all the viruses followed by norovirus that accounted for 42.5% of the viruses. In the current study rotavirus accounted for 18.5% of all cases of diarrhoea. This prevalence is relatively low compared with other findings in Tanzania which reported rotavirus to account up to 43% of diarrhoea cases [5, 55, 56]. The differences could be explained, at least in part by seasonal differences and by the fact that the previous studies used agglutination tests which have been reported to be less specific and may lead to false positive results compared to ELISA [66].

The frequency of norovirus found in this study (13.7%) is in keeping with numerous other studies conducted in both developing and developed countries that have reported norovirus to be responsible for 10 to 15% of all gastroenteritis [31].

We found a relatively low rate of enteric adenovirus (2.6%) which is in agreement with a previous report of 2.3% in Dar es Salaam [55]suggesting that adenoviruses play little role in causing diarrhoea among children in Tanzania.

In this study only one astrovirus was detected (0.4%).This report is the first to examine the epidemiology of astrovirus in patients with gastroenteritis in Tanzania, and our finding suggests that the virus accounts for only a minority of diarrhoea cases.

This is the first report on molecular epidemiology of rotavirus in Tanzania. The results showed unexpectedly high proportion of G9 serotype (85.1%). This is in contrast with findings of other African countries such as Kenya, Malawi and Ghana, where the highest reported prevalence of G9 was 26.9% [76-80]. The complete absence of serotypes G2 and G4 in this study group is in marked contrast to the global situation, where these strains are often the most common [81]. This variation in strain distribution underlines the importance of active rotavirus strain surveillance. The predominance of G9 has significant implication for rotavirus vaccine introduction in Tanzania. Evidently, the two vaccines that are in use namely RotaRix® and RotaTeq® cover only G1 to G4 [54].

Notably P[8] which is the most prevalent genotype globally [54] was detected in 87.2% of all strains. The combinations of [P] and G genotypes was mainly P[8] and G9 because of the predominance of serotype G9.

The present study also searched for some of the intestinal parasites that are associated with diarrhoea in children namely *C.parvum* and *G.lamblia*. *G.lamblia* was detected in only minority of diarrhoea cases in this study group probably indicating their limited role in childhood diarrhoea among children of Dar es Salaam. The prevalence of cryptosporidiosis was 18.9%, a prevalence that is higher than 8.5% reported in 1999 in children with chronic diarrhoea in Dar es Salaam [61]. The higher prevalence can partly be explained by inclusion of acute diarrhoea cases in the present study which is the most common type of diarrhoea.

In this study bacteria contributed equally as viruses in causing diarrhoea accounting for 33.2% and 31.9% of all the cases, respectively. However when age stratification is done diarrhoea in children aged 7-12 months was mainly due viruses, while bacteria, predominantly DEC, were the main cause of diarrhoea in children of the age 0-6 months. *V.cholerae* affected mainly chidren that were above two years of age. This age related pattern of pathogens is consistent with that reported in other studies conducted elsewhere [6,7].

In the present study only six children were exclusively breast fed. Due to small number of children who were exclusively breast- fed it is difficult to comment on whether exclusively breast fed children were not protected against diarrhoea from shigellosis, cholera, cryptosporidiosis or infection with DEC which was observed in this study. A worrying possibility is that breast feeding might be associated with higher HIV seroprevalence and thus predispose to these infections but there is no

direct evidence of this since HIV was not tested in this study group. It still remains to be emphasized that breast feeding is the safest and best option for mothers.

7. CONCLUSIONS

1. Acute watery diarrhoea is the most common type of diarrhoea among underfive in Dar es Salaam and that most cases occurred in children under two years of age.

2. DEC, *C.parvum,* rotaviruses and noroviruses were the most common enteric pathogens , in addition to that mixed infection was high (20%) in children with diarrhoea.

3. Among DEC strains typical EAEC and typical EPEC were the most common.

4. The genotype G9 which is an emerging rotavirus strain that is not included in the two available vaccines was by far most predominating among group A rotavirus.

5. Bacteria isolated from children with diarrhoea showed high resistance to the first line antibiotics, including those currently recommended for empiric treatment of diarrhoea in children in Tanzania i.e. erythromycin and cotrimoxazole.

8. RECOMMENDATIONS

Based on the results obtained during this study, the following recommendations are suggested:

1. To continue with rotavirus molecular characterization in Dar es Salaam since the period of surveillance of this was limited to a period of 3-months. In addition, rotavirus survailance should be conducted in other regions of Tanzania in order to have a comprehensive picture of strain distribution in the country in terms of regional and seasonal variation

2. The futur e candidate vaccines for rotavirus should provide protection against serotype G9, which is an emerging serotype.

3. There is a need to establish molecular diagnostic methods for detection of DEC since these are most common cause of diarrhoea in children. In addition there is a need to update our laboratory techniques by including the use of rapid methods and immunoassays techiniques to enhance identification of multiple infections.

4. In view of the antimicrobial resistant pattern found in this study the current IMCI guidelines for routine treatment of diarrhoea among children should be revisited.

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