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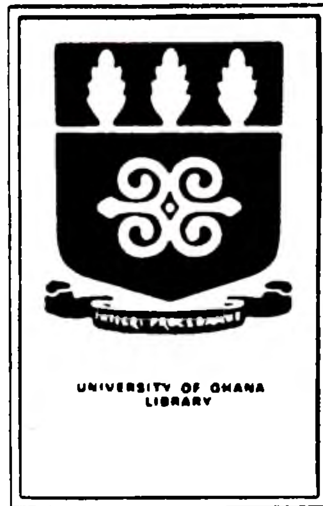

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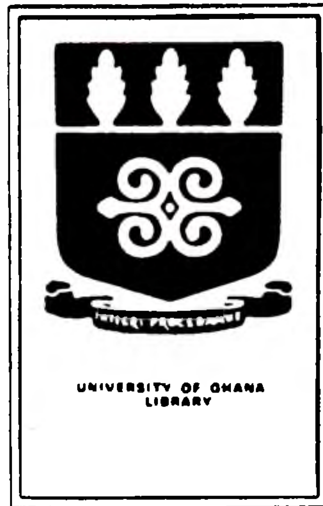

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INCIDENCE AND MOLECULAR CHARACTERIZATION OF NEONATAL HUMAN ROTAVIRUS STRAINS IN ACCRA.

A THESIS SUBMITTED BY

AKWASI AGYEMAN

TO THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF GHANA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF PHILOSOPHY (MPHIL) DEGREE.

SEPTEMBER, 2000.

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DECLARATION

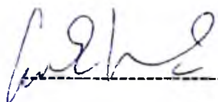
I certify that the work described in this research report was carried out by me at the Electron Microscopy Unit, Noguchi Memorial Institute for Medical Research and the Biochemistry Department of the University of Ghana, Legon under the supervision of Dr. G. E. Armah and Dr. J. P. Adjimani.

Akwasi Agyeman

----------------------Date ----------------------

Student

Dr. G. E. Armah



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Supervisor

Dr. J. P. Adjimani

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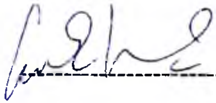
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DEDICATION

To the memory of my late father Nana Akwasi Agyeman and to my mother Akua Benewaah. To my siblings, Konadu, Bonsu, Dwomoh, Badu, Tutu, Oheneafrewo and Saah

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I would also like to thank all the lecturers at the Biochemistry Department especially Mr. Christian Clement for their tremendous assistance.

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My heartfelt appreciation goes to Messrs Dodoo, Ayim, Asmah, Odoi and Sister Suzie, all of the electron microscopy unit of the Noguchi Memorial Institute for Medical Research for the co-operation, love and contributions they offered during the period. My appreciation also goes to the management of the Noguchi Memorial Institute for Medical Research for allowing me to use their laboratory and facilities. Sincere appreciation is also expressed to all the technical staff of the Biochemistry

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To all my friends and all those whose names were not mentioned but who helped in diverse ways towards the completion of this work, I say thank you and God bless you all.

Finally, to members of my wonderful family, from whom my attention and services were withdrawn in order to have a complete concentration on this work, I would like to say that I appreciate your love, understanding and care.

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LIST OF ABBREVIATIONS

APS	- ammonium persulphate
TEMED	- N, N, N' - N - tetramethylethylene diamine
Mabs	- monoclonal antibodies
ELISA	- enzyme linked immunosorbent assay
SDS-PAGE	- sodium dodecyl sulphate polyacrylamide gel electrophoresis
RT-PCR	- reverse transcription polymerase chain reaction
RNA	- ribonucleic acid
µM	- micromolar
ml	- millilitre
µl	- micromolar
µg	- microgram
U.V	- ultraviolet
EDTA	- ethylenediamine tetra acetic acid

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ABSTRACT

Specific and sensitive tests for the detection and typing of rotavirus strains are essential for a more assessment of the epidemiology of rotaviral infection in a community. In this study, 200 stool specimens obtained from October, 1999 to March, 2000 from neonates at the Babies Unit of the Korle-Bu teaching and Legon Hospitals were examined. Group A rotavirus was detected in 30% and Non-group A in 2.5% of the samples tested by either enzyme-linked immunosorbent assay (ELISA) for the detection of VP6 antigen and/ or polyacrylamide gel electrophoresis (PAGE) of double-stranded RNA respectively.

Reverse transcription-PCR (RT-PCR) was used for the amplification of the VP7:G (1,062 bp) and VP4:P (876 bp) genes. Five positive specimens were positive by PAGE but negative by ELISA. G and P typing was carried out by nested amplification of variable sequences of the VP7 and the VP4 genes with six G- and five P-type-specific primers (multiplex PCR). The observed P and G types were as follows: G2, 57.1%; G3, 7.1%; G9, 10.7%; G2G3, 3.6%; P6, 67.9%; P8, 3.6%; P6P10, 14.3%. 21.4% of G type and 14.3% of P type were nontypeable. The G-P type combination most frequently found was G2P6 (42.9%), which is the most commonly found in neonates worldwide. Unusual strains of the type G3P6, G9P6 accounted for 7.1% and 3.6% respectively while mixed infections with more than one type were found in 17.9% of the samples typed. Samples whose either P and/ or G could be typed accounted for 28.6%.

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CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

Diarrhea is the second most common cause of death and morbidity in children in developing countries. It is of great economic importance, causing millions of lost working days each year, as well as much discomfort (Bern *et al.*, 1992).

Diarrhea is defined as an abnormally frequent discharge of semisolid or fluid faecal matter from the bowel. It is recognised in humans as one of major disease of public health concern world wide (WHO., 1990). In poorly developed countries where conditions such as inadequate means of disposal of human faecal waste, poor personal hygiene and lack of education exist, the various pathogens that cause diarrheal disease are readily transmitted to young children often resulting in high rates of morbidity and mortality (Bern *et al.*, 1992). Diarrhea continues to cause a great number of deaths, with a disproportionately high mortality in infants and young children in developing countries. In many developing countries, a child suffers six to eight episodes of diarrhea per year before the age of 2 (Bern *et al.*, 1994). Most of the life experience of these young children are spent suffering from diarrhea (Gurwith *et al.*, 1981). In a majority of cases, these episodes of diarrhea are mild and do not require a visit to a health care facility. However about 10% of these diarrheas results in clinical dehydration and 0.5 to 1% culminate in severe life threatening dehydration (Mata *et al.*, 1983).

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Studies carried out by Walsh and Warren (1979) estimated that 3 to 5 billion cases of diarrhea occurred in 1978 in Asia, Africa and Latin America resulting in 5 to 10 million deaths. Another estimate which reviewed data from several longitudinal studies in children revealed that 4.6 million diarrheal deaths and 744 million to 1 billion episodes of diarrhea occurred in children less than 5 years of age in the same regions excluding China (Synder and Merson, 1982). It has been reported further that 3-3.2 million children still die each year from diarrhea (23 deaths per 1000 live births), making diarrhea a major contributor to infant mortality in developing world (Flores *et al*., 1986).

Although there has been a decrease in incidence globally, it is still a major cause of death in children in Africa. In the impoverished rural communities of Africa, up to 25% of infants aged 6-11 months may suffer from diarrhea in any two week period (Bern *et al*., 1994). Worldwide, it is estimated that each child under five may suffer an average of 2.6 episodes of diarrhea per year (Oelsofen *et al*., 1990).

Bacteriological and parasitological advances made during the past century have led to the discovery of the etiology of some of the diarrhea illnesses, but a lot still remains unsolved (Ramachandran *et al*., 1996). Diarrhea can be caused by a variety of different germs, including bacteria, viruses and parasites. It can also be caused without an infection by food allergies or as a result of taking medicines such as antibiotics. The vast majority of diarrheal cases are due to acute self-limiting intestinal infection by bacteria and other parasites and are successfully managed at home without recourse to medical care.

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The symptoms of bacterial diarrhea are mostly of the milder forms and in most of these cases, administration of a large volume of water and electrolytes can prevent dehydration and cure the diarrhea. On the other hand, the life threatening forms of diarrhea requiring hospital visits or stay are caused mostly by viruses.

Rotavirus is the single most important viral etiologic agent of severe and dehydrating diarrhea of infants and young children world-wide and have been isolated in more than 50% of cases (Kapikian and Chanock, 1996). They were first observed in the duodenal epithelium of children with diarrhea by electron microscopy in 1973 by Bishop *et al.*, (1973) and subsequently designated rotavirus (Latin, *rota* = wheel) because of its appearance.

In various studies conducted in Africa, rotavirus has been shown to be the most common cause of diarrhea in young children under the age of five years. Rotavirus infection often occurs at an early age with virus shedding observed in infants under 12 months of age. Studies in Ghana have also shown the rotavirus to be a very important pathogen of diarrhea in children and to be responsible for over 20% of diarrhea in children aged 18 months or less peaking after the first year and then declining during the third year of life (Armah *et al.*, 1994). Infection is highest during the dry and hot seasons with peaks in September and February.

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Infections with rotavirus are widespread in both developed and developing countries as evidenced by the prevalence of serum antibodies in approximately 90% of infants and young children by three years of age (Kapikian *et al.*, 1996). Although rotavirus

infections are known to be transmitted by the faecal-oral route, the extremely high rate of infection in both developed and developing countries, regardless of sanitary conditions, has led to speculation that respiratory transmission might also occur (Kapikian *et al.*, 1996). For example, it is estimated that: (i) three million infants and young children develop rotavirus diarrhea yearly in the United States of America; (ii) 82,000 are admitted to the hospital with rotavirus diarrhea and (iii) rotavirus diarrhea causes about 150 deaths (Institute of Medicine. Prospects of immunizing against rotavirus infections, 1990). In contrast, the toll of the rotavirus infection and death is staggering in developing countries. Estimates indicate that each year 18 million cases of moderately severe or severe rotavirus diarrhea occur in children under five years of age and that more than 870,000 children in this age group die because of rotavirus disease (Unicomb *et al.*, 1989).

Rotaviruses are members of the family *Reoviridae* and are 70nm in diameter, spherical in shape, non-enveloped, and possess a distinctive double-shelled icosahedral outer shell (Fig.1). The virion consists of three shells: the outer shell, the inner capsid and the core (Fig. 2c), which encloses 11 discrete double stranded RNA genes (Fig.2a and 2b). Each of these genes encodes specific viral proteins, which are either structural or nonstructural.

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Viral protein (VP) 1, VP2, and VP3 are encoded by gene segments 1, 2, and 3 respectively and are located in the core. Viral protein 6 (encoded by gene segment 6) forms the inner capsid. VP4 (encoded by gene 4) and VP7 (encoded by gene segment 7, 8 or 9 depending on the strain) make up the outer capsid of the rotavirus particles and are known to induce neutralizing antibodies (Kapikian *et al.*, 1996).

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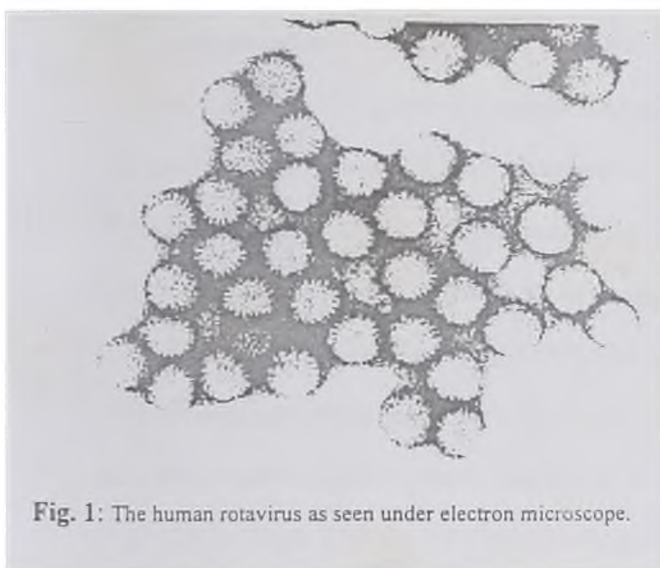

Fig. 1: The human rotavirus as seen under electron microscope.

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VP7 is a glycoprotein (G for glycoprotein and thus G-type) with a molecular weight of 34, 000 which is one of the two major neutralization antigens located on the outer shell. The other outer shell protein VP4 with a molecular weight of 84, 000 is protease-cleaved, and is hence called a P-protein and thus P-type. It protrudes from the outer surface as 60 spikes, each 10-12nm in length. The product of gene segment 4 (VP4) is a nonglycosylated protein (Arias *et al.*, 1982). Knowledege of the diverse properties of the VP4 has resulted in an increased awareness of the importance of this protein in the biology of rotaviruses. VP4 is the hemagglutinin in many rotavirus strains (Kalica *et al.*, 1983) and probably the cell attachment protein (Ruggeri *et al.*, 1992). Proteolytic cleavage of VP4 to VP5 [molecular weight (M_r) approximately 60 000 (60K)] and VP8 (M_r aproximately 28K) results in enhancement of viral infectivity (Espejo *et al.*, 1981; Estes *et al.*, 1981). Cleavage of VP4 enhances penetration (but not binding) of virus into cells (Fukuhara *et al.*, 1988). VP4 is also associated with restriction of growth of certain rotavirus strains in tissue culture cells (Crawford *et al.*, 1994) and with protease-enhanced plaque formation (Kalica *et al.*, 1983). Features of VP4 with potential biologic relevance have been found by analyzing nucleotide and predicted amino acid sequence data (Lopez *et al.*, 1985). Both VP4 and VP7 have blocked NH_2-termini, and VP5 is composed of two polypeptide species with slightly different amino acid sequences at their NH_2-termini. Comparison of these data with the nucleotide sequence of VP4 identified two trypsin cleavage sites (arginine 241 and arginine 247), with position 247 being the preferred cleavage site (Lopez *et al.*, 1985).

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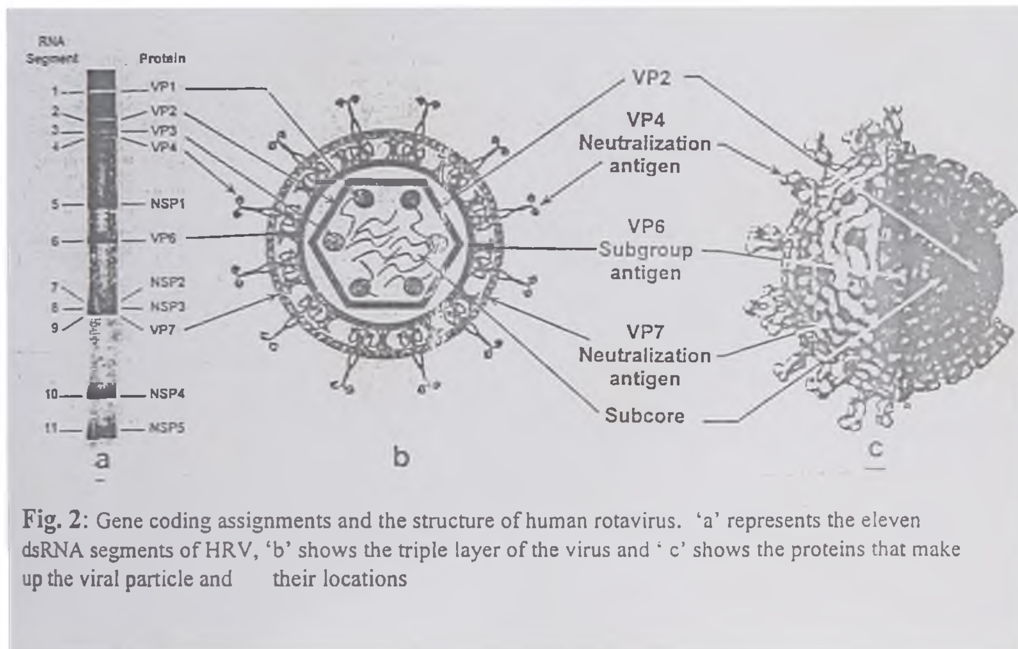

Fig. 2: Gene coding assignments and the structure of human rotavirus. 'a' represents the eleven dsRNA segments of HRV, 'b' shows the triple layer of the virus and ' c' shows the proteins that make up the viral particle and their locations

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Replication of HRV

Rotaviruses infect the small intestine after oral ingestion and spread through the faecal route. Multiplication occurs in the mature epithelial cells at the tip of the villi of the small intestine (Estes, 1996). The initial stages of replication have been examined by biochemical and morphologic (EM) procedures. Only triple-layered particles containing VP4 attach to cells when monitored by EM (Petrie *et al.*, 1981) or by cell binding (Crawford *et al.*, 1994) or infectivity assays (Bridger *et al.*, 1976). Increasing direct evidence indicates that virus attachment occurs through VP4, although early studies reported that VP7 and nonstructural protein (NSP) 2 had cell binding activity (Fukuhara *et al.*, 1988). Initial binding is sodium-dependent, pH insensitive and is dependent on sialic acid residues in the membranes. Virus entry can be receptor-mediated endocytosis or direct penetration. The virus-associated transcriptase is latent in triple-layered particles (full particle) and can be activated *in vitro* by treatment with a chelating agent or by heat shock treatment (Spencer *et al.*, 1983). Such treatment results in the removal of the outer capsid protein with conversion of the triple-layered particle to double-layered particle (Cohen *et al.*, 1979). Early EM studies suggested that virus entry occurs by endocytosis (Petrie *et al.*, 1981) and that incoming particles are rapidly transported to lysosomes and that uncoating might occur by the effect of lysosomal enzymes (Ludert *et al.*, 1987).

The synthesis of rotaviral transcripts is mediated by an endogenous viral RNA-dependent RNA polymerase (transcriptase) that has a number of enzymatic activities. After removal of the outer capsid, the viral RNA-dependent RNA polymerase is activated, and

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large numbers of single stranded mRNAs are produced which leave the single-shelled particle via its pores. Transcripts function both to produce proteins and also templates for the production of virus strands. Once the complementary minus strand is synthesized, it remains associated with the plus strand. The dsRNA segments are formed within nascent subviral particles, and free dsRNA or free minus strand ssRNA segments are never found in infected cells. Subviral particles VP1, VP2, VP3 and VP6 form in association with viroplasms, and these particles mature by budding through the membrane of the endoplasmic reticulum. In this process, particles acquire their outer capsid proteins, VP7 and VP4. Cell lysis releases particles from infected cells as shown in Fig. 3.

Pathogenesis of HRV

The infected duodenal villi cells lyse with increasing necrosis at the villous tips which eventually leads to atrophy and reduction of absorption. This is followed by a reactive crypt cell hyperplasia accompanied by an increased secretion, which also contributes to the severity of diarrhea. Viral factors determining pathogenicity of rotaviruses have been investigated in several animal models. The protein product of RNA segment 4, VP4, has been found to be a major pathogenicity determinant in several systems. In addition, the products of other structural genes (VP3, VP7) and also of nonstructural genes (NSP1, NSP2, NSP4) have been implicated (Hoshino *et al.*, 1995). NSP4 has also recently been described as a viral enterotoxin (Ball *et al.*, 1996).

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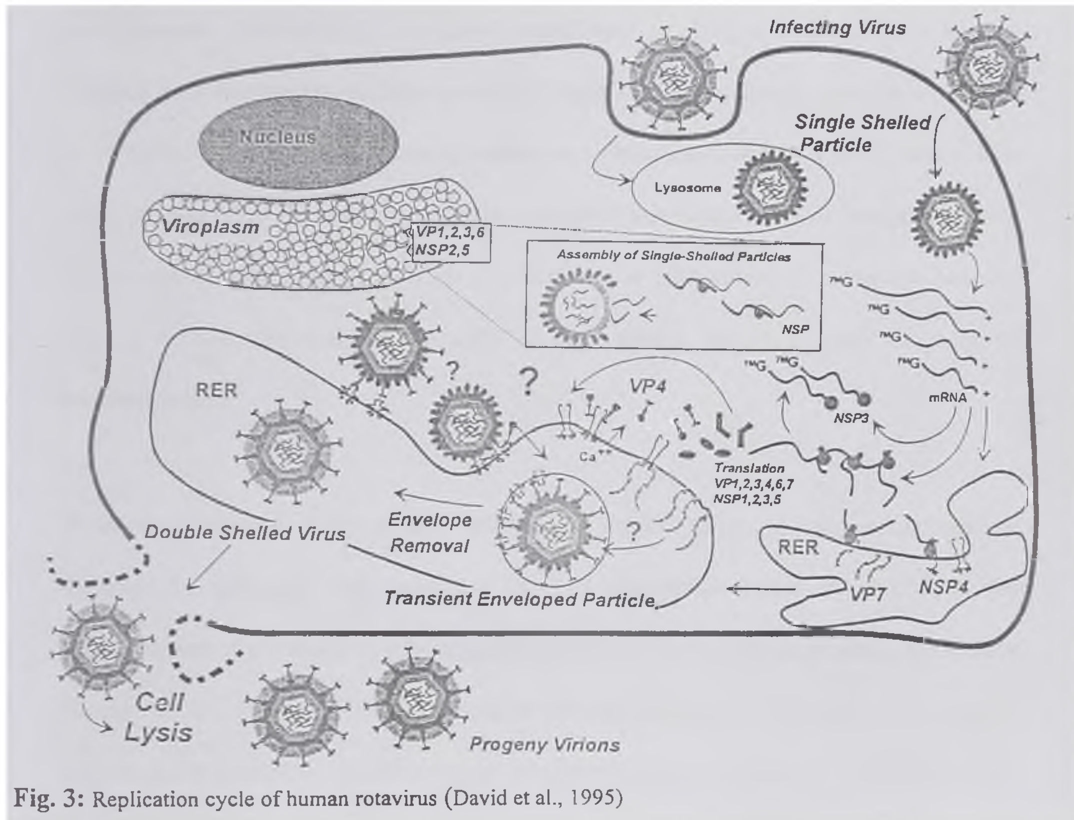

Fig. 3: Replication cycle of human rotavirus (David et al., 1995)

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Clinical Manifestations and Treatment

The typical clinical picture of a child with rotavirus gastro-enteritis is for all practical purposes indistinguishable from diarrhea of other etiology. In general, rotavirus gastroenteritis is commonly associated with fever, vomiting and dehydration than in children with diarrhea due to other etiological agents. In most severe cases the illness has an abrupt start and the child is usually taken to the hospital within the first 36 hours after onset of symptoms. In most cases the resolution of the illness is rather benign since the patient can be rehydrated within the first hours after admission. The average hospital stay is 4 days, however, up to 20% of the patients require 7 days or more of hospitalisation.

In immunodeficient children and children or adults receiving immunosuppressive therapy, the infection with rotavirus imposes additional threats ranging from the establishment of a chronic or prolonged diarrhea to severe cases associated with fatality (Yolken *et al.*, 1982). Thus the spectrum of rotavirus infection is rather broad, from subclinical infections to serious cases of dehydration leading to death. Although a series of syndromes have been associated with rotavirus diarrhea including intussusception, Reyes syndrome, sudden death, necrotizing enterocolitis, etc., a direct cause-effect relationship has not been established (Flores *et al.*, 1986).

Epidemiology of Rotavirus Infection

The importance of rotaviruses as the major etiologic agent of gastro-enteritis in children under 2 years of age has been well documented in more than 30 countries (Rodriquez *et*

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al., 1980). In most of these studies, rotaviruses have been associated with between 20% and 60% of diarrhea cases requiring hospital admission.

Not many prospective studies have been carried out to assess the actual incidence of rotavirus illnesses at the community level. One study in Washington, DC, estimated that 1 in 272 infants under 1 year of age and 1 in 451 children 1 to 2 years of age would be hospitalised with rotavirus diarrhea during a one year period (Rodriguez *et al.*, 1980). Another study in Michigan (Koopmans *et al.*, 1984) estimated that children experienced 0.15 episode of rotavirus diarrhea during the first year of life and 0.05 episode during the second year; 11% of those children required hospitalisation. These figures contrast with longitudinal studies carried out in Bangladesh and Guatemala in which estimates of 0.5 and 0.8 episodes, respectively, of rotavirus diarrhea per child per year were observed during the first 2 years of life (Mata *et al.*, 1983).

In areas with temperate climates, rotavirus diarrhea is seasonal and peaks during the cooler months of the year whilst in the tropical areas, rotavirus diarrhea occurs throughout the year, with peaks in the cool dry months. This seasonal distribution may be responsible in part for some differences (observed in some epidemiological studies) such as the modes of transmission, the rate of reinfection and the age of occurrence of the disease.

The age distribution of rotavirus diarrhea in developing countries differs somewhat from that observed in developed regions. In the developed countries, rotavirus infection is

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usually seen after the age of 12 months peaking usually at 18 months. However, in the developing countries, a rotavirus infection usually occurs before the age of 12 months. This early infection in developing countries may be attributed to an early exposure to rotavirus especially in prevailing unsanitary conditions in most developing countries. It could also be related to the seasonal variations observed in developing countries where the cyclic character of the disease would spare many young susceptible children for at least one season (Flores *et al.*, 1986).

Symptomatic rotavirus illness in neonates has however been observed to be low generally (Perez-Schael *et al.*, 1984). What makes the new-borns refractory to rotavirus illness is however not clear. The phenomenon may be related to the stage of intestinal maturation, passive protection afforded by breast-feeding or transplacentally acquired antibody or the preferential infection of the new-born by naturally existing attenuated rotavirus strains (Flores *et al.*, 1986).

Factors Implicated in Attenuation of Rotavirus Infections in the New-borns

Host Factors

Numerous authors have documented the presence of antibodies to rotavirus in human milk worldwide (Haffejee and Moosa, 1990). Antibodies to rotavirus, which are secretory IgA (s-IgA) antibodies produced in the mammary tissue (Rahman *et al.*, 1987), have been detected in 60%-100% of lactating mothers tested, except in South Africa, where the prevalence was only 3% and 13% among Asian and black mothers, respectively (Haffejee *et al.*, 1990; Cook *et al.*, 1978). The high early concentrations

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probably serve to protect the new-born at a stage when the total intake of milk is small (Yolken *et al.*, 1978; Cukor *et al.*, 1979).

Differences in sensitivities of the tests used may account for these discrepancies. Prolonged antibody production was found in studies that employed ELISA or solid-phase radioimmunoassay (Cukor *et al.*, 1979), whereas in those that used neutralisation assays or immunoelectrophoresis, antibodies were detected only during the first week of life (Cook *et al.*, 1978). None of the fluorescent-antibody or complement-fixation tests are sufficiently sensitive to detect antibodies to rotavirus in breast milk (Haffejee *et al.*, 1990). The antibodies certainly protect the neonates from becoming infected with rotavirus if present at a sufficiently high titer (Jayashree *et al.*, 1988). A British study, however, found no correlation between the presence of IgA in human milk and protection from rotavirus infection (Totterdell *et al.*, 1980). Hence, both the prevalence of lacteal antibodies to rotavirus and their protective effects have not yet been ascertained.

Breast-fed infants develop a bacterial flora that is transient and consist of approximately 99% bifidobacteria by the third week of life. These organisms are absent from the gut of formula-fed infants. In a prospective study Duffy *et al.*, (1986), showed that in breast-fed infants, those with moderate-to-large numbers of intestinal bifidobacteria were at lower risk of developing rotavirus-related illness than were those with low numbers. The latter infants, in turn, though at risk for rotavirus-associated illness, developed symptoms that were very mild in comparison to those in infants who were fed formula, none of whom were colonised by bifidobacteria.

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Although breast-feeding is associated with mild nature of neonatal rotavirus infections, the various factors discussed above do not explain the frequently asymptomatic character of the infection that occurs in new-borns who do not receive breast milk (Chrystie *et al.*, 1978).

Role of Breast-Feeding in Prevention of Rotavirus Infection

The effects of breast-feeding in the prevention of rotavirus infection are not clear. Banatvala *et al.*, (1978) have demonstrated that the rate of neonatal rotavirus infections was significantly lower in babies who were breast-fed. Similarly the number of rotavirus particles in the stools was lower in breast-fed babies. Prospective studies by Gurwith *et al.*, (1981) have not demonstrated a significant protective role of breast-feeding against rotavirus illness. British and Australian studies have found a significantly lower rate of rotavirus infection, as well as a lower number of rotavirus particles in the stools of neonates who were breast-fed than in formula-fed new-borns (Duffy *et al.*, 1986). In Japan a fivefold-lower prevalence of rotavirus infection in breast-fed infants less than 6 months old was observed compared to a control group of formula-fed infants (Konno *et al.*, 1978). Rotavirus infections were uncommon among breast-fed infants in a longitudinal Costa Rican study (Simhon *et al.*, 1985). These findings are in support of studies in Australia, Britain, and the United States, where breast-feeding was associated with reduction in rotavirus-associated morbidity (Weinberg *et al.*, 1984). In Gabon, Africa, where infants are usually breast-fed until they are 1 year old, the highest prevalence (23.5%) of rotavirus diarrhea, as determined by EM of stool specimen, is seen in infants less than 6 months of age (Sitbon *et al.*, 1985). Duffy *et al.*, (1986) also

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found no difference between the rates of rotavirus-associated gastro-enteritis in breast-fed and formula-fed babies and emphasized that the clinical disease was much milder in the breast-fed group. This, they attributed to colonisation of the gut by bifidobacteria in the breast-fed infants. Thus, although there is some controversy regarding the role of human milk in the prevention of rotavirus infection, there is ample proof that human milk does protect the infant from rotavirus diarrhea, and diarrhea if it does occur, is mild and resolves rapidly. Various factors in breast milk have been found to afford this protection. Neonates who receive breast milk with low concentration of two of these factors, i.e antirotaviral secretory IgA and α_1-antitrypsin, are likely to become infected with rotavirus as are formula-fed babies (McLean *et al.*, 1981)

Classification of Human Rotavirus

Early investigation into antigenic relationships suggested that all rotaviruses shared a common group antigen. This was believed to be situated within the inner capsid layer of rotavirus particles. Thus, it was thought that any one of the viruses from any species could be used for the preparation of antigen or antibody for diagnostic tests, and that this would aid in the diagnosis of rotavirus infection in humans and animals (Woode *et al.*, 1976). As investigations proceeded, viruses with rotavirus morphology, but without the common group antigen were found in humans and animals (Nagesha *et al.*, 1988). Serological studies have also confirmed that there are viruses which carry group antigens (group epitopes) which are different from the original rotavirus group antigen and rotaviruses to date, have been divided into seven groups A through G based on this.

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Group A, B, and C are those currently found in both humans and animals, whereas viruses in Groups D, E, F and G have been found only in animals to date. Viruses within each group are capable of genetic reassortment, but reassortment does not occur among viruses in different groups (Yolken *et al.*, 1988). The group A rotavirus have been most extensively studied. The group specific epitopes of rotaviruses are located in VP6 (Ramig, 1994).

Rotaviruses have been classified into groups, subgroups and serotype/genotype based on antigenic specificity and genetic structure. They are classified serologically by a scheme that allows for the presence of multiple groups (subgroups) and the existence of serotypes within each group. A rotavirus group (or subgroup) includes viruses that share cross-reacting antigens detectable by a number of serologic tests such as immunofluorescence, enzyme linked immunosorbent assay (ELISA), and immune electron microscopy (IEM) (Estes *et al.*, 1985). The group antigenic determinants, or common antigens, are found on most if not all of the structural proteins as well (David *et al.*, 1995). However, cross-reactive epitopes on the inner capsid protein (VP6) are those usually detected in diagnostic enzyme linked immunosorbent assays.

Subgroup specificity is also determined by VP6, the most abundant viral protein, which is the target of group diagnostic assays, and contains the antigen used to further classify rotaviruses into subgroups I and II. Two different rotavirus subgroups have been recognised in humans and animals. Subgroup specificity of human rotavirus (HRV) is intimately related to the migration pattern of viral RNA in polyacrylamide gel

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electrophoresis (PAGE). HRVs with subgroup I specificity mostly have 'short' RNA patterns characterised by slow-migrating gene 11, while those with subgroup II specificity have 'long' RNA patterns characterised by rapidly-migrating gene 11 (Fig. 4). In humans, rotavirus subgroup II strains are more common than subgroup I strains (Nakagomi *et al.*, 1985). Most of the animal rotavirus studied to date belong to subgroup I.

Within each group, rotaviruses can be further classified into serotypes defined by reactivity of viruses in plaque reduction (or fluorescent foci reduction) neutralization assays using hyperimmune serum prepared in antibody-negative animals (Hoshino *et al.*, 1984). Using such assays, 14 VP7 serotypes have been identified and strains of human and animal origin may fall within the same serotype. Neutralization assays can measure reactivity of antibody with the two outer capsid-neutralizing antigens (VP4 and VP7). However, in most cases, the predominant reactivity measured is with the glycoprotein VP7. This may be because VP7 comprises a greater percentage of the virion outer capsid, or alternatively with hyperimmunization, VP7 selectively induces highly specific antibodies. Identical classification of the same virus isolates using MAbs to VP7 uneqivocally demonstrate that plaque reduction neutralization assays with hyperimmune serum primarily measure reactivities with VP7 (Coulson *et al.*, 1987).

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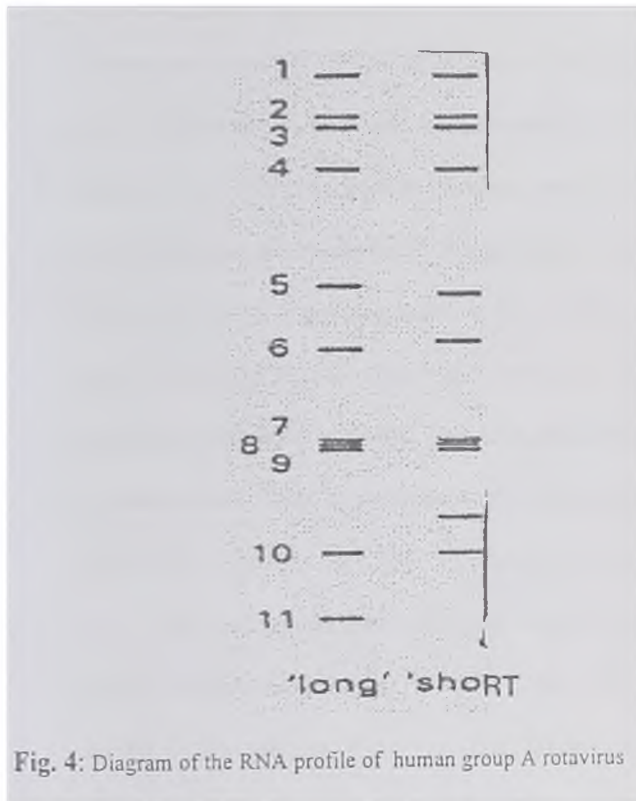

Fig. 4: Diagram of the RNA profile of human group A rotavirus

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Classification of rotaviruses by a binary system, in which distinct serotypes VP4 and VP7 are named, has been proposed and accepted (Graham and Estes, 1980). However, a lack of readily specific typing serum or MAbs to different VP4 types have hampered classification of VP4 (P) by serology. Properties of VP4 types have however been studied by sequence analysis and current evidence suggests the existence of at least 19 different genotypes of VP4. Genotypes of VP4 and VP7 are determined by sequence analysis, whereas serotypes are determined by reactivity with polyclonal or monoclonal antisera. For VP7, a correlation between genotype and serotype has been established. Such correlation is less clear for VP4, although sequence variation between amino acids 84 and 180 have been suggested to be useful in defining P type-specific epitopes (Larralde *et al.*, 1991). Serotype designation thus reflects the expression of neutralizing epitopes on both VP4 and VP7. Increasingly, epitope expression on these outer capsid proteins has been found to be dependent on the specific combinations of VP4 and VP7 in this complex (Chen *et al.*, 1994). Although the molecular basis of these interactions is not yet well understood, new structural information clearly documents the presence of specific interactions between VP4, VP6 and VP7 within this complex capsid. The serology of the epitopes in proteins that interact in this complex is clearly complicated and likely will not be understood fully until high-resolution structural data on the capsid are available (David *et al.*, 1996).

Non Group A Human Rotaviruses

Several 'rotavirus-like' organisms also known as 'pararotavirus' or 'non-Group A rotavirus' were identified initially in piglets, calves and later in chicken, human and rats (Vonderfecht *et al.*, 1984). They are morphologically indistinguishable from

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rotavirus but do not share any of the rotavirus antigens, including the common epitopes of the VP6. They also have a segmented genome with 11 double stranded RNA genes, but their electrophoretic RNA patterns are different from those of conventional rotaviruses. The prevalence of these rotavirus-like viruses has not yet been determined and the extent of their association with human diarrheal illness remains to be established although there was an outbreak of acute diarrhea associated with group C rotavirus in China (Tao *et al.*, 1984).

Comparative studies on the amino acid sequences of the VP7 proteins of the various serotypes have identified six discrete regions (A through F) with significant amino acid divergence (Glass *et al.*, 1985). These regions, although very distinct among different serotypes, are highly conserved within each serotype (Green *et al.*, 1987). Furthermore, the serotype of a given rotavirus isolate could be predicted from the sequence of two of these divergent regions (Green *et al.*, 1988).

Each of the 11 rotavirus genomic segments has been shown to possess unique sequence at both the 3'- and 5'- ends, and are highly conserved among strains (Glass *et al.*, 1985). Six variable regions have been detected within the gene 9 segment of rotavirus and have been exploited for the genetic classification of rotavirus VP7 (Fig. 5). Within the variable regions, unique sequences common to group A viruses and specific to the viral genotype and which correspond to serotype, exists. These short sequences (primers) have been used for the genotyping of the rotavirus VP7 in PCR.

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Similarly, five genetically distinct human rotavirus gene 4 groups have been described on the basis of comparative nucleotide sequencing and the predicted amino acid sequences, and at least four of them represent distinct VP4 antigenic types. Within the gene 4 of the human rotavirus are 5 hypervariable regions which have unique sequences corresponding to the group A rotavirus. These sequences have been found to be specific to the viral genotype and correspond to the serotype. Thus, these regions have been used for the construction of primers which are used for the genotyping of VP4 using PCR. Figure 6 shows the VP4 gene illustrating the variable regions, primer sequence and PCR products.

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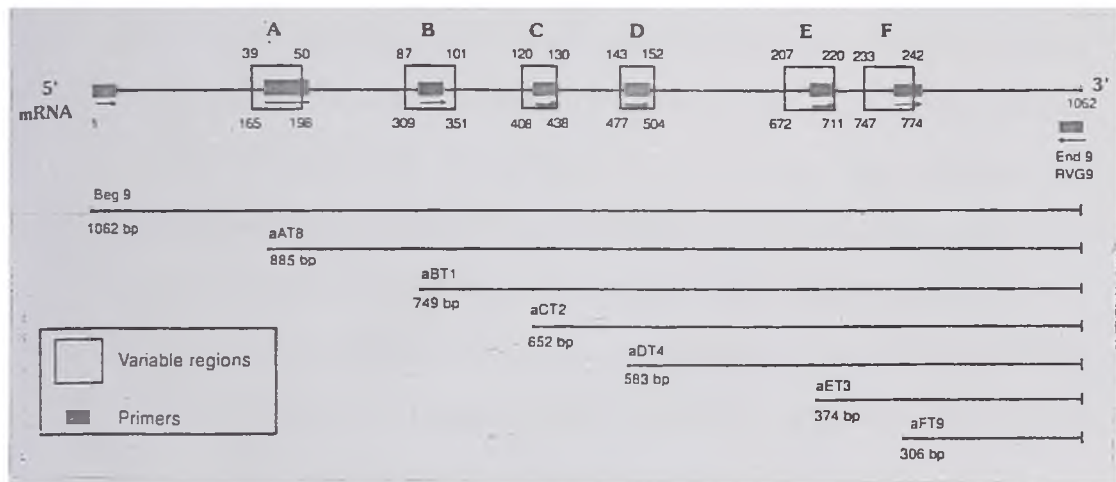

Fig. 5: Rotavirus gene 9 showing locations of variable regions, PCR primers for typing and expected length of amplified segments.

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Prevalence of HRV P and G Types (of Rotaviruses) in Infected Infants

Surveys of rotavirus P genotypes in more than 500 fecal specimens from seven countries revealed 2 major types, P[8] and P[4]. Ten other worldwide studies involving more than 2700 specimen identified four major neutralisation antigen gene combinations. Eighty three percent of the specimens were genotypes P[8] with G1, G3, or G4 specificity and P[4] with G2 specificity (Steele *et al.*, 1995). When specimens that could not be typed for one or both genes and specimens with mixed infections were excluded, this figure rose to 95.9%. The single most common strain reported was P[8], G1 (53%), followed by P[8], G4 (14.3%); P[4], G2 (10.7%); and P[8], G3 (5.4%). Other genotypes with unusual P and G types included P[6] with G1, G2, G3, or G4 specificity (0.8% of the total); P[9], G1 or G3 (1.1%); P[4], G1 or G4 (0.4%); P[8], G9 (0.3%); and P[3], G1 or G3 (0.3%). Of note, although uncommon in specimens analysed so far, P[6] and P[9] strains and the natural reassortants P[4], G1 have been detected in faecal specimens from many countries, suggesting that they may have a global distribution.

Studies in Bangladesh and Brazil have also revealed a significant prevalence of unexpected strains. In Bangladesh, although a small number of strains were typed, 10% of them were either natural reassortants (P[4], G1 and P[4], G4) or uncommon P[6], G1) rotaviruses (Bern *et al* 1992). Studies in Brazil showed 5 uncommon serotypes (P[6], G1, P[6], G3; P[6], G4; P[3], G1, and P[3], G3) representing one third of the single infections (Timenesky *et al.*, 1994). Strains with the P[6] serotype had the highest prevalence isolates among all the studies surveyed, while the P[3] genotype, the

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prototype of which is rhesus rotavirus, had previously been reported only among infants from Israel and the United States (Silberstein *et al*., 1995). Genotype P[8], G5 was found in specimens from several different years and from all five regions studied in Brazil and it represented 12.6% of the single infections. This made it the most common strain identified. The prevalence of mixed infections (21%) was unusually high, suggesting a complex rotavirus infection pattern in Brazil. This result coupled with other observations from another report in which G5 strains were detected but the P genotype could not be specified reported (Timenetsky *et al*., 1994), suggesting that Brazilian P[8], G5 strains may represent reassortants between human and animal rotaviruses.

Although both the Brazilian and Bangladeshi studies were limited because of small number of completely typed strains, they represent one of the first reports of rotaviruses with P genotypes other than P[8]or P[4] that could be of major public health importance.

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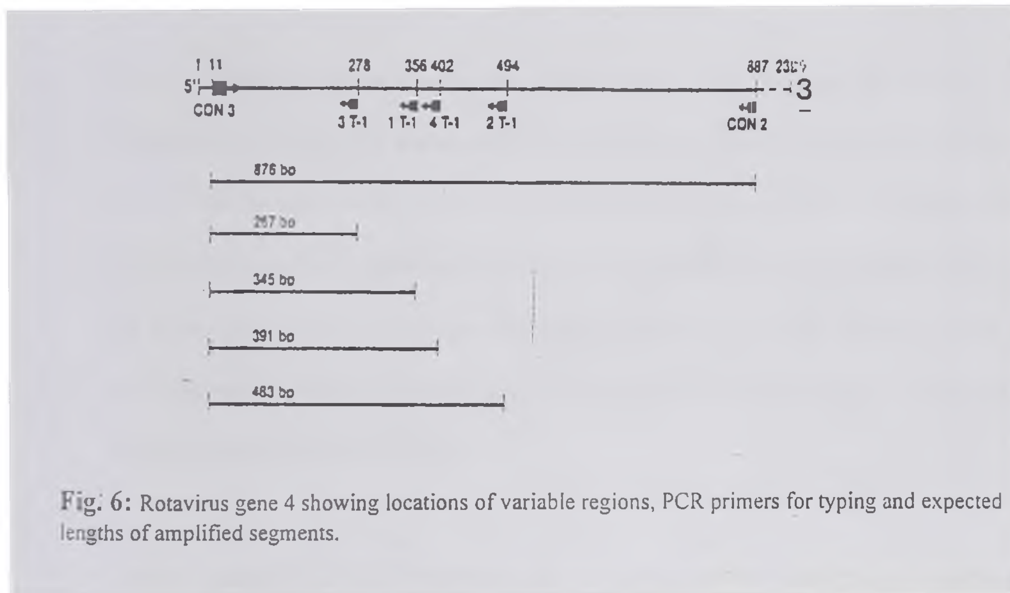

Fig. 6: Rotavirus gene 4 showing locations of variable regions, PCR primers for typing and expected lengths of amplified segments.

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Genetic Variation in Rotavirus

The RNA segments of the rotavirus when run on polyacrylamide gel electrophoresis (PAGE) appear as 11 distinct bands on the gel. Based on the migration patterns of the 11th- gene segment on PAGE, rotaviruses are classified into 'long', 'short' and 'super-short' electrophoretypes as previously described. There is however considerable variation of electrophoretypes within any given community at any given time, although usually one strain predominates and the virus population in a given area changes with time (Rodger *et al.*, 1981).

Before human rotaviruses could be propagated in cell cultures, the analysis of the electrophoretic migration pattern of the virus genomic RNA segments was an important tool for the epidemiologic studies. A common observation made was the existence of numerous strains with differing RNA migration patterns even among strains obtained on the same day in a given geographic location (Flores *et al.*, 1982). This suggested that a high degree of genetic variability exists that may allow the generation of new serotype specificity and/or altered virulence.

Genetic variation among rotavirus can be explained by two major mechanisms; accumulation of successive mutation (genetic drift) and reassortment of genes (genetic shift). Point mutations in the genes of the rotavirus could certainly occur as the virus is amplified and propagated from one individual to another. Changes in the base pair sequence of genes may result in altered migration without any size changes. On the other hand the segmented nature of the rotavirus genome allows for the occurrence of

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reassortment when two strains coinfect simultaneously a given host. Silent mutations among equivalent genes from different strains have been observed in rotaviruses (Flores *et al.*, 1985). The number of such mutations is however relatively small.

The existence of two distinct genetic 'families' of human rotavirus based on genomic cross-hybridisation studies of over 100 human rotavirus isolates obtained in Venezuela has been proposed (Flores *et al.*, 1985). The two families are represented by the well-characterised laboratory strains Wa and DS-1. Most of the Venezuelan isolates hybridise at high stringency with the genes of the prototype strain Wa; a few of the isolates however hybridised strongly to the DS-1 prototype strain. Some isolates exhibited a high degree of homology to Wa in most of their genes while simultaneously presenting one or two genes homologous to DS-1. This observation would suggest that reassortment may have take place under natural conditions during coinfection by strains belonging to different rotavirus 'families' Furthermore, the reported finding of two mixed rotavirus with different RNA electrophoretic patterns in the same stool of ill children provides further support to this hypothesis.

The consequences that may derive from natural development of rotavirus reassortants are unclear. Similarly the consequences of successive accumulation of mutations in one or more of the rotavirus genes are impossible to predict. Under experimental conditions selective pressures can induce reassortants with certain gene constellation, however, it is not known whether such selective pressures act under natural conditions and to what extent they may force the generation of reassortants or the induction of new serotypes.

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Neonatal Rotavirus Infection

Rotavirus infections in neonates are generally mild or asymptomatic as stated earlier. Generally, fewer than one-third of rotavirus-infected neonates have diarrhea, although higher rates have been reported in some hospital nursery populations (Haffejee *et al.*, 1991). Infections usually occur during the first week of life and generally invokes a mucosal antibody response without a concomitant serologic antibody response. Neonatal rotavirus-infections appear to incite an immune response that affords significant protection against future severe rotavirus associated diarrhea, although not necessarily against a symptomatic rotavirus infection (Haffejee *et al.*, 1991). The outer capsid protein VP4 of neonatal strains are highly conserved, a property that probably plays a key role in their attenuated virulence. Immaturity of proteolytic enzymes in the neonatal gut and presence of secretory anti-rotavirus IgA and trypsin inhibitors in breast milk are other factors that could account for the asymptomatic nature of rotavirus infections in new-borns.

Genotype Associated with Neonatal Rotavirus Infection

New-born infants born in hospital nurseries sometimes become infected with rotavirus, usually in the absence of symptoms of diarrhea (Bishop *et al.*, 1983). Thus, infection of neonates with these strains may have important implications for the success of vaccination against rotavirus. A number of studies have shown differences between strains of rotavirus that infect neonates and strains that infect older infants and children. Studies done in Venezuela and South Africa have identified strains related to P[6] with G1, G3, or G4 specificity (Genstch *et al.*, 1996). Isolates from New Delhi and Bangalore were first characterised molecularly and serologically and shown to belong to

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serotypes G9 and G10 respectively, serotypes not previously identified in neonates (Dunn *et al.*, 1993). These strains had related but distinct VP4 genes, both of which were closely related to the prototype bovine rotavirus B223 which has a P8[11] and G10 specificity. RNA-RNA hybridisation experiments supported the hypothesis that both strains had arisen by reassortment between human and bovine rotaviruses (Das *et al.*, 1993). Subsequent genotype analysis identified neonatal strains circulating in the New Delhi area as P[11], G9 (70.2% of total). About one-third of these were isolates obtained during the same period from the same hospital as the prototype strain of this group, 116E (Cicirello *et al.*, 1994). A second novel neonatal rotavirus (P[6], G9, 13.5% of total) was identified at this hospital and also at another hospital in 1993 during an epidemiologic study of neonatal rotavirus prevalence. A low prevalence of 2 other strains, P8[11], G3 (2.7% of total) and P[4], G2 (1.4 of total) was identified among culture-adapted strains but not in faecal specimens, therefore, their importance as causes of neonatal infections is unknown. The implications from these findings is that these differences may be responsible for mild or asymptomatic infections. Australian investigators, who analysed RNA patterns of rotavirus by electrophoresis, isolated two strains of rotavirus that were almost identical from new-borns in nurseries. The two strains were distinct from other rotavirus strains isolated from older ill children in the community (Rodger *et al.*, 1981). This electrophoretic dissimilarity of strains again was demonstrated also by Albert *et al.*, (1984). Perez-Schael and colleagues (1997) also observed a similar constancy of RNA patterns in rotavirus strains isolated from neonates. Hoshino *et al.*, (1985) showed that three English strains recovered from asymptomatic new-borns had identical RNA profiles; the same conclusion applied to five strains from Sweden. On the other hand,

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during two outbreaks of neonatal rotavirus infection in a tertiary care nursery, 11 different electrophoretic strains were identified (Rodriguez *et al.*, 1983).

The similarities found in neonatal electrophoretypes do not, however, hold for serotypes. All four human serotypes (Totterdell *et al.*, 1976) that cause symptomatic infection in infants and young children also cause asymptomatic or mild infections in neonates. Similarly, both serotype subgroups I and II have been implicated in neonatal infections (Hoshino *et al.*, 1985). It appears, therefore, that there is no correlation between a specific serotype or subgroup and the occurrence of asymptomatic or mild infections in neonates. It is of interest that although four serotypes were implicated in neonatal infections in the study of Hoshino *et al.*, (1985) each individual serotype was endemic in the neonatal nurseries of only one of the four different countries at any given time. The mutation rate among nursery strains of rotavirus with regard to VP7 gene is very low, i.e., these strains appear to be genetically stable (Flores *et al.*, 1988). The VP4 gene is highly conserved in neonatal rotavirus strains and differs from that of strains found in older children with diarrhea (Flores *et al.*, 1986). Since enzymatic cleavage of VP4 is required for infectivity, it is conceivable that the relatively immature proteolytic enzymes that occur in new-born babies may 'process' the VP4 in a manner that would allow replication of the virus without causing clinical illness.

Thus, although both breast milk and the natural attenuation of the nursery rotavirus strains probably account for the general asymptomatic nature of neonatal rotavirus infection, it appears that the second factor, the inherent attenuation of nursery strains, is

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most important. This property has led to the consideration of these strains as vaccine candidates (Edelman *et al.*, 1987). If efficacious, such a vaccine would be an important advance in the reduction of the morbidity and mortality from infantile diarrhea, since it is estimated that about 870,000 children less than 5 years of age die annually from rotavirus diarrhea worldwide, the majority in developing countries (Ho *et al.*, 1988). Other promising vaccines are the bovine WC3 vaccine and the combined rhesus-human quadrivalent reassortment vaccine (Flores *et al.*, 1990). The WC3 vaccine gave a high protection rate in small field trials in the United States (Clark *et al.*, 1988), although larger trials in different settings are clearly indicated. The quadrivalent or multiserotype vaccine produced a high rate of febrile reactions in a small Venezuelan trial, but no long-term follow-up results are yet available to assess protection if any against rotavirus diarrhea (Flores *et al.*, 1990).

Development of a vaccine against rotavirus might reduce the high infant mortality rates due to diarrheal illness in developing countries as well as the high endemicity observed in both developed and developing countries. Before such a vaccine can be developed, the different strains in circulation must be characterised and documented especially the neonatal strains since they make good candidates for rotavirus vaccines. Whilst there exist some data on neonatal strains in the developed countries, such data is almost non existent in the developing countries. The aim of of this study therefore is to characterise by molecular methods rotavirus strains in neonates in Accra. This is the first study of its kind in Ghana and will provide valuable data that would be very essential in the development and formulation of vaccines against rotavirus strains.

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The specific objectives are as follows:

(i) To isolate rotavirus from stools of neonates in Accra.

(ii) To determine the incidence of rotavirus infection in neonates.

(iii) To characterise by molecular methods rotavirus isolates in neonates.

Justification for the work

The determination of the incidence of rotavirus infection in neonates and the characterisation of the various strains would provide valuable local information on rotaviruses in neonates. This would contribute to the global development and formulation of vaccine against the different rotavirus strains, by providing epidemiological data on rotavirus strains in neonates.

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CHAPTER TWO

MATERIALS AND METHODS

Materials/Reagents

Acetone, sodium chloride, hydrochloric acid, sodium dodecyl sulphate, sucrose, bromophenol blue, glacial acetic acid, sodium hydroxide were obtained from Fluka Chemical Company (Buchs) and were of analytical grade.

Sodium dihydrogen phosphate, dipotassium hydrogen phosphate, N N N' N' tetramethelene diamine, ammonium persulphate and glycine were obtained from Wako Chemical Company (Japan).

N' N' methelene bisacrylamide was from Serva Fienbiochemica Company Ltd. (N.Y). Ethanol and disodium hydrogen phosphate were obtained from Riedel-deHaen (ph. Eur) and extraction buffers L2 and L6 were from Severn Biotech Ltd. Rnase-free sterile distilled water was obtained from RNAsin, Promega, Madison, WI. Primers were from Pharmacia Biotech and Tris-HCl, KCl, $MgCl_2$, Reverse transcriptase and dNTPs were obtained from Life technologies, Gaitherburg, MD. The IDEIA Rotavirus kit was from Dako Diagnostics Ltd, Cambridgeshire, UK and agarose was obtained from Seakem, Flowen, USA.

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Methods

Study Area:

This study was conducted in the Greater Accra region, the capital of Ghana. Accra lies in the coastal belt of West Africa and has two main seasons-a rainy season from April to June and a dry season from December to March. In between these major seasons, there are minor rainy and dry seasons from September to November and July to August, respectively. The Korle-Bu Teaching hospital, where the study was conducted, is the main referral facility in the city of Accra. It serves as the medical center for all zonal classes and has a daily out patient population of approximately 1500 people.

Two hundred neonates at the selected hospitals (Korle-Bu Teaching Hospital and the Legon Hospital) were recruited into the study and the basic data collected by questionnaire (Appendix IV). Stool samples were collected from neonates who were on admission at the babies unit of the hospital. The stool samples were collected in plastic cups and transported to the laboratory at 4°C where they were stored under glycerol at −20°C for processing within 10 days.

Detection of Rotavirus in stools

Approximately 10% suspension of stool samples were prepared by homogenization in phosphate buffered saline (PBS) pH 7.4 for 3 mins. and used for analysis. Rotavirus detection was carried out using Enzyme Linked Immunosorbent Assay (ELISA). The 10% suspension of stool samples were tested for rotaviruses with the IDEIA Rotavirus kit following the manufacturer's instructions. Briefly, two drops (100µl) of test samples

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(supernatant from 10% suspension) were added to each well of the 96 well plate followed by two drops of conjugate. The plate was then incubated at 30°C for 60 minutes, after which time the wells were washed five times using working strength buffer (Appendix II). A drop (50µl) of substrate Part A (Appendix II) was added to each well followed by one drop of substrate Part B (Appendix II), and incubated at 30°C for 10 minutes. The reaction was stopped by the addition of one drop of stopping solution (Appendix II) to each of the wells. Each plate included two negative controls and a positive control. The absorbance was read at 450nm and any samples which had a value equal to or greater than the sum of 0.1 and the absorbance value of the negative control were considered positive. All assays were in duplicate.

Characterisation by Polyacrylamide Gel Electrophoresis

RNA Extraction for PAGE:

The 10% suspension of the faecal samples were spun down and RNA was extracted from the supernatant of the faecal samples using the method of Flook *et al.*, (1992). Two hundred microliters of purified virus solution was mixed with 200ul of Bender buffer (Appendix I) and incubated at 65°C in a water bath for 30 mins. Sixty microliters of 8M potassium acetate was added, mixed by inverting the tube a couple of times, and allowed to stand on ice for 45 mins. The mixture was clarified by spinning at 10 000 rpm and twice the volume of cold absolute alcohol was added to the supernatant in another tube and allowed to stand for a few minutes. The RNA precipitate was pelleted by centrifugation and re-suspended gently in 200µl of Tris-EDTA buffer (TE) pH 8.0 and left for 30 mins at room temperature. The viral genomic RNA was finally precipitated

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with alcohol (420μl) and 10μl of NaCl, dried under vacuum and re-suspended in 15μl of loading buffer or TE. The extracted RNA was then analysed by polyacrylamide gel electrophoresis using the SDS-tris-glycine discontinuous buffer system (Laemmli., 1970) on 10% acrylamide gels and run overnight at a constant voltage of 100V for 18 hours. Samples were applied to separate lanes of polyacrylamide slab gels and electrophoresed in an Atto (Japan) mini-gel system and the gels stained by the method of Herring *et al.*, (1982).

Silver staining of page gels:

After removal of the gel from the glass plates, it was fixed in solution 1 (Appendix I) for 30 minutes. The gel was then transfered into solution 2 (Appendix I) for another 30 minutes, and then placed in 200ml of silver nitrate stain and swirled slowly for 30 minutes. The stain was decanted after 30 minutes and the gel washed twice with water to remove any trace of excess silver nitrate that may darken the gel. The gel was then washed with 50ml of developer (Appendix I) for 10secs. and developed by adding 250ml of developer (Appendix I). The gel was allowed to stand for approximately 5 minutes, until the nucleic acid bands were visible. The developer was afterwards poured off and replaced with 200ml of solution 3 (Appendix I) to fix the gel. The gel was stored in solution 2 and later photographed.

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Genotyping of Rotavirus

All the PAGE positive rotavirus samples were characterized by molecular methods using the polymerase chain reaction (PCR) techniques.

(i) *RNA Extraction for PCR*:

Five hundred microlitres of lysis buffer L6 (Appendix III) was added to 100ul of 10% faecal extract in a 1.5ml eppendorf tube and 10µl of size fractionated silica was added to the mixture. The mixture was then vortexed for 10 seconds, left to stand at room temperature for 15min. before pelleting by centrifugation at 13, 000 rpm for 15 seconds. The supernatant was discarded and 0.5ml of lysis buffer L2 (Appendix III) was added. The pellet was washed by gently resuspending with pipette and then pelleted again by centrifugation at 13,000 rpm for 60 seconds. The supernatant was discarded and the washing process was repeated as above. The pellet was further washed twice with 0.5ml of 70% ethanol, followed by 0.5ml of acetone and then the tubes were placed with caps open in a dry heating block at 56°C for 5 minutes. The nucleic acid was eluted from the silica by adding 25µl of distilled water, vortexed, incubated at 56°C for 15 minutes in the heating block and centrifuged at 13,000 rpm for 2 min to recover the supernatant.

(ii) *Reverse Transcription to Generate cDNA:*

The extracted RNA was used for RT-PCR after random priming with hexamers or specific priming with VP7 and VP4 consensus primer pairs (Beg9/End9 and Con3/Con2 respectively) by the method of Gentsch *et al.*, (1996). Briefly, 1µl of random primer Pd(N)$_6$ or 1µl of either specific primer pair (Beg9/End9 for VP7 or Con3/Con2 for VP4), was added to 20µl of the extracted RNA, heated at 97°C for 5 min. to denature the

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dsRNA followed by annealing of the primers for 5 min at 70°C for random priming, 42°C for VP7 or 50°C for VP4 and chilled on ice for 2 min. Fourteen microliters of the RT reaction mix was added, to yield a total volume of 30ul consisting of 20mM Tris-HCl pH 8.4, 50mM KCl, 5mM $MgCl_2$, 50uM of each dNTP and 200U of MuLV reverse transcriptase. The RT reaction was carried out by incubation at 37°C for 1hr, and the reaction terminated at 95°C for 5 min followed by chilling on ice for 2 min.

The RT-PCR for determining the G-type used Beg9 and End9 primers (Gouvea *et al.*, 1990). The sequence of primers (5' to 3') and the nucleotide positons are as follows: Beg 9 with sequence (nt 1 to 28) GGCTTTAAAAGAGAGAATTTCCGTCTGG; and End 9 with sequence (nt 1062 to 1036) GGTCACATCATACAATTCTAATCTAAG.

The RT-PCR for determining the P-type used Con2 and Con3 primers (Gentsch *et al.*, 1992). The nucleotide (nt) positions and sequences of these (5' to 3') are as follows: con 3 (nt 11 to 32), TGGCTTCGCCATTTTATAGACA; con2 (nt 868 to 887), ATTTCGGACCATTTATAACC.

(iii) *Genotyping:*

(a) *G-typing*

G-typing was performed using a semi-nested PCR and adapted from the method of Gouvea *et al.* (1990). The first round PCR amplified the whole length of VP7 gene using primers Beg 9 and End 9. The second round typing PCR was a multiplex PCR and incorporated the primer End 9 and the G-type specific primers, aBT1 (G1-specific), aCT2

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(G2- specific), aET3 (G3-specific), aDT4 (G4-specific), aAT8 (G8-specific) and aFT9

(G9-specific). The aAT8 maps to variable region A with nucleotide position (nt) 178 to 198 and sequence, GTCACACCATTTGTAAATTCG, with product size, 885bp, the primer aBT1 maps to the region B of nucleotide position and sequence (nt 314 to 335, CAAGTACTCAAATCAATGATGG), with product size 749bp, the primer aCT2 maps to region C of nt and sequence (nt 411 to 435, CAATGATATTAACACATTTTCTGTG), with product size 652bp, the primer aDT4 maps to region D of nt and sequence (nt 480 to 498, CGTTTCTGGTGAGGAGTTG), with product size 583bp, the primer aET3 maps to region E of nt and sequence (nt 689 to 709, CGTTTGAAGAAGTTGCAACAG) with product size 374bp and the primer aFT9 maps to region F of nt and sequence (nt 757 to 776, CTAGATGTAACTACAACTAC) with product size 306bp. These primers were selected to be pooled as a primer mix and used in combination with the common primer RVG9 (nt1062 to 1044, GGTCACATCATACAATTCT), 19 nucleotide at the 5' end of the primer End 9.

The PCR reaction mix for the first consisted of 18mM Tris-HCl pH 8.4, 45mM KCl, 2mM $MgCl_2$, 50uM of each dNTP, 1µl of Taq polymerase and 2µM of each primer. The first round PCR was performed by using 5µl of the random primed cDNA to 45µl of the PCR mix. After denaturation at 94°C for 5 min, 30 PCR cycles each consisting of 94°C for 1min, 42°C for 2 min and 72°C for 1 min were performed, followed by an extension at 72°C for 7 min. The second round PCR was performed using the same protocol but with 1ul of the first round reaction product as the template in a final reaction volume of

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50µl, and reducing the number of cycles to 15. All amplified products were examined by gel electrophoresis in 2% agarose gels containing 0.5µg/ml ethidium bromide under standard conditions.

(b) *P-typing*:

P-typing was performed using a semi-nested PCR adapted from the method of Gentsch *et al.*, (1992). The first round PCR amplified an 876bp fragment of the gene of group A rotaviruses using the consensus primers Con2 and Con3. The second round typing PCR incorporated Con3 and the P-type specific primers 1T-1 (P[4]-specific), 3T-1 (P[6]-specific), 4T-1 (P[9]-specific and 5T-1 (P[10]-specific). 1T-1 (nt 339 to 356), TCTACTTGGATAACGTCG and product size, 345bp; 2T-1 (nt 474 to 494), CTATTGTTAGAGGTTAGAGTC and product size 483bp; 3T-1 (nt 259 to 278), TGTTGATTAGTTGGATTCAA, with product size 267bp; 4T-1 (nt 358 to 402), TGAGACATGCAATTGGAC with product size 391bp and 5T-1 (nt 575 to 594), ATCATAGTTAGTAGTCGG with product size 583bp. A second set of genetic group-specific primer pairs was employed to confirm the results obtained with typing primers. Their nucleotide positions on gene 4 and sequences (5' to 3') are as follows: 1C-1 (nt 314 to 331), GGACTGCAGTAGTTGCTA; 1C-2 (nt 474 to 494), TTAGTATCAGAAGTTAGTGTA; 2C-1 (nt 1324 to 1344), ATACGAACACGTACAATAAAC; 2C-2 (nt 1809 to 1828), CATCATTTACTGAGTCAGTT, 3C-1 (nt 261 to 278), GAATCCAACTAATCAACA; 3C-2 (nt 446 to 467), TGTTGAAATTCGGCACTAACA; 3C-3 (nt 288 to 312), AGAGGGTACCAATAAAACTGATAT; 3C-4 (nt 589 to 606), TGCAGTTTCTACTTCAGA; 4C-1 (nt 223 to 242), ACCTCACTCAACTTAGT;

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and 4C-2 (nt 464 to 484), ATAATGTTGAATATTGAGTGT. The reaction mix for the first and second round amplification was the same as that of the G-typing except for the primer concentrations (1μM Con2 and Con3 for the first round PCR and 2μM of each typing primer for the second round). Forty PCR cycles were performed with annealing performed at 50°C for a minute. The second round PCR cycle was reduced to 25 cycles. PCR products were examined as described above. The products were analysed by 2% agarose gel electrophoresis and stained with ethidium bromide (0.5μg/ml). The products were run along side with a 1kbp marker at 80 volts for 30 minutes before observation under U.V light.

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CHAPTER THREE

Results

During the period of the study (October, 1999 to March, 2000), a total of 200 neonatal faecal specimens were collected from the Legon and Korle-Bu teaching Hospitals. These were tested by Enzyme-linked Immunosorbent Assay (ELISA) for the detection of group A rotavirus as described in materials and methods. Out of the 200 neonatal samples tested, 60 samples (30%) were found to be positive for rotavirus antigen. Rotavirus was detected in all age groups, with the exception of the age groups 16-19 and 24-27 days. The age distribution of neonatal rotavirus infection during the rotavirus season is shown in Table 1. The infection was highest in the 0-3 days age group which recorded 37.9% HRV positives out of the total of 29 samples. A 33.3% HRV positivity was observed for the age group 20-23 days. There was a 32.2% infection among children in the age group 4-7 days and 30.8% among children in the age group 12-15 days. Children in the age group 8-11 days and neonates who were more than 27 days old had 29.4% and 17.4% HRV infection respectively.

PAGE

The neonatal stools were tested by PAGE for rotavirus as well as determining the electrophoretic pattern. This method also afforded us the opportunity of detecting the various electrophoretypes as well as selecting samples with adequate intact virus (enough dsRNA) for genotyping by PCR. In addition, it also allowed for the detection of non group A human rotaviruses. Out of the 60 (30%) stool samples that were positive by ELISA, 23 were positive by PAGE. In all 28 (14%) of the 200 samples turned out to

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be positive by PAGE. Of the ELISA positive samples, only 24 (40%) had diarrhea (Table 2). Eleven (45.8%) of the 24 neonates who had diarrhea were rotavirus positive whiles 13 (54.2%) had diarrhea but were rotavirus negative. All the neonates who had diarrhea and were rotavirus positive were observed to be infected with group A rotavirus. All the group A rotaviruses were detected by ELISA and the non-group A by PAGE

Table 1: Neonatal Rotavirus infections in Accra according to age

Age (days)	Total number of children	Diarrhea cases +ve HRV(%)	Non Diarrhea cases +ve HRV (%)
0-3	29	2 (0.0)	27 (40.7)
4-7	66	7 (14.3)	59 (32.2)
8-11	17	4 (50.0)	13 (23.1)
2-15	39	7 (57.1)	32 (25.0)
16-19	0	0 (0.0)	0 (0.0)
20-23	24	2 (100.0)	22 (27.2)
24-27	0	0 (0.0)	0 (0.0)
>27	25	2 (50.0)	23 (13.0)
0-27	200	24	176

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Table 2: Relative frequency of human rotavirus types identified

Virus	Total number of positive samples	Number tested Diarrhea (%)	Non Diarrhea (%)
Type A rotavirus	60	24 (40)	36 (60)
Non type A rotavirus	5	0 (0)	5 (0)

Two distinct rotavirus RNA electrophoretypes were observed to occur. Seven (25%) were of the long electrophoretic pattern and 22 (75%) of short type. No unusual patterns such as mixed infections were observed as depicted in Figure 7.

G and P characterization

The 28 isolates detected by the PAGE were the ones which had enough RNA for the RT-PCR. These 28 isolates were genotyped but a total of 8 samples (28.6%) could not be genotyped; there were four samples (14.3%) whose G-types could not be determined, but their P-genotypes could be determined and two samples (7.1%) could not be P-typed but their G-types could be identified. Two (7.1%) of the samples were completely untypable, although gene products could be obtained after reverse transcription and amplification, indicating the possibility of unusual strains. The predominant genotype was VP4 P6 accounting for 19 (79.2%) of the P-typed (24) specimens. Four samples with mixed P-type specificities (P6P10) accounted for 16.7% of the P-typed specimens. These samples were P[6]P[10] types, while only one (4.2%) was found to be the P[8] type. The

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predominant VP7 G-type was G[2] (72.7%) followed by G[9] (12.5%), and G[3] (8.3%). The mixed G-type was (G[2]G[3]) (4.2%). No VP7 G[4] and G[1] strains of human rotavirus were isolated in this study.

Three distinct single rotavirus strains with a combination of VP7 genotype and VP4 genotype, were found among neonates in Accra as shown in Figure 8. Strains of the type P[6]G[2] were the most predominant (42%). There was 7.1% of type P[6]G[3] strains and 3.6% of P[6]G[9] strains. There was one mixed G-type (3.6%)and four mixed P-type strains (10.7%). The P and G-typing of the strains after the PCR amplification are as shown in figures 9 and 10 respectively.

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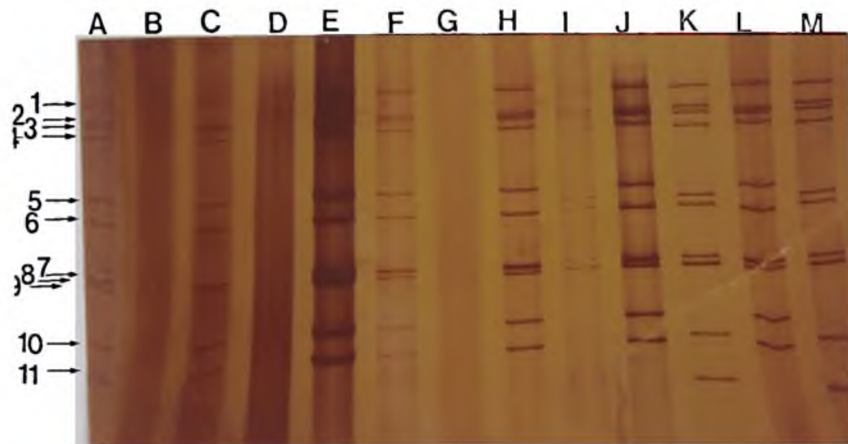

Fig. 7. The electrophoretic pattern of human rotavirus strains from neonates in Accra. All samples (lanes A-M) exhibited the typical 4-2-3-2 pattern of group A rotavirus. Lanes I, K and M show the long pattern whilst lanes A-H and L show the short pattern. Numbers denote positions of dsRNA segments.

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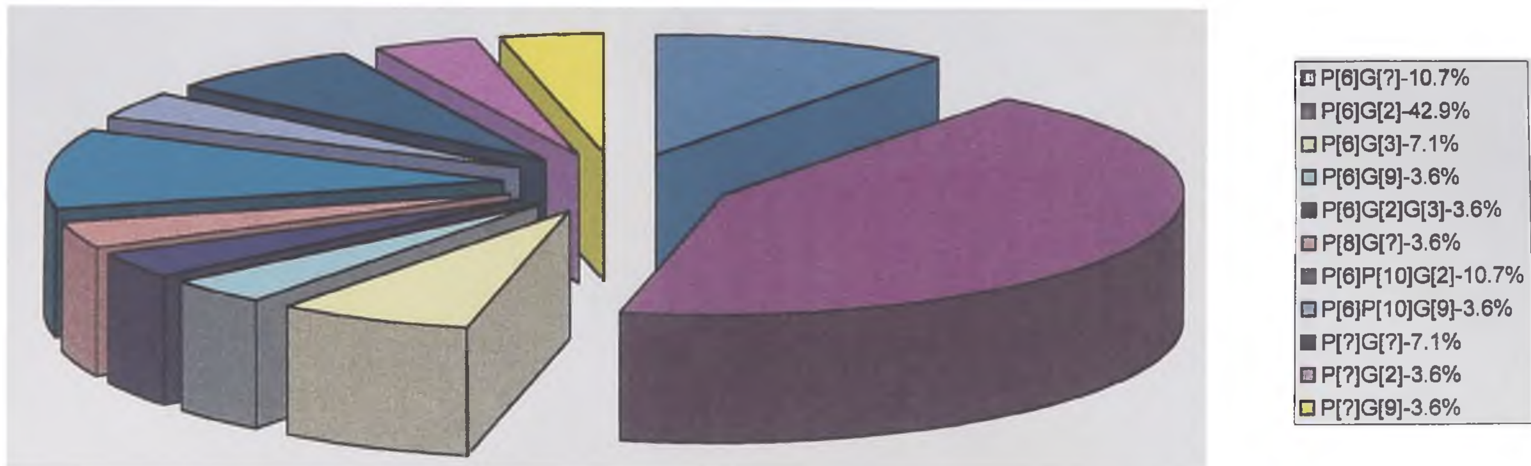
Fig. 8: Neonatal HRV Genotype Distribution in Accra from Oct., 1999 - March, 2000



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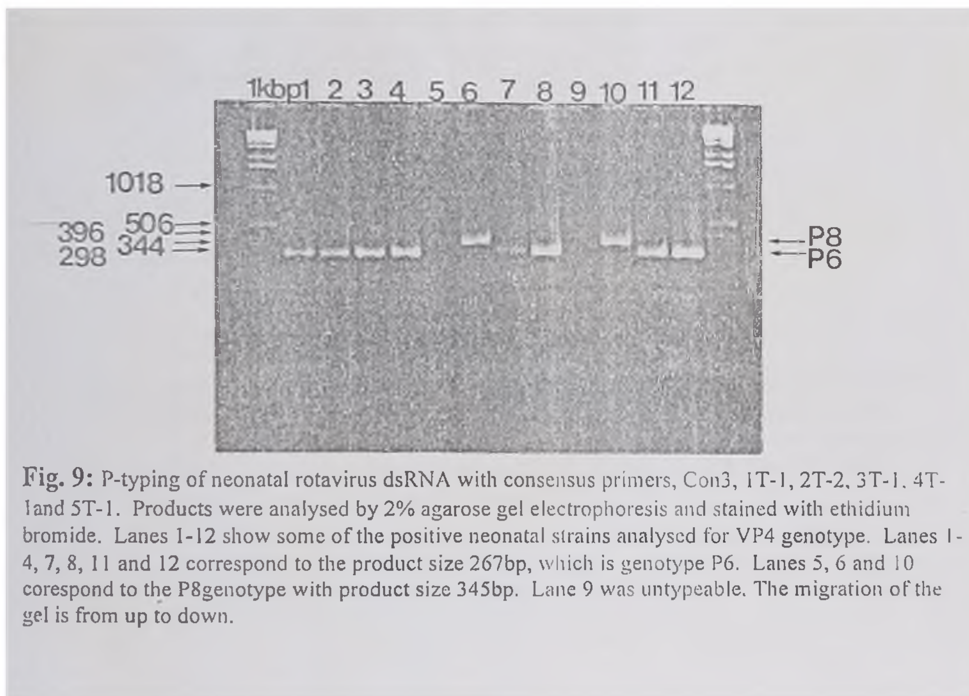

Fig. 9: P-typing of neonatal rotavirus dsRNA with consensus primers, Con3, 1T-1, 2T-2, 3T-1, 4T-1and 5T-1. Products were analysed by 2% agarose gel electrophoresis and stained with ethidium bromide. Lanes 1-12 show some of the positive neonatal strains analysed for VP4 genotype. Lanes 1-4, 7, 8, 11 and 12 correspond to the product size 267bp, which is genotype P6. Lanes 5, 6 and 10 corespond to the P8genotype with product size 345bp. Lane 9 was untypeable. The migration of the gel is from up to down.

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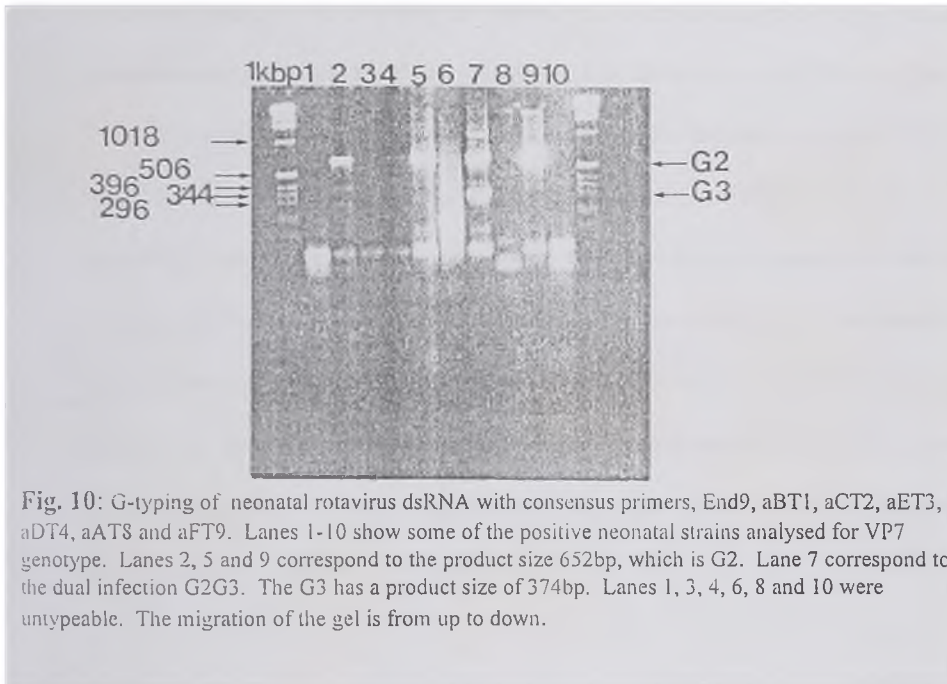

Fig. 10: G-typing of neonatal rotavirus dsRNA with consensus primers, End9, aBT1, aCT2, aET3, aDT4, aAT8 and aFT9. Lanes 1-10 show some of the positive neonatal strains analysed for VP7 genotype. Lanes 2, 5 and 9 correspond to the product size 652bp, which is G2. Lane 7 correspond to the dual infection G2G3. The G3 has a product size of 374bp. Lanes 1, 3, 4, 6, 8 and 10 were untypeable. The migration of the gel is from up to down.

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CHAPTER FOUR

DISCUSSION AND CONCLUSION

Discussion

Rotavirus infection in neonates is common and has been reported to be generally asymptomatic, although diarrheal illness has also been recorded. In this study, about 50% of neonates who were infected with rotavirus exhibited one or more of the following symptoms, fever, bloody stool, watery stool, diarrhea and vomitting. Out of a total of 24 babies who had diarrhea only, 11 (45.8%) were positive for human rotaviruses. The presence of rheumatoid-like factor in some neonatal faecal specimens has been suggested to give false-positve results with ELISA (Yolken *et al.*, 1979). This may probably explain why 36 (60%) of the neonates positive by rotavirus ELISA were asymptomatic but tested negative by PAGE. Another explanation could also be that the amount of RNA was so small to be detected by the PAGE.

In this study, a high incidence of 30% was recorded with the use of ELISA for the detection of the rotaviral antigen. The methods of choice for detection of rotavirus in stool samples should be very sensitive, specific and reproducible, ensuring consistency in the laboratory assays. ELISA for the detection of viral antigens is the method commonly employed in many laboratories in combination with either electrophoretype determination by PAGE or detection of viral particles by EM. The overall sensitivities of these methods are in the range of 10^8 to 10^9 viral particles/ml for PAGE. The most sensitive ELISA described detects as few as 10^5 to 10^6 viral particles/ml, whereas for a

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positive EM reaction 10^8 viral particles/ml are required (Brandt *et al.*, 1981). Data obtained from the comparison of diagnostic sensitivities and specificities of commercial ELISA kit, and PAGE for direct detection of group A rotavirus in stool samples, with RT-PCR as the standard method by Arguelles *et al* (1999), indicate that for the rapid screening of a large number of samples, the ELISA is able to detect rotavirus in stool samples with sensitivity (98%) and specificity (100%) similar to those of RT-PCR at a considerably lower cost and without previous treatment of the samples (Coulson *et al.*, 1991).

To select samples with enough dsRNA for the RT-PCR and to detect other rotavirus groups as well as determine the electrophoretic patterns, polyacrylamide gel electrophoresis was performed on all the rotavirus positive stools. The detection of some rotavirus strains by PAGE which could not be detected by ELISA is an indication of the presence of rotaviruses of other groups other than group A. The overall detection of rotavirus in 30% of neonatal samples was in agreement with findings of a similar study in South Africa where rotavirus incidence of 30% was observed. Possible reasons for the high incidence of rotavirus infection in this study could be the poor feeding practices of the babies by the mothers and poor hygiene. At present, faecal-oral route and air-borne mechanisms are the suspected modes of transmission of rotaviruses (Henry *et al.*, 1990), and both are favoured by overcrowding, low socio-economic status and poor sanitation. These might explain the high incidence of rotavirus infection observed in this study as most inhabitants in Accra find themselves in such living conditions.

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All rotavirus strains regardless of the group to which they belong have 11 distinct RNA gene segments which appear as 11 discrete bands when run on a polyacrylamide electrophoretic gel. Based on the electrophoretic migration pattern, rotaviruses have been classified as subgroup I or subgroup II depending on migration pattern of the 11th gene. Subgroup I rotavirus have a short pattern and subgroup II, a long pattern (Nakagomi *et al.*, 1985). These two rotavirus RNA patterns (long and short) were detected in our study. The short pattern was observed to occur in 21 of the samples whiles the long pattern was observed to occur in 7 of the samples. There was no unusual pattern observed.

Rotaviruses were found in stools of neonates who were less than a day old. Neonatal rotavirus infection typically occurs very shortly after birth. This early infection may be attributed to an earlier exposure to rotavirus especially in conditions such as contaminated sources and overcrowding as was observed for the Babies Unit of the Korle-Bu teaching hospital. Most infected neonates excrete rotavirus during the first week of life and some newborns shed rotavirus less than 24 hours after birth (Haffejee *et al.*, 1990).

Using RT-PCR techniques to assess the distribution of VP7 and VP4 genotypes of neonatal rotavirus field specimens from Accra, the VP4 P[6] genotype was found to be most prevalent. This observation supports previous findings in a study in Nigeria where P[6] was observed to be the most prevalent genotype in neonates (Adah *et al.*, 1997). Rotavirus strain P[6] genotype have been associated with asymptomatically-infected

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neonates in newborns nurseries (Silberstein *et al.*, 1995), but recent reports from India, South Africa and Brazil have indicated that this genotype could be constantly detected in symptomatically-infected neonates and children (Timnesky *et al.*, 1994). Four of the samples (14.3%) had mixed infections and all had P[6]P[10] specificities. Cases of mixed infection specificity have been reported also by several workers across the globe (Ramachandran *et al.*, 1996). However, in South Africa, the most common dual infection was found to occur between strains bearing a P[8] and a P[4] genotype (Steele *et al.*, 1995). This was not the case in this study as none of the 4 specimens with mix infection specificities had this combination. A possible explanation for the high preponderance of the unusual dual infection strain P[6]P[10] genotype could be attributed to the emergence of a new strain P[10] in the study area. However, it should be noted that no strain bearing the single P[10] genotype was isolated, and only one strain with a P[8] specificity was detected during the study. This study has shown clearly that strains with G[2] genotype specificity are the only circulating neonatal strain in Ghana. The detection of G[9] strain as a mixed infection could be a recent emerging strain.

Previous hybridization and sequencing studies of unusual human rotavirus isolates revealed that naturally occurring reassortants between human and animal rotaviruses can occasionally emerge under natural conditions (Urasawa *et al.*, 1993). Bovine rotaviruses are particularly known to be conspicuously involved in the generation of such reassortants. In India, two strains of rotaviruses, 1321 and 116E (Das *et al.*, 1993) were shown to contain either a VP4 gene (116E) or both VP4 and VP7 genes (1321) of probable bovine rotavirus origin. In these instances, however, no direct evidence for dual

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infection of an individual subject with bovine and human rotaviruses were demonstrated. A recent study using RNA-RNA hybridization and sequencing techniques has demonstrated the occurrence in infants of an apparent dual infection with human and bovine rotaviruses (Nakagomi *et al.*, 1994). Most of the big towns in the Greater Accra region have very poor sanitation due to the lack of toilet facilities and proper waste management, and animals and humans live in close contact. Under these conditions, it is tempting to speculate that the possibility exists that naturally occurring reassortment events may be taking place in this region of the country.

Summary of conclusion

This study has thus clearly demonstrated that:

(i) the P[6]G[2] strain predominates in neonates in Accra at this time. Other unusual strains such as P[6]P[10]G[2], P[6]G[3], P[6]G[9], P[6]G[2]G[3] and P[6]P[10]G[9] were also detected.

(ii) The high prevalence of mixed infection among different human rotavirus genogroups suggests the potential for reassortment between viruses from these genogroups. The presence of some untypeable rotavirus strains in this study may be an indication of the existence in Ghana of strains with unusual genotypes.

The study provides the first characterization of G and P type of neonatal strains in Ghana and also the first reported presence of novel P[6]P[10]G[2] and P[6]P[10]G[9] genotype combinations. It also indicates the presence of unusual human rotaviruses in circulation in Ghana. The emergence of these strains strengthens the need to continue

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surveillance of circulating strains especially in Africa for inclusion in a future rotavirus vaccine formulation.

The results of this study has important implications for vaccine application in the country. Many studies have shown that rotavirus is less common in the first 6 months of life and that maternally-acquired antibodies in breastmilk contribute to the relative infrequency of rotavirus in this age group (McLean *et al.*, 1981). This hypothesis no longer seems to hold as recent work including this study has shown that neonates are highly susceptible to rotavirus infections. Thus, the target age group for any such vaccination should be looked at more critically.

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APPENDICES

APPENDIX I

Reagents for PAGE

Reagents for extraction process:

(i) 0.1M NaCl (5.844g dissolved in 1000ml of water)

(ii) 0.2M Sucrose (68.4g of sucrose dissolved in 1000ml of water)

(iii) 0.1M Tris-HCl, pH 8.0 (12.1g of Tris-HCl dissolved in 1000ml of water and pH adjusted to 8.0 with NaOH)

(iv) 0.05M EDTA, pH 8.0 (18.612g of EDTA dissolved in 1000ml distilled water and pH adjusted to 8.0 with NaOH)

(v) 0.5% SDS (0.5g of SDS in 100ml of distilled water)

(vi) Bender Buffer;

100ml of 0.5M EDTA, pH 8.0

100ml of Tris-HCl, pH 8.0

5.844g of NaCl

68.46g of Sucrose

5g of SDS

1000ml of distilled water.

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(vii) POTASSIUM ACETATE (KAc) solutions:

5M KAc (490.75g in 1000ml of distilled water)

8M KAc composed of;

600ml of 5M KAc

115ml of glacial acetic acid

285ml of distilled water.

(viii) TE BUFFER pH 8.0:

0.2ml of 0.5M EDTA pH 8.0

1ml of 1M Tris-HCl

98.8ml of distilled water

Autoclaved.

Stock Solutions:

(i) 0.5M EDTA:

1 86.12g of NaOH

1000ml of water

(ii) 1M Tris-HCl:

121.11g of Tris-HCl

1000ml of water

(ix) PHOSPHATE BUFFERED SALINE (PBS) pH 7.4:

0.2M monobasic sodium phosphate

0.2M dibasic sodium phosphate

0.14M sodium hydroxide

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(x) *Reagents for loading of samples*:

1. SAMPLE BUFFER:

10ml of stacking gel buffer

4ml of glycerol

20mg of Bromophenol blue

6ml of distilled water

Autoclaved

(xi) *Reagents for casting of gels*

Stock Solutions;

(a) Separating Gel Buffer (3M Tris-HCl pH 8.8)

36.33g Tris-HCl dissolved in 100ml of distilled water and pH adjusted to 8.8 with NaOH

(b) Stacking Gel Buffer (0.5M Tris-HCl)

6.05g Tris-HCl dissolved in 100ml of distilled water and pH adjusted to 6.8 with NaOH.

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(xii) 30% Acrylamide solution:

30g of acrylamide

0.8g of Bisacrylamide

100ml of distilled water

filter through a 0.45uM filter

Store in dark bottle at 4°C

(xiii) Ammonium Persulphate:

1mg of ammonium persulphate

1ml of double distilled water

(xiv) 5X SDS Electrophoresis Running Buffer:

151.1g of Tris Base

5g of SDS

72g of Glycine

1000ml of distilled water

Reagents for silver staining:

Solution 1:

Absolute ethanol	40ml
Glacial acetic acid	5ml
Double distiled water	55ml

Solution 2:

Absolute ethanol	10ml
Glacial acetic acid	0.5ml

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Double distilled water	89.5ml

Solution 3:

Glacial acetic acid	5ml
double distilled water	95ml

Silver Nitrate solution:

0.4g of silver nitrate and 200ml of distilled water

Developing solution:

7.5g of NaOH pellets, 2ml of 36% formalin and 250ml of distilled water

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APPENDIX II

Reagents for ELISA

Diluent:

Tris buffered saline containing antimicrobial agent and red dye

Positive control:

Inactivated bovine rotavirus (calf rotavirus strain 3209176)in buffer containing antimicrobial agent and red dye.

Conjugate:

Rotavirus specific rabbit polyclonal antibody conjugated to horseradish peroxidase in a buffered protein solution containing antimicrobial agent and blue dye.

Washing buffer concentrate (x25).

Tris buffered solution containing antimicrobial agent and detergent.

Substrate part A:

25% N N Dimethylformamide, TMB and antimicrobial agent.

Substrate part B:

Hydrogen peroxide and antimicrobial agent.

Stopping solution:

0.46mol/L sulphuric acid.

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APPENDEX III

Reagents for the Extraction process

(i) Lysis Buffer L 2

180g of Guanidinnium isothiocyanate

0.1M Tris-Hcl pH 6.4

(ii) Lysis Buffer L 6

60g of Guanidinnium isothiocyanate

50ml of 0.1M Tris-Hcl pH 6.4

11ml of 0.2M EDTA pH 8.0

1.3g of Triton X-100

(iii) Fractionated silica

(iv) Absolute ethanol

(v) Acetone

(vi) Random primer $Pd(N)_6$

(vii) 10X buffer II

(viii) 50mM $MgCl_2$

(ix) 10mM dNTPs

(x) 200/ul M-MLV

(xi) Taq poplymerase

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APPENDIX 1V

Questionaire:

1. Name of Child	....		NEMCHD
2. Registration number	/__/__/__/__/__/__/		REGNUM
3 Age (days)	/__/__/		AGE
4. Is child breastfeeding	Yes/__/	No/__/	BRESFED
5. Is child formula feeding	Yes/__/	No/__/	FORFED
6. Has child diarrhea	Yes/__/	No/__/	DIAHEA
7. Has child fever (>37.5)	Yes/__/	No/__/	FEVER
8. Admin Temp	/__/__/./__/		ADTEMP
9. Temp. at collection	/__/__/./__/		COLTEM

10.Other comments...

....