

STUDY OF ROTAVIRAL DIARRHOEA IN PAEDIATRIC PATIENTS

THESIS SUBMITTED
UNIVERSITY OF MUMBAI

FOR THE DEGREE OF
MASTER OF SCIENCE
IN
MICROBIOLOGY

SUBMITTED BY
ANURADHA A. KIRTIKAR

UNDER THE GUIDANCE OF
DR. (MRS.) R. A. DESHMUKH

DEPARTMENT OF VIROLOGY
HAFFKINE INSTITUTE FOR TRAINING, RESEARCH & TESTING
MUMBAI

Disclaimer

This research work belongs to

Anuradha Kirtikar

No content of this work can be
circulated or used for any
commercial purpose without the
consent of respective researcher

**STUDY OF
ROTAVIRAL DIARRHOEA IN
PAEDIATRIC PATIENTS**

**A
thesis
submitted to**

THE UNIVERSITY OF MUMBAI

for the degree of

***MASTER OF SCIENCE
IN MICROBIOLOGY***

By

**ANURADHA KIRTIKAR,
DEPARTMENT OF VIROLOGY,
HAFFKINE INSTITUTE,
PAREL, MUMBAI-12.**

2000

STATEMENT UNDER UNIVERSITY RULE # 2190

2190

As required by the University rule number 2190, I state that the work presented in the thesis is original and has not been submitted for any degree of this or other University. Whenever reference has been made to the work of others, it has been clearly stated along with the source of information in "Bibliography".



**Signature of the research guide
Dr.(Mrs.)R.A.Deshmukh,
M.D., Ph.D.,
Officer-in-Charge,
Dept. of Virology,
Haffkine Institute for Training,
Research, and Testing,
Parel, Mumbai – 12.**



**Signature of the candidate
Ms. Anuradha Kirtikar.**

Acknowledgement

As I have come towards the end of my work, I recall all the help that has been offered to me and it gives me great pleasure in thanking all the people who have made this achievement possible.

The two most important people without whose guidance I would have been incapable of completing my work are Dr.(Mrs.)R.A.Deshmukh and Dr.(Mrs.)T.Krishnan. My guide, Dr.(Mrs.)R.A.Deshmukh, Officer-in-Charge, Dept. of Virology, Haffkine Institute, has always been encouraging in all my endeavors. Besides giving me the necessary insights into the ways of science, she has been very accommodating and has given me enough space and freedom to implement any new ideas. I also thank her for the support that I have always taken for granted.

Dr.(Mrs.)T.Krishnan, Div. of Virology, National Institute of Cholera and Enteric Diseases, has been a beacon of light showing me the way all through my work. I have learnt a lot from her including techniques in molecular biology and the quest for perfection. I hope that I have imbibed even a fraction of her zest and enthusiasm for everything around her.

I thank the former Directors of Haffkine Institute, Dr.V.L.Yemul and Dr.B.G.Khadse, for providing the necessary infrastructure required for my work.

Dr.S.V.Gadre, Assistant Director and Head, Dept. of Bacteriology, Haffkine Institute, has been like my guardian. His suggestions have always been invaluable and I have turned to him for help from time to time. I

also thank his family for giving me a sense of belonging in an environment that was at times hostile.

I am grateful to Dr.J.Deshpande, Director, Enterovirus Research Centre, for providing me training in cell culture techniques in his institute.

I thank Mrs.M.V.Joshi, Librarian, Haffkine Institute, and the library staff for providing library facilities that were nothing short of excellent. Mrs.M.V.Joshi has been ever helpful and has taught me efficient ways of using various sources of information.

Mr.J.V.Sawant, Instruments Engineer, Haffkine Institute, has been ever ready to help with the maintenance of instruments.

I owe thanks to Mr.A.Arekar, Biostatistician, Haffkine Institute, for the statistical analysis of my data. I also thank Dr.Mahajani, Head, Dept. of Pharmacology, Haffkine Institute, Dr.Mandke, Statistician, Enterovirus Research Centre, and Prof.(Ms.)A.Chakraborty, Prof. Of Statistics, St.Xavier's College, for -going beyond their calling and helping me understand the subject of statistics.

I thank Dr.(Mrs.)M.V.Kulkarni, Prof. and Head, Dept. of Pediatrics, Lokmanya Tilak Memorial Hospital, Dr.(Mrs.)J.Kamat, Prof. and Head, Dept. of Pediatrics, K.E.M. Hospital, and Dr.Aigle, Medical Supervisor, Kasturba Hospital for Infectious Diseases, for permitting me to collect samples from their patients and in their help in doing the same.

I wish to express my gratitude towards my colleagues and friends in the Dept.s of Chemotherapy and Bioavailability for helping me in small but significant ways each day of my work. My friends Ms.Smita Patil and Mr.Viraj Kulkarni have been encouraging seniors on whose help and

support I could always count on. I thank Mr.Arun Iyer for his help during the initial stages of my work.

My friend Ms.Vandana Rajgopalan has helped me in every way conceivable, scientific or otherwise.

The staff of the Dept. of Virology has been friendly and helpful and I thank each one of them.

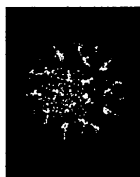
I can only inadequately thank my friend, Pooja, my sister, Vaijayanti, and my mom, for their constant emotional support and faith in me. Though they could not help me in my work, it was only their love and prayers that have given me the strength and confidence to bring this project to completion.

Finally, as I reach the first major step in my academic career, I remember my late father and feel that he would have been proud of me.

Anuradha.

INDEX

CHAPTER	PAGE NO.
INTRODUCTION	1
AIMS & OBJECTIVES	5
REVIEW OF LITERATURE	6
MATERIALS & METHODS	23
RESULTS	30
DISCUSSION	34
SUMMARY & CONCLUSION	41
APPENDIX	43
BIBLIOGRAPHY	46
SYNOPSIS	



INTRODUCTION

It has long been recognized that diarrheal disease is a leading cause of morbidity and mortality, especially in developing countries and acute infectious gastroenteritis is one of the commonest causes of childhood illness throughout the world. In 1982, Snyder and Merson published the first estimate of global morbidity and mortality from diarrheal disease based on active surveillance data collected from longitudinal studies of children. The study estimated the total annual number of acute diarrheal episodes for children under five years old in Africa, Asia (excluding China), and Latin America alone to be 744 million and the total annual number of deaths from acute diarrheal illness for children under five years of age all over the world was estimated to be 4.6 million for 1980 (1). An update of this study reported a decline in diarrheal mortality over time with 3.3 million deaths per year from diarrhea. But diarrheal morbidity continued to be endemic with an estimated 1 billion episodes of diarrhea occurring in children per year, globally (2).

Since then, diarrheal disease control programs have been established all over the world, with oral rehydration therapy as the keystone. Yet, diarrhea remains one of the commonest illnesses of children, and one of the major causes of infant and childhood mortality in developing countries. Malnutrition is a major factor contributing to this. A well-nourished child suffering from acute diarrhea is likely to have an excellent outcome with the use of oral rehydration therapy. But diarrhea in a severely malnourished child poses a major and quite different clinical challenge. The outcome in these children, even with the use of oral fluids, is much less certain. Several studies have linked malnutrition to the high incidence of diarrhea. (3). And since malnutrition continues to prevail in developing countries, so does diarrhea. Another factor contributing to the unchecked spread of diarrhea is poor hygiene and sanitation. Coupled with ignorance and poverty, it makes the developing countries a virtual breeding ground for diarrheal diseases. Diarrheal diseases thus assume phenomenal proportions in developing countries and demand immediate action for their control.

Despite the magnitude of this problem, infectious agents like *E.coli*, *Salmonella*, *Shigella*, *Campylobacter*, protozoa and other parasites could be implicated in only upto 35% of diarrhea cases while in 65% of cases, such agents could not be isolated. (4). This scenario continued till the early 1970s until the discovery of the Norwalk virus by Kapikian *et al* in 1972 (5) followed by the discovery of rotavirus in 1973 by Bishop *et al*. (6). Within five years of its discovery, rotavirus was recognized as the most common cause of severe vomiting and diarrhea in infants and young children worldwide, accounting for approximately one third of cases of severe diarrhea requiring hospitalization (7,8,9). Rotaviruses cause childhood diarrhea not only in humans, but also in birds like chickens, turkeys and in animals like calves, piglets, foals, dogs, cats, deers, rabbits and mice (10). Rotavirus is, thus, ubiquitous in nature.

However, rotaviruses infecting this wide range of birds and animals belong to different strains and each strain is specific for a species. Over time, though, there were instances where an animal rotavirus was found to be infecting humans. Rotaviruses affecting humans were once thought to be limited to one antigenic family termed Group A, whereas other antigenic groups (B-G) were thought to be strictly zoonotic. In 1982, however, an epidemic of Group B rotavirus affected millions of persons in China (including adults, children, and neonates) (11), and since then outbreaks have recurred, although affecting fewer persons. Studies of immunoglobulin pools from Shanghai suggest that the Chinese population had been exposed to this pathogen in the past (12). Since Group B rotavirus is a common diarrheal pathogen for swine, and since all rotaviruses have a segmented genome capable of antigenic changes through reassortment of genes, it may be possible that this human Group B epidemic arose by a reassortment that allowed the swine virus to propagate in the human gut. Group C rotaviruses are also primarily swine pathogens but have been detected among humans in all parts of the world. Outbreaks have occurred in Japan and England, but the importance of Group C rotaviruses in endemic disease is unknown (13,14). The Group B

epidemic was not known to have spread beyond China until Krishnan T *et al* reported the detection of this virus in Calcutta in 1999 (15). Therefore, it becomes necessary to monitor the epidemiology of rotavirus infection in a population. This would not only enable us to foresee a possible epidemic of unusual rotavirus gastroenteritis but also to ascertain the prevalent strains with a view towards vaccine production.

Rotavirus infects virtually all children by five years of age. Some of these infections are severe and many children are infected more than once, although severity of disease decreases with each infection (16). Studies of natural rotavirus infections indicated that initial infection protects against subsequent severe diarrheal disease, although subsequent asymptomatic infections and mild disease still occurred (17,18). Thus, immunization early in life, which mimics a child's first natural infection, is not expected to prevent all subsequent disease but to prevent cases of severe rotavirus diarrhea and its sequelae (e.g. dehydration, physician visits, and hospitalizations).

Research to develop a safe and effective rotavirus vaccine began in the mid-1970s when investigators demonstrated that previous infection with animal rotavirus strains protected laboratory animals from experimental infection with human rotaviruses (19). Several trials of live, oral vaccines demonstrated that they were safe and could prevent diarrhea in young children (20,21,22,23). However, the efficacy of these vaccines varied. Then exploiting the inherent property of rotaviruses to reassort their genome, multivalent vaccine candidates were developed (24). On August 31st, 1998, a tetravalent rhesus-based rotavirus vaccine was licensed in the United States for vaccination of infants (25). This vaccine was produced incorporating a rhesus rotavirus strain along with three human-rhesus reassortant strains. These three strains were obtained from the ones found to most commonly infect humans. Post-licensure studies, however, reported several cases of intussusception and the vaccine was hence withdrawn (26).

Because the clinical features of rotavirus gastroenteritis are nonspecific, confirmation of rotavirus infection in children with gastroenteritis by laboratory testing of fecal specimens is necessary for reliable rotavirus surveillance (27). The most practical method taking into consideration accuracy and time required for the test is antigen detection by enzyme immunoassay. But this method is expensive. Another practical method that is also inexpensive is antigen detection by latex agglutination. This method, however, may compromise on accuracy. Other techniques including electron microscopy, reverse transcription-polymerase chain reaction, nucleic acid hybridization, polyacrylamide gel electrophoresis and culture are mainly used in research settings. Thus there is a lack of an appropriate diagnostic tool. A definitive viral diagnosis can reduce indiscriminate use of antibiotics, eliminate necessity for other tests in search of causative agents and remove uncertainty, allowing the physician to make proper prognosis and remain alert to potential complications.

Taking all these points in consideration we undertook the present study.



*AIMS
AND
OBJECTIVES*

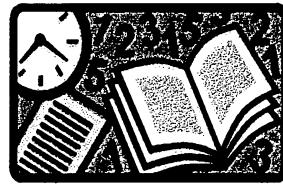
Taking into consideration the lack of a systematic surveillance and good data, we undertook the present study to gauge the severity of the problem posed by rotavirus infections in Mumbai.

The study was planned keeping the following aims and objectives in mind:

To determine the prevalence of rotavirus infection in pediatric cases of diarrhea.

To study the prevalence of enteric bacteria in pediatric cases of diarrhea.

To compare three methods for detection of rotaviruses, namely latex agglutination, polyacrylamide gel electrophoresis and virus isolation in cell cultures.



*REVIEW
OF
LITERATURE*

HISTORY

Major strides have been taken in elucidating the etiological agents of diarrhea in infants and young children in whom gastroenteritis or infantile diarrhea is frequently a serious disease requiring hospital treatment to correct dehydration. Prior to the 1970s, not a single viral agent could be implicated as an important etiological agent of infantile diarrhea. Moreover, bacterial and parasitic agents known at that time failed to fill this etiologic void. The advent of tissue culture technology encouraged research on henceforth-undiscovered viruses. A number of animal viruses were discovered in the 1960s using tissue culture techniques.

The epizootic diarrhea of infant mice (EDIM) virus that causes diarrheal disease in suckling mice was discovered in 1963 (28). The EDIM virus was observed by electron microscopy in the intestinal tissue of infected mice. Immediately afterward, Malherbe *et al* reported the isolation of a 70-nm virus with similar morphology (29). This virus, being morphologically similar to reovirus particles, did not serologically cross-react with reovirus (30). This virus was isolated in vervet monkey kidney cell culture and was obtained from a rectal swab from a healthy vervet monkey and was designated as SA11 (simian agent 11). Then in 1967, O (Offal) agent was described by Malherbe and Strickland-Cholmley as being very similar to SA11 (31). This virus was isolated in vervet monkey kidney cell culture from the mixed washings of intestines of cattle and sheep. In 1969, the presence of another 70-nm virus was demonstrated in stool from calves with severe diarrheal illness. It was also demonstrated that this virus caused disease when passaged serially in calves. In 1971, this virus designated neonatal calf diarrhea virus (NCDV), was successfully cultivated in primary fetal bovine cell cultures by Mebus *et al* (32). NCDV though similar to reoviruses was shown to be distinct antigenically (33).

After many years of lack of success of conventional virus isolation techniques for incrimination of viruses as causes of infantile enteritis, the use of electron microscopy led to the recognition of new agents.

The Norwalk virus was detected by immune electron microscopy from infectious stools in 1972 by Kapikian *et al* (5). This 27-nm virus was associated with outbreaks of a self-limiting form of gastroenteritis affecting primarily adults and older children; these illnesses only rarely led to dehydration, and although this virus was associated with mild gastroenteritis in infants and young children, it was not identified as the cause of severe diarrhea in this age group (34).

Close on the heels of this discovery came the discovery of rotaviruses. Bishop *et al* first discovered rotaviruses with the electron microscope in 1973 (6). They observed these virus particles in the duodenal mucosa from children with acute non-bacterial gastroenteritis and based on the morphology, size and location of the particles, deduced that it belonged to the orbivirus group and was closely related to the reovirus group. Shortly afterward, several studies identified this virus in feces by electron microscopy (35,36,37,38) and in a short period, rotaviruses were recognized as the major viral etiologic agents of severe diarrhea of infants and children in both developed and developing countries (39,40). Rotaviruses were found to be morphologically similar to the viruses mentioned above and were also shown to share a group antigen with them (7,39,41,42). Rotaviruses, which are ubiquitous agents, infecting virtually all children by 24 months of age, have consistently emerged as the single most important etiologic agents of severe diarrhea of infants and children in both the developed and developing countries.

MORPHOLOGY

Rotaviruses have a distinct morphologic appearance. Studied under an electron microscope, three types of particles can be observed. Complete particles measure ~70nm and resemble a wheel with short spokes and a well-defined smooth outer rim. Owing to this appearance, it acquired the name rotavirus (from the Latin *rota*, meaning wheel) (43). The complete particles are also called *smooth particles* because it has a well-defined, smooth circular appearance. They have a distinctive double-layered icosahedral protein capsid that consists of an outer and an inner layer. Within the inner capsid is a third layer, the core that contains the virus genome. The complete infectious particles, thus, have three layers. They were earlier thought to have only two layers and were hence called double-shelled particles. Double-layered particles previously called single-shelled particles, lack the outer shell and are described as *rough* particles because their periphery shows projections of the inner capsid. Single layered core particles usually lack genomic RNA and are seen infrequently.

Several studies have given insights about the structural features of rotaviruses (44,45,46).

NOMENCLATURE and CLASSIFICATION

Based on common morphologic and biochemical properties, rotaviruses are classified within the family *Reoviridae*. The general properties of rotaviruses are:

They are triple layered 65-75nm icosahedral particles with a double stranded RNA genome that is segmented into eleven segments, each segment coding for one protein. (Table A)

The genus Rotavirus is further divided serologically into a number of groups and further into multiple serotypes within each group. Early serologic studies

Table A. Rotavirus proteins

Genome segment	Protein product	Location in virus particles	Function
1	VP1	Inner core	RNA polymerase?
2	VP2	Inner core	RNA binding
3	VP3	Inner core	Guanylyltransferase
4	VP4	Outer capsid	Hemagglutinin, neutralization antigen, protease-enhanced infectivity, virulence, putative fusion region, cell attachment
5	NSP1 (NS53)	Nonstructural	Slightly basic, zinc finger, RNA binding
6	VP6	Inner capsid	Hydrophobic trimer, subgroup antigen
7	NSP3(NS34)	Nonstructural	Slightly acidic, RNA binding
8	NSP2(NS35)	Nonstructural	Basic, RNA binding
9	VP7	Outer capsid	RER integral membrane glycoprotein neutralization antigen , bicistronic gene, two hydrophobic NH ₂ -terminal regions
10	NSP4(NS28)	Nonstructural	RER transmembrane glycoprotein, role in morphogenesis, three hydrophobic NH ₂ -terminal regions
11	NSP5(NS26)	Nonstructural	Slightly basic, rich in serine and threonine, RNA binding

revealed that rotaviruses from birds and a variety of mammals possessed a common group antigen so these viruses were classified as different serotypes within the same group. A serogroup has viruses that share cross-reacting antigens detectable by a number of serological tests like immunofluorescence, ELISA and immune electron microscopy (47,48,49). Rotaviruses which are serologically unrelated and carrying their own distinct group antigen are classified as separate groups. There are seven such groups named group A to group G. Group A, B, and C rotaviruses are currently associated with diarrhea in both humans and animals, while group D, E, F, and G rotaviruses have been found only in animals to date.

Within each group, rotaviruses are classified into serotypes defined by reactivity of viruses in plaque reduction neutralization assays using hyperimmune serum prepared in antibody-negative animals (50,51). It was assumed that all assays detected the same or similar specificities, and hence the term "serotype" was applied to the differences defined by these assays. However, some animal viruses reacted like members of the "serotype 1" of human rotavirus (52). This was surprising, since animal rotaviruses had been found to have neutralization specificities different from those observed in humans (53). Studies of reassortant viruses eventually showed that "serotype" designation was distinct from the neutralization specificity and that different genes coded for these two specificities (54) and the specificity coded by the sixth gene (VP6) was referred to as the subgroup, and the neutralization specificity coded by the ninth gene (VP7) as serotype. Thus, strains of animal and human origin may fall within the same serotype. 14 VP7 serotypes have been identified. 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. In some cases, a rotavirus strain does not react clearly when being classified. This is usually because although the two viruses being compared have similar VP7, they possess distinct immunologic forms of VP4.

A binary system of classification of rotaviruses has been proposed in which both serotypes of VP4 and VP7 are named (50,55).

GENOME

The rotavirus genome contains eleven segments of double stranded RNA which range in molecular weight from 2×10^5 to 2.2×10^6 Dalton (56,57,58). All these eleven segments are contained within the virus core capsid. The RNA segments are said to fall into four classes based on length measurements by electron microscopy and gene sequencing. Also, polyacrylamide gel electrophoresis (PAGE) of these RNA segments is used to number them in order of their migration, with the slowest RNA segment designated *gene 1*, and so on. Group A rotaviruses have four large segments, two medium sized segments, three smaller segments and two smallest segments. In some human rotavirus strains, the 11th RNA segment migrates more slowly than usual, and is thus located between the 9th and 10th segment forming a "short" pattern which is characteristic of almost all antigenic subgroup 1 human rotaviruses (59,60). Similarly, a "long" 10th and 11th segment pattern of human rotaviruses is characteristic of almost all subgroup 2 human rotaviruses. Several "super-short" patterns of RNA migration have also been observed in which the 11th segment migrated even more slowly than the "short" pattern rotaviruses (61,62). Since initially rotaviruses could not be propagated in cell culture, comparison of the migration patterns of RNA during PAGE was an important technique for characterization of strains (63,75). The term *electropherotyping* was applied to this method of differentiating strains (75). A wide variety of electropherotypes was observed in human rotaviruses and all these differed from those observed in animal rotaviruses. However, the electrophoretic pattern was found to be unrelated to antigenic specificity as defined by serotype. Studies indicate that viruses of the same serotype exhibit different electropherotypes and viruses of the same electropherotype belong to different serotypes (64,65,66).

PROPAGATION IN CELL CULTURE

Various animal rotaviruses like SA11 and the O agent grow readily in cell culture (29,31). However, the human rotaviruses are rather fastidious agents that were not grown efficiently in tissue culture until relatively recently (67,68). Initially, human rotaviruses were observed to induce an abortive infection of cells in culture; viral antigens were produced, but infectious virus could not be passaged serially. The first human rotavirus to be grown efficiently and reproducibly in cell culture was mutant of the Wa human rotavirus (69). This strain was grown to relatively high titer through passages in primary cultures of African green monkey kidney cells. Efficient growth of the mutant required pretreatment of virus with trypsin. The infectivity of most rotaviruses is enhanced by, and in some cases is dependant on, trypsin treatment (70). It was found that trypsin did not increase levels of infectious virus by dispersion of aggregates or affect the efficiency or rate of attachment of virus to cells. Trypsin is thought to convert a noninfectious fraction of the virus into infectious virus by allowing this fraction to uncoat in the infected cell. It is possible that trypsin present in the intestine of the host may contribute to the increase of the severity of disease (70). Sato K *et al* then isolated three strains of human rotavirus in cultures of MA104 cells, which is a stable cell line derived from embryonic rhesus monkey kidney (71). With minor modifications, the method of Sato K *et al* was adopted for isolation of rotaviruses in cell culture (72,73). Susceptibility of MA104 cell line and that of a human colon carcinoma cell line (HT-29) to infection by different rotaviruses was compared (74). HT-29 cells appeared to be more sensitive to human rotavirus infection, whereas Ma104 cell line was found to be more susceptible to animal rotavirus replication. Human rotavirus has also been recently propagated in a human colon carcinoma cell line (CaCo-2) and although the cells did not show any cytopathic effect, virus particles were detected in the cell cytoplasm by immunoflourescence staining (75).

PHYSICOCHEMICAL PROPERTIES:

Rotavirus particles are infectious so long as they are enveloped in the outer capsid i.e. infectivity depends on the presence of the outer capsid (76). Calcium chelating agents like EDTA and EGTA remove the outer capsid and in turn cause the virus particle to lose infectivity (77,78). Rotavirus infectivity is stable within the wide pH range of 3 to 9 (77,79,80). Virus infectivity is also stable for months at 4° to 20°C when stabilized by CaCl₂ (81). Rotaviruses are also stable to ether but repeated freezing and thawing diminishes their infectivity (82,83). Disinfectants like phenols, formalin, chlorine and ethanol inactivate virus infectivity (82,84).

PATHOLOGY AND PATHOGENESIS

Rotaviruses have limited tissue tropism, infecting only the villus epithelium of the small intestine. Shortening and atrophy of the villi takes place, along with mononuclear cell infiltration in the lamina propria. Mitochondrial swelling and sparse and denuded microvilli were also observed (83,84). The various pathological effects were observed in experimental animals that developed diarrheal illness (84,85). A study comparing the severity of infections in malnourished and well-nourished animals reported that malnutrition increased the severity of symptoms and mucosal damage (88).

CLINICAL FEATURES

Rotavirus produces a spectrum of responses that vary from subclinical infection to mild diarrhea to severe and occasionally fatal dehydrating disease. The incubation period of rotavirus diarrheal disease has been estimated to be less than 48 hours (89). In experimental animals, the incubation period can be as short as 19 hours (90). The incubation period is influenced to some extent by the dose of virus and by the age of the child. In

a newborn, many cells may not need to be destroyed before diarrhea sets in. The duration of virus excretion in neonates is found to vary from 1 to 9 days (91,92). Occasionally, virus excretion does persist longer. A study found that vomiting was more frequently associated with rotavirus diarrhea than with non-rotavirus diarrhea (93). This led to greater dehydration. Rotavirus diarrhea started later than the vomiting but lasted longer than non-rotavirus diarrhea. Diarrhea is usually most profuse on the second or third day of disease, and diminishes fairly quickly thereafter, although a few children may continue to excrete rotavirus and have mild diarrhea for longer periods (94,95).

EPIDEMIOLOGY

MORBIDITY AND MORTALITY

Although high morbidity is attributed to rotavirus infections, the mortality is low in the developed countries. The large proportion of morbidity that rotaviruses are responsible for even in the developed nations is demonstrated by a study in Germany. Rotaviruses were found to be the leading cause of diarrhea in hospitalized pediatric patients and were detected in 31% of the cases (96). A study carried out all over the United States which involved 99 laboratories reported that 20% of pediatric diarrhea patients tested positive for rotavirus. This study was carried out by the CDC during the years 1989-90 as a National Rotavirus Surveillance System to monitor national patterns in the epidemiology of rotavirus (97). Another laboratory based surveillance for rotavirus in the United States carried out from July 1997 to June 1998 by the CDC found the rotavirus positivity among pediatric diarrhea patients to be 23% (98). In the U.S. alone, 1 million cases of diarrhea occur annually and up to 150 deaths in children (99,100,101,102). This high morbidity but low mortality can be attributed to the effective replacement of fluids and electrolytes lost during the infection. These means of rehydration solutions

are used more routinely in the developed countries, but in the developing countries, rotaviruses are usually the leading cause of life-threatening diarrhea in infants and children.

Rotaviruses have been proved to be the major causative agent of diarrheal diseases in developing countries where such diseases are prevalent. A study in Cairo, Egypt, determined that rotaviruses accounted for 34% of fatal diarrhea cases (103) demonstrating the importance of rotaviruses in severe dehydrating diarrheal illness in the developing countries, while in Kenya, a recent study implicates rotavirus in 16.1% of diarrhea cases (104). A short study in South Africa identified rotavirus as the causative agent in 12.8% of pediatric diarrhea patients (105). A study carried out over a period of 13 months in western Saudi Arabia reported the occurrence of rotavirus in 46% of specimens that were collected from infants and young children suffering from diarrhea (106). In Brazil, rotaviruses were implicated in 19% of diarrhea cases (107). Human rotavirus was detected in 39% of diarrheal stool samples examined in China (108). In Bangkok, 18% of pediatric diarrhea patients were found to be infected by rotavirus (109). A study in Bangladesh found that rotaviruses were the most frequently detected pathogen in children with diarrheal disease. 46% of the study subjects were found to be rotavirus positive (110). These studies demonstrate the magnitude of rotavirus diarrhea all over the world.

In India, several studies indicate that rotavirus is an important etiologic agent of diarrhea in infants and young children. A study in rural Northern India was carried out for a year wherein more than 900 children below 71 months of age were followed prospectively (111). 2.3% of the children suffering from persistent diarrhea were found to be infected with rotavirus. Another study was conducted in rural, semi-urban and urban communities at Chandigarh from 1988 to 1991. Stools were collected from children with diarrhea and it was found that 11.5% of total diarrheal episodes were caused by rotavirus

(112). A very low prevalence of 1.07% was found in children studied in a rural area near Calcutta (113). In 1994, four hundred Delhi children below the age of three years and suffering from acute watery diarrhea were studied over a period of a year (114). Rotavirus antigen was detected in 6% of the stools, which is much lower than earlier studies. Even during the peak season for rotaviruses, rotavirus antigen was detected only in 13% of the cases. A similar study in 1992 in Delhi among hospitalized children suffering from gastroenteritis had reported that rotavirus was detected in 15.28% of the patients (115). A similar rate of incidence of rotavirus was found in an earlier study of pediatric diarrhea patients in northern India (116). The etiologic role of rotavirus in acute diarrheal illness in children under five years of age was studied by Nath G *et al* over a period of one year in an urban slum community in Varanasi (117). Rotavirus was detected in 17.7% of 376 children with diarrhea. In south India, rotaviruses were detected in 17.8% of the specimens examined (118). This study was carried out from August 1983 to July 1985 and the subjects were 916 children under three years of age. Another two-year study from 1981-1982 was undertaken in Pune to determine the role of viruses in the causation of diarrhea in hospitalized children (119). Of the 426 children included in the study, rotavirus was detected in 28.6%. While in Mumbai, a study carried out from 1984 to 1986 reported that rotavirus was detected in 23% of pediatric diarrhea patients (120).

TRANSMISSION

Rotaviruses being viruses belonging to the gastrointestinal tract appear to be transmitted mainly by the fecal-oral route. However, the respiratory route of transmission of rotaviruses is not ruled out (121,122). This theory gains credence from circumstantial evidence like the acquisition of rotavirus antibodies in the first few years of life in all settings regardless of hygienic standards (7,123) and the occurrence of respiratory symptoms in a few patients with rotavirus diarrhea (124). A study utilized the mouse model of

rotavirus infection to investigate the experimental transmission of rotavirus by respiratory droplets (125). Efficient transmission of rotavirus infection by means of aerosols was documented in all exposed animals. Gastrointestinal illness was also demonstrated proving that rotavirus infection can be transmitted by aerosol droplets under experimental conditions.

Rotaviruses are shed in large amounts by infected persons. A person with rotavirus diarrhea may excrete approximately 1 trillion infectious particles/ml of stool (126) and since rotaviruses are resistant to physical inactivation, they are transmitted efficiently. Also, rotaviruses are amazingly stable in the environment. Calf rotavirus present in feces was found to retain its infectivity even after being kept at room temperature for seven months (127,128). A young infant who is infected but who is not in contact with other infected infants and young children may have acquired the infection from an older sibling or parent with subclinical infection (129) since rotaviruses are known to be shed in high concentrations even before onset of disease and also after cessation of diarrhea (130,131). All these factors ensure that most infants and young children are infected by rotavirus at least once.

TEMPORAL DISTRIBUTION

Rotaviruses are known to display a seasonal pattern of infection, with epidemic peaks occurring during the cooler months of the year. This pattern is well documented in various studies (127,132). Inpatients as well as outpatients have exhibited this pattern consistently. Thus, a maximum of rotavirus infections occur during the months of December, January, and February and were virtually absent from July through October. This pattern, however, is more obvious in the developed countries and several studies report the occurrence of rotavirus infections throughout the year. Also peaks of infection during the rains and the summer have been observed in other settings (132).

AGE, SEX, AND SOCIOECONOMIC STATUS

Since preliminary infection with rotavirus confers immunity on the child, further episodes of rotavirus diarrhea are less severe and hence rotavirus gastroenteritis severe enough to require hospitalization occurs most frequently in infants and young children from approximately 6 months to 2 years of age (133,134). Infants under 6 months of age, though protected by maternal antibodies, are shown to experience the second highest frequency of rotavirus gastroenteritis. This age group has also shown to have the highest frequency of illness by some studies (135). In India, 96.7% of victims of rotavirus have been reported to be 6-24 months old (136). However, neonates who shed the virus show a low frequency of clinical illness (137).

It has also been shown that susceptibility to rotavirus infection is gender biased. It was observed that rotavirus was more prevalent in boys than in girls (138,139).

Malnutrition plays an important role in increasing the severity of clinical manifestations of diarrhea in general (3) and hence also of human rotavirus infections (140). Repeated diarrheal infection leads to malnutrition and vice versa. The factor of malnutrition is associated closely with lower socioeconomic status and hence there is an increased occurrence of rotavirus infections in children belonging to the lower strata of society. Other factors also associated with lower socioeconomic status are poor hygiene and sanitary conditions, which also promote the spread of the infection. Also, since the level of literacy in this class is low, ignorance leads even mild infections to progress to severe gastroenteritis and dehydration due to lack of proper diagnosis and treatment.

DIAGNOSIS

Rotavirus infection leads to clinical manifestations that are associated with diarrhea in general. Since they are not sufficiently distinctive, diagnosis cannot be based on these alone. Epidemiologic pattern of disease and the signs and symptoms exhibited by the patient may suggest the diagnosis, but laboratory confirmation is required. Diagnosis requires detection of virus or viral antigen or a serologic response. Rapid diagnosis of rotavirus illness provides the physician useful information for formulating prognosis and may prevent unnecessary use of antibiotic therapy and prolonged hospitalization. It also allows for timely institution of appropriate infection control measures and for gathering of epidemiological and clinical information which is vital for vaccine development.

For the detection of rotavirus from clinical specimens it is important to collect the specimens during the period in which rotavirus is being shed. Specimens from the first to about the fourth day of illness are ideal for virus detection. However, shedding of the virus may continue for up to 3 weeks, depending on the duration of symptoms. Viral shedding usually continues during the course of diarrhea, but diarrhea can continue for an additional 2 to 3 days (141).

When discovered, rotavirus was observed by an electron microscope (6). Since rotaviruses have a distinctive morphologic appearance, electron microscopy (EM) has high specificity. It was hence initially employed for the detection of rotaviruses. Early studies on rotaviruses were also carried out using EM. Although EM has remained a research tool more than a diagnostic one, when only a few samples are to be examined for rotavirus, EM is the most rapid technique. Direct EM examination of stools permits detection of rotavirus in 80% to 90% of the virus positive specimens (142), but if the specimen is centrifuged and the pellet is examined after staining with a suitable method, EM is as efficient as any other method for rotavirus detection

from a stool sample (143). It also has the added advantage of being able to detect non-group A rotaviruses (144). Immune electron microscopy is not necessary for the detection of rotaviruses because of their distinctive appearance, which can be readily identified without the use of an immune serum.

Many assays have been developed for the detection of rotaviruses from stool specimens. These include immunological techniques like ELISA, counter-immuno-electro-osmophoresis, reverse passive hemagglutination assay, and latex agglutination. A variety of techniques like IEM, CF, IF, IAHA, ELISA, neutralization, HI, and inhibition of reverse passive hemagglutination are used for measuring the serologic response to rotavirus infection (145). Molecular biology techniques used for detection of rotaviruses include electrophoresis of rotavirus RNA in gel, and PCR. These techniques are also useful for the detection of non-group A rotaviruses. Cell culture can also be used with reasonable efficiency for the detection of rotaviruses.

Of the above-mentioned techniques, most are used mainly as research tools as they are cumbersome to perform, involve long procedures and expertise, or require reagents that are expensive. Only some of the techniques are suitable as diagnostic aides to be used by laboratories in all parts of the world. Commercial antigen-detection kits for rotavirus permit rapid viral diagnosis. Only small amounts of stool are required for these tests, and samples may be frozen before testing. These kits vary widely in range of sensitivities (70-100%) and specificities (50-100%) (146,147). These kits mainly are enzyme immuno assays and latex agglutination tests. The ELISA kits are too expensive to be used as an everyday diagnostic tool in developing countries like India. Latex agglutination kits are more widely available and inexpensive. In this test, latex particles are coated with anti-rotavirus antibody. Stool specimen is prepared in given buffer and added to

one drop of latex particles coated with anti-rotavirus antibody. This suspension is mixed and rotated and observed for agglutination.

Another method for the detection of rotaviruses that is much more accurate is electrophoresis. Rotavirus multiplies in the gut with such efficacy that, during infection, its genome dominates the RNA content of stool. When this RNA is extracted from the stools and run on a gel, an electric field causes the separate rotavirus genes to migrate in characteristic patterns. The presence of these patterns is diagnostic of rotavirus infection. Pattern variations also provide insight into strain differences. The term *electropherotyping* was applied to this method of differentiating strains (148). Because human rotaviruses could not initially be propagated in tissue culture, electropherotyping became an important laboratory and epidemiologic technique for characterization of strains (149). Polyacrylamide gel electrophoresis (PAGE) detection of rotavirus from stool is highly specific, sensitive and inexpensive means of virus detection with the added ability to detect both group A and non-group A rotaviruses (150).

TREATMENT

Gastroenteritis caused by rotavirus is a self-limiting syndrome and as such requires no treatment to stop the infection from progressing. The manifestations of the infection however, require treatment, which is mainly the replacement of fluids and electrolytes lost by vomiting and diarrhea. To treat severe dehydration, intravenous fluid administration has been used successfully for many years. In other cases oral rehydration salts solutions are effectively used. Since administration of rehydrating fluids parentally is not always possible in all parts of the world, the efficacy of oral fluid replacement therapy has been evaluated by many studies (151,152). Oral rehydration therapy with electrolyte solutions containing either glucose or sucrose has been shown to be highly effective for treatment of rotavirus gastroenteritis

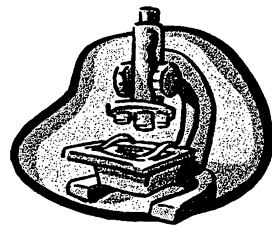
(153). The WHO has described the concentration of various salts in the oral glucose electrolyte solution that has been found to be effective (154). This solution was compared with another WHO preparation that was augmented with alanine. Their effect was studied in 3–48 month old children with mild dehydration that was associated with rotavirus infection. The alanine-fortified solution was absorbed more readily but the reduction in diarrheal stool output or duration of diarrhea was not significant and hence it was not preferred over the previously recommended formula (155). Some studies have even compared rice based solutions with glucose-based oral rehydration solutions in infants and young children suffering from mild dehydration due to diarrhea caused by various pathogens including rotavirus (156). Both were found to be effective with minor differences in total stool output and retention of fluids. Thus even a simply formulated oral rehydration salts solution is adequate treatment for a child suffering from dehydration due to rotavirus infection. However, if the patient does not respond favorably to the oral rehydration regimen or if the patient is severely dehydrated or in shock, intravenous fluids become a necessity.

PREVENTION AND CONTROL

All the data collected about the epidemiology of rotavirus infections all over the world indicate that the prevention of rotavirus disease is necessary. Improved environmental hygiene (i.e., food, water, and sanitation), although essential in outbreak management, may be ineffective in endemic control of gastroenteritis caused by rotavirus, perhaps because person-to-person transmission is the principal mechanism for the spread of infection. Because improvements in environmental hygiene may not be accompanied by reductions of endemic rotavirus diarrhea, immunization may play an important role in control of the disease. Since natural infection does not protect the child completely against subsequent reinfection or associated mild disease, prevention of rotavirus disease can be achieved by means of a vaccine,

which should ideally prevent severe rotavirus gastroenteritis during the first 2 years of life. Progress has been made in developing various vaccine candidates, each of them, however, has some drawback.

Following the discovery and recognition of rotavirus as an important human enteropathogen, work towards the development of rotavirus vaccines was begun in the USA and Europe. The first candidate rotavirus vaccine was studied in clinical trials in Finland, Yugoslavia, Italy, Switzerland, Austria and the UK (157). Efficacy trials in Finland of a live oral bovine rotavirus vaccine showed that protection was acquired against disease rather than infection; better protection against severe than mild disease (157), i.e. it had low efficacy in prevention of disease. This vaccine was also found to be weakly immunogenic in infants in developing countries (158). A review of rotavirus vaccine trials in Finland observes that the maximal protective efficacy against any rotavirus gastroenteritis in subjects with demonstrable vaccine immunogenicity was approximately 75% and to achieve similar protection in all vaccinees, efforts should be focused on enhancing the immunogenicity of oral rotavirus vaccines (159). A tetravalent rhesus-based rotavirus vaccine was licensed in the United States for vaccination of infants (25). But this vaccine was found to cause intussusception in several cases and was hence withdrawn (26). Thus despite extensive experience with rotavirus vaccines, the vaccines and the need for such vaccines still require further assessment.



MATERIALS
AND
METHODS

STUDY SUBJECTS

In this study, 225 patients and 25 control subjects were included.

The study subjects were admitted in the pediatric wards of Lokmanya Tilak Memorial Hospital, Sion, K.E.M.Hospital, Parel, and Kasturba Hospital for Infectious Diseases, Mumbai Central. All subjects were infants and children under 5 years of age.

SPECIMEN COLLECTION

PATIENTS

Stool samples were collected from children suffering from diarrhea and admitted in the pediatric wards of the hospitals. Children were excluded if they had an associated complicating illness. The samples were collected on the day of admittance.

CONTROLS

Control patients were children admitted to these hospitals with other illnesses and who showed no symptoms of gastroenteritis. Stools from these children were collected on any day after admittance to the hospitals.

The clinical features and personal history of all patients including controls was recorded. These include features like age, sex, family income, nature of current illness etc. The specimens were collected in sterile 15ml containers with wide mouths and screw caps. The specimens were labeled and stored at 4 °C until processed.

DETECTION OF ENTERIC BACTERIA

The specimens were plated on basic and selective and differential media like nutrient agar, MacConkey's agar and Salmonella-Shigella agar within an hour of sample collection. The plates were observed for distinctive colonies. All the isolates were subjected to identification on the basis of their morphological, cultural, and biochemical characteristics. Some of the tests included were Gram's nature, colony characteristics, motility, utilization of various sugars like glucose, lactose, sucrose, and mannitol, utilization of citrate and urea, tests for production of Indole and PPA, the methyl red and Voges-Proskauer tests, growth on KCN and oxidase test as per the standard protocol (160).

The media and reagents were prepared as per the standard protocol

DETECTION OF ROTAVIRUS

Detection of rotavirus was done using three methods, namely, latex agglutination (LA), polyacrylamide gel electrophoresis (PAGE), and isolation of the virus in cell culture.

LATEX AGGLUTINATION

Commercially available latex agglutination kit for the detection of rotavirus manufactured by Sanofi Pasteur diagnostics was used. The kit was stored at 2-8 °C as described. It is a rapid test based on latex agglutination, intended to detect rotavirus from feces in acute gastroenteritis.

The samples to be tested were kept at room temperature. The kit was also removed from the fridge to allow the reagents to reach room temperature. The stool specimens were mixed properly and the specimens were diluted 1:10 in dilution buffer and mixed well with vortex mixer for 1min. This suspension was then centrifuged at 1,000 r.p.m. for 10 mins.

Two separate drops of about 50µl were taken on disposable slide (provided in the kit) with a micropipette. Contents of both latex reagent vials were well suspended before use by gently rolling the vials. One drop of reagent 1 was put on one of the sample drops with micropipette. Control suspension was added on to the other drop. Mixture was carefully stirred by using fresh wooden applicator for each drop. The drops were spread to cover the entire area of circle of test slide for interpretation of results. The slide was continuously tilted back and forth and observed against dark background for eventual development of precipitate. The detection of agglutination is done by the naked eye.

The result was considered to be negative if no agglutination was observed in the sample drop containing reagent 1 within 2 mins. Result was considered to be positive if agglutination was detected in sample drop containing reagent 1. Agglutination was either complete resulting in granules and a clear background, or partial when granules could be detected, but the background remained opaque. If the results were doubtful and the test could not be interpreted, the test was repeated. If agglutination was observed in the drop containing control latex reagent, the particular specimen could not be studied by this method as instructed in the procedure with the kit. These samples were further tested by PAGE and cell culture.

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

The method followed for PAGE is a modification of the protocols standardized by AJ Herring *et al* (161) and SM Rodger *et al* (55). The modification was carried out by Krishnan T, Div. of Virology, National Institute for Cholera and Enteric Diseases, Calcutta. This method was considered to be the standard method of detection for rotaviruses against which the other methods of detection were compared. It was considered as the standard because of its high specificity and sensitivity (150).

The procedure for detection of rotaviruses by PAGE is separated into three sections as follows:

- A. Extraction of rotavirus dsRNA from fecal material.
- B. Electrophoresis of dsRNA in polyacrylamide gels.
- C. Silver-staining of dsRNA bands.

A. Extraction of rotavirus dsRNA from fecal samples

The stool samples were vortexed. In case of solid samples, a minimum amount of phosphate buffered saline (PBS) was added to the samples and the mixture was homogenized by vortexing. 0.5ml of the sample was added to 0.5ml of 0.1M sodium acetate buffer with 1% sodium dodecyl sulfate (SDS) in an eppendorf tube. To this mixture was added 0.5ml of phenol-chloroform mixture and vortexed. The tubes were then centrifuged at 10,000 rpm for 10 mins. The aqueous supernatant was collected in fresh eppendorf tubes and refrigerated until tested by PAGE.

B. Electrophoresis of rotavirus dsRNA in PAGE

Bio-Rad electrophoresis apparatus was used for PAGE and a discontinuous system of buffers was used. Separating gel mixture of 10% polyacrylamide was prepared and this solution was poured within glass plates with dimensions 14cm X 16cm. Spacers that were 0.75mm thick separated the glass plates. When the polyacrylamide was set, a 3% stacking gel solution was prepared. This solution was poured over the separating gel till the glass sandwich was filled. A comb was placed onto the glass sandwich. When the gel was set, the comb was removed and the wells were washed with reservoir buffer. Approximately 30 μ l of the extracted supernatants were loaded in the wells. A current of 25mA was applied to each slab gel for 16hrs.

C. Silver staining

After the gel run was over, the spacers were removed from in between the glass plates. The stacking gel was removed and discarded. Also, the bottom

left corner of each gel was cut off for orientation of the gel. The gel was gently lifted off the glass plate and placed in fixing solution in a glass tray. After rocking the tray for 30mins., the fixing solution was drained off and the silver stain was added. The tray was again rocked for 30mins., after which the stain was drained off. The gel was then rinsed with double distilled water to make sure that no traces of the silver stain were left behind. The developing solution was then added and the tray was rocked till the RNA bands were satisfactorily stained. To stop the reaction, the developing solution was drained off and the stopping solution was added. The bands were visualized against white light.

CELL CULTURE

The cell line used for the isolation of rotavirus was MA-104. This is an established cell line derived from the kidneys of embryonic rhesus monkeys. The cell line was supplied by The National Center for Cell Sciences, Pune.

Inoculation of flasks for cell culture:

The cells from the surface of the flask were dislodged by trypsin treatment. The medium from monolayer culture of cells in tissue culture flasks was aspirated. 1ml of trypsin solution was added and the flask was rocked 4-5 times quickly back and forth so that the trypsin coated the cells in the flask, and traces of medium and serum were diluted. The cells were not detached at this stage. The trypsin solution was aspirated. The flask was then kept at 37°C in an incubator for 2-5 mins. Cell detachment was checked by tapping the flask against the hand so that the small amount of fluid in the flask sheared the loosely attached cells off the surface. If no cells were detached, the flask was put back at 37°C for a few more minutes and the procedure was repeated until the cells were completely detached from the flask. The cells were then suspended in growth medium (MEM with 10% FCS) and triturated gently to disrupt cell clumps. The cell number was determined by counting the

cells using a hemocytometer and viability staining was done with trypan blue. The cells were then diluted with growth medium to an approximate concentration of 2×10^4 to 4×10^5 /ml. Aliquots of the cell suspension were then added to flasks containing growth medium. The cell suspension was rotated and evenly distributed on the surface of the culture flasks. The flasks were then incubated at 37°C until the formation of monolayers of cells. The growth medium was changed twice a week.

Isolation of the virus:

The isolation of rotavirus was done in tissue culture flasks and the protocol standardized by Kutsuzawa T *et al* was followed (73). The stool specimens that were positive for rotavirus by PAGE were used for virus inoculation.

The stool specimens were brought to room temperature. A 10% suspension in MEM of the specimens was then prepared. After centrifuging at 10,000rpm for 10mins., the clear supernatant fluids were collected. These supernatants were mixed with an equal amount of 20µg of trypsin per ml for 20 mins. at 37°C. This mixture was then diluted 1:20 with MEM before inoculation. Before these samples were inoculated, confluent MA104 cell monolayers were washed three times with MEM without serum. Excepting the controls, each well was inoculated with 0.1 ml of the diluted mixture. The plates were then incubated at 37°C for 60mins. The cultures were then washed three times with MEM without serum and fed with MEM without serum and containing 0.5µg of trypsin per ml. The flasks were incubated at 37°C. Media were changed every other day. The flasks were inspected everyday for the occurrence of cytopathic effects (CPE). When CPE was observed, the culture flasks were frozen, thawed, and then the culture lysates were centrifuged. The supernatants were collected and tested for the presence of rotavirus by PAGE. When no CPE was observed even after eleven days after inoculation, cultures were lysed by freeze-thawing and supernatants were obtained by centrifugation. These lysates were trypsin treated as described above and inoculated into fresh MA104 cell cultures. This procedure was carried out till

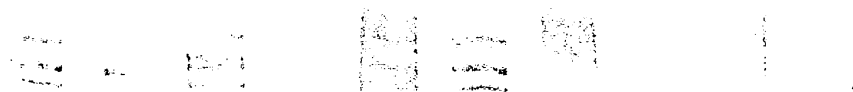
passage 6. All cell lysates were then tested by PAGE after each passage for rotavirus positivity regardless of the demonstration of CPE.

STATISTICAL ANALYSIS

All statistical analysis was done by using the chi square test with Yate's correction (continuity correction).



RESULTS

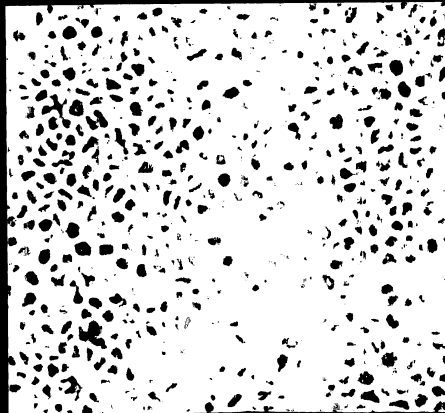


throughout the

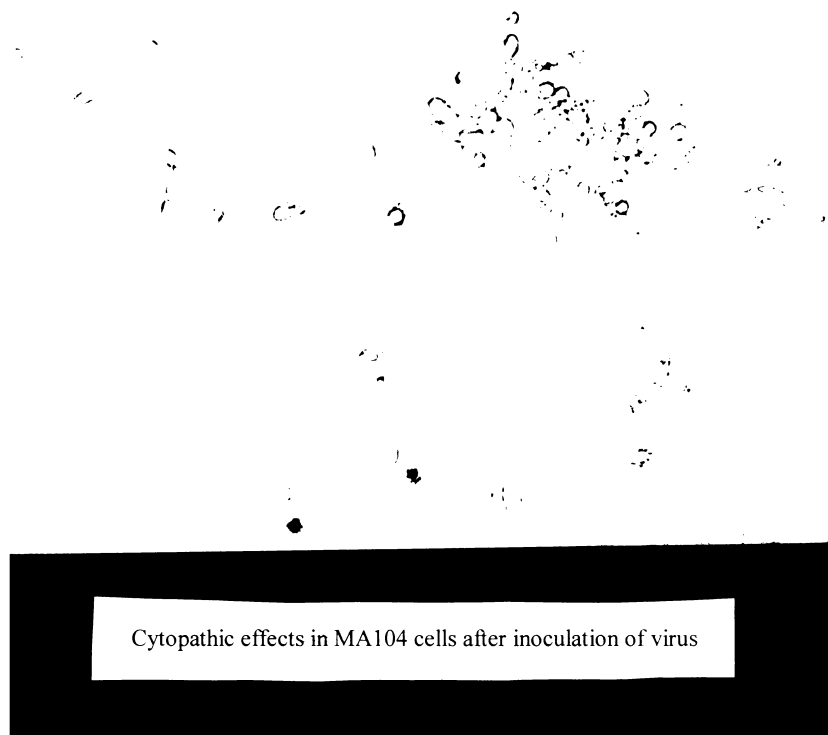
21

Standards: lanes 1 and 10

A light micrograph of MA104 cells in MEM



A light micrograph of a monolayer of MA104 cells



Our study included a total of 225 pediatric diarrhea patients, which comprised the study group, and 25 pediatric patients who were not suffering from diarrhea which comprised the control group.

The demographic characteristics and symptoms of the group suffering from diarrhea is shown in **Table 1**. 59.11% of the patients were males and 40.88% were females.

12.44% of the patients were below the age of 6 months, 47.55% belonged to the age group of 6-12 months, 16% were from the age group of 12-18 months, 9.33% from the age group of 18-24 months, and 14.66% were above 2 years of age.

36% of the diarrhea patients were passing 10-12 stools per day, while 64% of the patients were passing 1-2 stools per hour.

29.33% of the patients vomited 1-2 times per day, 37.77% of the patients showed a vomiting frequency of 10-12 per day, and 12.88% of the patients vomited 1-2 times per hour. 20% of the patients did not suffer from vomiting.

60.88% of the patients suffered from mild fever (upto 100°F), 21.77% of the patients had moderate fever (100-101°F), while 4% of the patients had high fever (>101°F). 13.33% of the patients did not suffer from fever.

Of these patients, 19.55% were admitted to the Lokmanya Tilak Memorial Hospital, 23.55% were admitted to the King Edward Memorial Hospital, and 56.88% were admitted to the Kasturba Hospital for Infectious Diseases.

Age (months)	Sex		Hospital			Total	Frequency of diarrhea			Frequency of vomiting				Gradation of fever			
	Male	Female	LTMH	KEM	KHID		10 - 12/ day	1-2/ hour	Ab- sent	1-2/ day	10 - 12/ day	1-2/ hour	Ab- sent	Mild	Mod- erate	High	No fever
<6	17	11	12	6	10	28	11	17	--	18	2	--	8	17	8	3	--
6-12	65	42	19	24	64	107	47	60	--	40	34	12	21	77	28	2	--
12-18	21	15	3	8	25	36	16	20	--	6	10	11	9	23	7	2	4
18-24	11	10	3	4	14	21	3	18	--	2	12	4	3	7	3	1	10
>24	19	14	7	11	15	33	4	29	--	--	27	2	4	13	3	1	16
Total no. of patients	133	92	44	53	128	225	81	144	--	66	85	29	45	137	49	9	30

Table 1. Demographic characteristics and symptoms of patients
suffering from diarrhea.

DETECTION OF ENTEROPATHOGENS

E.coli was detected in 38.22% of the diarrhea cases. Species of *Vibrio* and *Proteus* were isolated from 8.0% and 4.0% of the cases respectively. Rotavirus was detected in 16.0% of the diarrhea cases, and in none of the controls. The detection of rotavirus was done by PAGE. All the detected cases of rotavirus belonged to group A. All these cases of rotavirus diarrhea showed no growth of any bacteria. An etiologic agent could not be identified in 33.77% of the cases. (Table 2)

Table 2. Enteropathogens detected from diarrhea patients.

Likely etiologic agent detected	Diarrhea cases	Percentage (n=225)	
Only <i>E.coli</i>	86	38.22	
<i>Vibrio</i> species (with and without <i>E.coli</i>)	18	8.0	
<i>Proteus</i> species (with and without <i>E.coli</i>)	9	4.0	
Rotavirus	36	16.0	
No agent isolated	76	33.77	

The bacterial isolates were identified by means of biochemical tests (Table 3).

Table 3. Key for Identification of the Clinical Isolates

Genus/ Species	Biochemical Reactions													
	Mot	Glu	Lac	Suc	Man	Dul	Ind	MR	VP	Cit	Gel	Ure	PPA	Oxl
<i>E.coli</i>	+	AG	AG	AG	AG	AG	+	+	--	--	NL	--	--	--
<i>P.rettgeri</i>	+	A	--	(A)	A	--	+	+	--	+	NL	+	+	--
<i>P.vulgaris</i>	+	AG	--	AG	--	--	+	+	--	+	(L)	+	+	--

Legend :

AG Acid and Gas

A Acid only

+

Positive

--

Negative

Letters in parenthesis indicate variable results

The demographic characteristics and symptoms of the patients who were detected to be rotavirus positive are given in **Table 4**. 63.88% of these patients were male and 36.11% were females.

8.33% of the rotavirus positive patients were under 6 months of age. 55.55% of patients suffering from rotavirus disease belonged to the age group of 6-12 months, 19.44% of the patients were from the age group of 12-18 months, and 5.55% of the patients were from the age group of 18-24 months. 11.11% of the patients were above the age of 2 years.

38.88% of these patients were passing 10-12 stools per day and 61.11% of them were passing about 1-2 stools per hour.

27.77% of the patients vomited 1-2 times a day, 36.11% of the patients vomited 10-12 times a day, and 11.11% of the patients vomited almost 1-2 times per hour. 25% of these patients did not exhibit the symptom of vomiting.

Mild fever was experienced by 58.33% of the patients from this group, 19.44% of them had moderate fever, and 5.55% of the patients suffered from high fever. No fever was experienced by 16.66% of the patients.

Of the 36 patients suffering from rotavirus disease, 22.22% were admitted to the Lokmanya Tilak Memorial Hospital, 27.77% were admitted to the King Edward Memorial Hospital, and 50% of the patients were from the pediatric ward of the Kasturba Hospital for Infectious Diseases.

Age (months)	Sex		Hospital			Total	Frequency of diarrhea			Frequency of vomiting				Gradation of fever			
	Male	Female	LTMH	KEM	KHID		10 - 12/ day	1-2/ hour	Ab- sent	1-2/ day	10 - 12/ day	1-2/ hour	Ab- sent	Mild	Mod- erate	High	No fever
<6	2	1	1	0	2	3	1	2	--	2	--	--	1	--	2	1	--
6-12	13	7	5	6	9	20	8	12	--	3	7	2	8	16	2	1	1
12-18	4	3	0	3	4	7	3	4	--	2	4	1	--	3	2	--	2
18-24	2	--	0	1	1	2	1	1	--	--	2	--	--	--	1	--	1
>24	2	2	2	0	2	4	1	3	--	3	--	1	--	2	--	--	2
Total no. of patients	23	13	8	10	18	36	14	22	--	10	13	4	9	21	7	2	6

Table 4. Demographic characteristics and symptoms of patients suffering from rotavirus diarrhea.

All the samples from patients suffering from diarrhea were also tested by LA for the presence of rotaviruses while only those samples which were positive for rotavirus by PAGE were inoculated onto MA104 cells for the isolation of the virus.

LA detected 33 cases of rotavirus diarrhea, of which 4 were false positive. Also, 7 false negative results were obtained as compared to the results by PAGE (Table 5).

In cell culture, only 19 of the 36 rotavirus samples could be isolated (**Table 5**). CPE was observed in the form of obscure cell borders, cell fusion, rounding of cells, and detachment. The growth of the virus in the cells was also confirmed by running the RNA extracts from MA104 cell lysates on polyacrylamide gels.

Table 5. Correlation of results by PAGE, latex agglutination, and in vitro isolation.

Samples	PAGE positive (standard test)	LA positive	False positive	False negative	Cell culture positive	False negative
Patients	36	33	4	7	19	17
Controls	Nil	Nil	--	--	--	--



DISCUSSION

Diarrhea is by far the major killer among all diseases affecting children worldwide. Acute diarrhea is a very frequent disease in developing countries and is the first cause of death in infants under 2 years of age. The magnitude of the impact of the diarrheic diseases on infantile morbidity and mortality rates in developing countries is represented by the more than one billion of episodes and approximately 3.3 millions deaths/year, respectively (2). Despite the fact that acute diarrhea continues to be an important public health problem in USA, the existence of an efficient and well established health system and high levels of sanitary conditions, as well as adequate food hygiene, have altogether been determinant factors for the drastic reduction in the morbidity and mortality rates brought about by diarrheic diseases, occurring since the beginning of the present century. The current hospital mortality rates have been reduced to levels lower than 2% (162). On the other hand, in the majority of the developing countries of the Americas, Asia and Africa, infantile mortality rates have always been associated with the frequency of diarrheic disease. In those more severe cases that need hospitalization, the risk of perpetuation of diarrhea is beyond the efficacy of oral hydration, since food intolerance and nutritional aggravation are the most frequent clinical complications. Persistence of diarrhea is most probably due to food intolerance after an episode of acute diarrhea presumably induced by an enteropathogenic agent. It is well known that persistence of diarrhea due to food intolerance is associated to malabsorption of nutrients, a very common cause of nutritional aggravation leading to severe malnutrition (163). Persistent diarrhea and malnutrition are the major clinical components of food intolerance as a consequence of the morphological and functional lesions that occur on the small bowel mucosa causing malabsorption of the nutrients of the diet. Thus enteric infection constitutes the triggering factor that generates a vicious cycle represented by persistence of diarrhea, food intolerance and severe protein-calorie malnutrition and malnutrition in its turn leads to decreased immunity and hence increased susceptibility to infections. The

presence of these major clinical complications is responsible for deaths in hospitalized infants due to severe acute diarrhea in developing countries.

In the present study, the study subjects are pediatric patients admitted to three municipal hospitals in Mumbai. Though all these hospitals are in central Mumbai, they serve patients from all over the city. Hence our findings may reflect the rate of incidence of rotavirus infections all over the city. But such a conclusion cannot be drawn based on a study that has taken only 3 major hospitals into consideration. Also, the patients admitted to these hospitals all belong to the lower socio economic strata of society, with low literacy levels, no source of potable water, and poor hygiene and sanitary conditions. Hence, no differentiation in the occurrence or severity of disease could be made based on these factors. The factors that could be used to study the role of the virus in causing diarrhea in children under 5 years of age, and those that are taken into consideration in this study are the age and sex of the affected children and the seasonality of the virus.

It was found that infection with rotavirus was more frequent among infants and children less than two years old, with a maximum incidence among children 13-15 months old (106). Other studies have also reported that rotavirus gastroenteritis severe enough to require hospitalization occurs most frequently in infants and young children from approximately 6 months to 2 years of age (89,164,165). Infants under 6 months of age have been observed to experience the next highest frequency of such illness. In our study, we found the maximum incidence of such disease in the age group of 6-12 months with 55.55% of the rotavirus diarrhea cases occurring in this group. A maximum of cases might have occurred in this group as it is at this age that maternal antibody has just been lost and any passing virus can cause disease. However, the rate of incidence in this group is not significantly higher than the occurrence of rotavirus disease in other age groups. Only 11.11% of rotavirus infections occurred in the age group above 24 months as

compared to 88.89% of infections in the age group below 24 months. However, these figures too are not significant ($\chi^2 = 0.16$, $p>0.05$) as the number of diarrhea patients in the age group under 24 months was also large.

No significant sex-related differences in the incidence of diarrheal disease was reported in a study by Bhan *et al* (111). While this observation was made for diarrheal disease in general, a Delhi based study found rotavirus to be more prevalent in boys than in girls (115). However, bearing in mind the excess of male births over females, the greater general resistance of the female, and the tendency in some communities to lavish more care and attention on males than females, it might be likely that there is no genuine difference between the susceptibility of the two sexes. In contradiction, another study by Nath G *et al* reported that girls (24.1%) were more susceptible to rotavirus infection amongst the diarrheal group in comparison to boys (13.8%) (117). In our study, although we have found that 63.88% of the rotavirus infections have occurred in males, the difference in the occurrence of rotavirus infections in males and females is not significant ($\chi^2 = 0.20$, $p>0.05$).

In the present study, though there is a slight increase in rotavirus disease in the winter months, it is not a significant one when compared to the number of cases occurring in the summer ($\chi^2 = 0.60$, $p>0.05$). The increase of rotavirus incidence in winter is significant when compared to the incidence of rotavirus in the rainy season ($\chi^2 = 4.07$, $p<0.05$). Although the cases of diarrhea in the rainy season were maximum, they had a different etiologic agent. When the number of cases of rotavirus infections in winter was compared with those occurring during the rest of the year, there was no significant difference ($\chi^2 = 3.25$, $p>0.05$). Considering that rotavirus disease is also called “winter disease”, this is surprising. A striking and well-recognized feature of rotavirus epidemiology is the annual peak of infection and illness in winter. But this is the case only in the temperate zones. Data from 34 studies of the etiology of

childhood diarrhea were compiled in order to investigate the seasonal patterns of rotavirus gastroenteritis (132). This report found that the incidence of rotavirus infections peaked in winter primarily in the Americas and that peaks in the autumn or spring are common in other parts of the world. In the tropics, the seasonality of such infections is less distinct and within 10 degrees latitude of the equator, some locations exhibited no trend. Throughout most of the world, rotavirus is present all the year round, which suggests that low-level transmission could maintain the chain of infection. A study carried out in hospitalized children in Delhi reported a peak of rotavirus infections in the early winter months (115). Diarrhea due to rotavirus was found to be more prevalent during the cooler months of November to February by another study carried out in an urban slum population (117). More of rotavirus diarrheal episodes were observed to occur during the winter season by Yaccha *et al* in a study carried out in Chandigarh (112). Even in Mumbai, a study has reported the increase in prevalence of rotavirus in cooler months of the year (120). November and December are the cooler months of the year when the maximum and minimum temperatures are low and humidity is also low. It has been found that there are more number of rotavirus cases as described above in these months. However, in our study we have found no significant increase in the occurrence of rotavirus disease during the winter months. This finding is in concurrence with that reported by Desikan *et al* in their study carried out in Bangladesh (166). This recent study has found no seasonal variation in the incidence of infection.

Other organisms detected in the patients in our study were *E.coli* and species of *Proteus* and *Vibrio*. A study by Bhan *et al* has reported the isolation of various bacteria from the stool specimens of children suffering from diarrhea (111). The children suffering from the disease were divided into two groups, namely, those suffering from persistent diarrhea and those suffering from acute diarrhea. Established enteric pathogens were isolated during the initial illness in 46.4% of persistent and 55.4% of acute diarrhea episodes.

Pathogens isolated during persistent episodes included enterotoxigenic *Escherichia coli* (ETEC) (9.3%), *Salmonella* species (4.7%), as well as *Campylobacter* (4.7%), *Shigella* species (2.3%) and *Entamoeba histolytica* (2.3%). Similar proportions of these pathogens were isolated also during episodes of acute diarrhea. Multiple pathogens were isolated in 7% of the persistent and 5% of the acute episodes. In a similar study carried out in Kenya, bacterial diarrhea was found in 27.7% of diarrhea patients under 5 years of age (104). The bacterial pathogens included *E. coli*, *Salmonella* species, and *Shigella* species. Intestinal parasites were found in 12.6% of the cases. Rotavirus was found in 16.1% of the cases. In our study, we have detected *E. coli* in a majority of cases (38.22%). However, since we were not able to type the strains detected by us, it cannot be said whether the isolated *E. coli* strains were the normal flora or were pathogenic. The other bacteria isolated were *Vibrio* species (8.0%) and *Proteus* species (4.0%). Rotavirus was detected in 16.0% of the samples. No mixed infection of rotavirus along with any bacteria was observed. An astonishing 33.77% of the samples showed no growth of any bacteria including normal flora of the gastrointestinal tract. This might be due to the drug therapy that the patients had undergone. Antibiotics like Ciprofloxacin, Ampicillin, Tetracycline, etc. are used as the first step of defense against enteropathogens. Indiscriminate use of antibiotics even before consulting a physician may have led to the complete destruction of the intestinal organisms. The etiologic agents in these 33.77% samples could be any of the other viruses causing gastroenteritis like adenoviruses, caliciviruses, coronaviruses, etc. Protozoa or parasites may also be responsible for the intestinal disease of these patients.

The rate of incidence of rotavirus observed in this study is less than that of 23% observed by Desai *et al* in Mumbai a few years back (120). But it is still high compared to some other areas in India and will continue to remain so until living conditions improve (111,112,113,114).

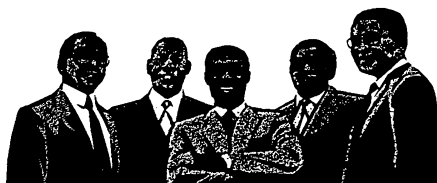
We found that the presenting symptoms of patients suffering from rotavirus diarrhea and those suffering from diarrhea which was not due to rotavirus were not distinctive. The frequency of diarrhea and vomiting as well as gradation of fever exhibited by patients from both groups was almost similar. Hence, diagnosis of rotavirus infection cannot be made on the basis of the symptoms alone. An appropriate diagnostic method is necessary.

In a comparative study of various methods for detecting human rotaviruses from stool specimens, Hendricks *et al* found that PAGE and EM were 100% specific while the specificity of latex agglutination was 81% (109). In this study, 103 children were assessed for human rotavirus infection in Bangkok. PAGE was found to be 90% sensitive, EM 84%, and latex agglutination 80%. Another study by Chakravarti *et al* carried out in children below 2 years of age reported the sensitivity of latex agglutination and PAGE to be 91.4% and 80% respectively, the corresponding specificity was 98.18% and 100%, respectively (167).

In our study we have found the rate of incidence of rotavirus to be 16.0% by PAGE. Since all the detected rotaviruses belonged to group A, we can safely assume that rotavirus infection in Mumbai as of yet is by the conventional strain. However, the absence of any atypical rotaviruses should not stop us from carrying out routine surveillance in order to keep a check on the strains of rotavirus circulating in the community. We have considered PAGE to be the standard one because its sensitivity is more than 90% and its specificity is 100% (150). Compared to this method, LA gave 4 false positive results and 7 false negative results, thereby giving a sensitivity of 87.87% and specificity of 97.88%. LA can therefore be used as a bedside method for preliminary testing of stools for rotavirus. However, results from this test need to be confirmed by another method. Also, viruses belonging to groups B and C go undetected by this method of diagnosis.

Another method of diagnosis that we have tested is isolation of the virus in cell culture. 17 false negative results were obtained; therefore the sensitivity of this method was calculated to be 52.77%. The specificity of this method could not be checked as only samples that were positive for rotavirus by PAGE were tested by this method. This method has proved to be cumbersome as after every passage of the virus in cells, PAGE had to be used to confirm the presence or absence of the virus as CPE is not always visible and hence is not very useful as a diagnostic tool. It is more suited to a research setting. Various studies have reported the efficiency of rotavirus detection by this method to be about 75% as compared to antigen detection test procedures (71,168,169).

PAGE has been used to detect rotaviruses in many studies and PAGE of rotavirus can provide information on variation in rotavirus strain prevalent in the community. A surveillance of this type can keep a check on the simultaneous co-circulation of multiple strains in the community, which may lead to extensive genomic variation in rotavirus strains. This information is vital for vaccine development. Also in developing countries like India, where the cost of diagnostic kits like EIAs is prohibitive, PAGE becomes a handy tool in the hands of laboratory technicians. Besides being extremely sensitive and specific, it is also inexpensive with no requirement for fancy reagents.



SUMMARY
AND
CONCLUSION

Since this study covers a period of only two years and is carried out in a limited locality and socioeconomic strata of the city, it is only suggested that rotavirus may be one of the commonest causes of acute gastroenteritis in children younger than five years.

This study was carried out from March 1998 to January 2000. In this period, of the total 225 diarrhea samples collected, 36 samples were found to be positive for rotavirus by PAGE, giving rotavirus positivity of 16%.

It was found that all the detected rotaviruses belonged to group A. No atypical rotaviruses were observed.

The age group of 6-12 months was found to be most susceptible to infection with 55.55% of the rotavirus cases occurring in this age group. Overall, the age group upto 24 months was more susceptible (88.88%) than the age group from 24 months to 5 years (11.11%).

Although rotavirus disease was recorded around the year, a slight seasonal variation was observed. 47.22% of the observed infections took place during the months of November to February.

E.coli was isolated from a majority of the 225 samples (38.22%). Other bacteria identified belonged to the *Vibrio* and *Proteus* species. 49.77% of the test samples showed no growth of bacteria out of which 16.0% tested positive for rotavirus. No etiologic agent could be identified for the remaining 33.77% of the patients.

Of the existing diagnostic methods, we found PAGE to be the most appropriate method taking into consideration its cost effectiveness and accuracy.

Rapid progress towards the development of rotavirus vaccines has prompted a reassessment of the disease burden of rotavirus diarrhea in developing countries and the possible impact of these vaccines in reducing diarrheal morbidity and mortality among infants and young children. Countries like India should, therefore, have an active and systematic surveillance for detection and epidemiological study of rotavirus infection.

APPENDIX

ISOLATION OF BACTERIA

Nutrient agar, MacConkey's agar, and Salmonella-Shigella agar were obtained from HI media in powdered form and were prepared according to instructions.

Reagents for biochemical tests were prepared according to standard protocol.

ISOLATION OF VIRUS

Minimum Essential Medium (MEM)

MEM powder with Earle's salts, L-glutamine and non-essential amino acids (Gibco cat. no. 4150-034) was dissolved in 900ml of deionised water. 2.2 g of NaHCO_3 was added, Penicillin, Streptomycin, and Kanamycin solution was added to a concentration of 200u/ml. pH was adjusted to 7.2 with 1N HCl and 1N NaOH. The volume was brought to 1000ml by adding water. The medium was then filter sterilized using a membrane with a porosity of 0.22 microns. The filtered medium was aseptically dispensed into sterile bottles and stored at 4°C till use.

Trypsin solution

200 mg of Trypsin, 400mg of EDTA, and 100mg of glucose were dissolved in 180ml of PBS. The pH was adjusted to 7.5 by 1N NaOH. 0.02ml of 0.1% Phenol red was added, and the volume was brought to 200 ml with PBS. This solution was then filter sterilized and stored at 4°C.

Phosphate buffered saline (PBS)

NaCl	: 8g
KCl	: 0.4g
$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	: 1.77g
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	: 0.69g
Glucose	: 2g
Phenol red	: 0.01g

Make volume to 1000ml with distilled water and filter sterilize.

ELECTROPHEROTYPING OF VIRUS

Sodium acetate buffer with 1% SDS (pH 5.0)

0.1M glacial acetic acid	: 60ml
Anhydrous sodium acetate	: 1.15g
SDS	: 2g
Distilled water	: 140ml

Phenol-chloroform mixture

Redistilled phenol	: 100ml
Chloroform	: 64ml
Isoamyl alcohol	: 2.67g
8-hydroxy-quinoline	: 0.25g

Separation gel buffer (4X)

Tris base : 18.17g
Dissolve in distilled water and adjust pH to 8.8 with 12N HCl, and make final volume to 100ml.

Stacking gel buffer (4X)

Tris base : 6.6g
Dissolve in distilled water and adjust pH to 6.8 with 12N HCl, and make final volume to 100ml.

Tris Glycine reservoir buffer (4X)

Tris base : 12g
Glycine : 57.6g
Dissolve in distilled water and adjust pH to 8.3 with 12N HCl, and make final volume to 1000ml.

Sample buffer (4X)

Stacking gel buffer	: 7.5ml
Glycerol	: 2.5ml
Bromophenol blue	: 10mg

Acrylamide solution (30%)

Acrylamide : 30g
Methylene bisacrylamide : 0.8g
Dissolve in 100ml distilled water.

Separation gel (10%)

Distilled water	: 8.06ml
Separation gel buffer	: 5.9ml
Acrylamide solution	: 6.65ml
TEMED	: 5 μ l
2% ammonium persulfate	: 0.3ml

Stacking gel (3%)

Distilled water	: 3.1ml
Stacking gel buffer	: 1.25ml
Acrylamide solution	: 0.5ml
TEMED	: 5 μ l
2% ammonium persulfate	: 150 μ l

Fixing solution

Distilled water	: 170ml
Ethanol	: 20ml
Acetic acid	: 10ml

Silver stain

Silver nitrate	: 0.37g
Distilled water	: 200ml

Developing solution

Sodium hydroxide	: 7.5g
Distilled water	: 200ml

Stopping solution

Acetic acid	: 10ml
Distilled water	: 190ml



BIBLIOGRAPHY

1. Snyder JD and Merson MH.
The magnitude of the global problem of acute diarrhoeal disease: a review of the active surveillance data.
Bull WHO 1982;60:605-613.
2. Bern C, Martines J, de Zoysa I, Glass RI.
The magnitude of the global problem of diarrhoeal disease: a ten year update.
Bull WHO 1992;70:705-714.
3. Rohde J, Northrup RS.
Diarrhoea: a nutritional disease.
JIMA 1987;7(85):196-202.
4. Thornton A, Zuckerman AJ.
The virus of acute diarrhoea.
Nature 1975;254:557-558.
5. Kapikian AZ, Wyatt RG, Dolin R, Thornhill TS, Kalica AR, Chanock RM.
Visualization by immune electron microscopy of a 27 nm particle associated with acute infectious non-bacterial gastroenteritis.
J Virol 1972;10:1075-1081.
6. Bishop RF, Davidson GP, Holmes IH, Ruck BJ.
Virus particles in epithelial cells of duodenal mucosa from children with viral gastroenteritis.
Lancet 1973;2:1281-1283.
7. Kapikian AZ, Cline WL, Mebus CA, *et al.*
New complement-fixation test for the human reovirus-like agent of infantile gastroenteritis. Nebraska calf diarrhea virus used as antigen.
Lancet 1975;1:1056-1061.
8. Kapikian AZ, Cline WL, Kim HW, *et al.*
Antigenic relationships among five reovirus-like (RVL) agents by complement fixation (CF) and development of new substitute CF antigens for the human RVL agent of infantile gastroenteritis.
Proc Soc Exp Biol Med 1976;152:535-539.
9. Yolken RH, Kim HW, Clem T, *et al.*
Enzyme-linked immunosorbent assay (ELISA) for detection of human reovirus-like agent of infantile gastroenteritis.
Lancet 1977;2:263-267.

10. Flewett TH, Woode GN.
The rotaviruses.
Arch Virol 1978;57:1-23.
11. Hung T, Chen G, Wang C, et al.
Waterborne outbreak of rotavirus diarrhea in adults in China caused by a novel rotavirus.
Lancet 1984;i:1139-1142.
12. Hung T, Chen G, Wang C, et al.
Seroepidemiology and molecular epidemiology of the Chinese rotavirus.
In: Bock G, Whelan J, eds. Novel diarrhea viruses. (Ciba Foundation Symposium;128). Chichester, UK: John Wiley & Sons, Ltd., 1987;49-62.
13. Bridger JC, Pedley S, McCrae MA.
Group C rotaviruses in humans.
J Clin Microbiol 1986;23:760-763.
14. Penaranda ME, Cubitt WD, Sinarachatanant P, et al.
Group C rotavirus infections in patients with diarrhea in Thailand, Nepal, and England.
J Infect Dis 1989;160:392-397.
15. Krishnan T, Sen A, Choudhary JS, Das S, Naik TN, Bhattacharya SK.
Emergence of adult diarrhea rotavirus in Calcutta.
Lancet 1999;353(9150):380-381.
16. Mata L, Simhon A, Urrutia JJ, Kronmal RA, Fernandez R, Garcia B.
Epidemiology of rotaviruses in a cohort of 45 Guatemalan Mayan Indian children observed from birth to the age of three years.
J Infect Dis 1983;148:452-461.
17. Velazquez FR, Matson DO, Calva JJ, et al.
Rotavirus infections in infants as protection against subsequent infections.
N Engl J Med 1996;335:1022-1028.
18. Ward RL, Berntein DI, and the US Rotavirus Vaccine Efficacy Group.
Protection against rotavirus disease after natural rotavirus infection.
J Infect Dis 1994;169:900-904.
19. Wyatt RG, Mebus CA, Yolken RH, et al.
Rotaviral immunity in gnotobiotic calves: heterologous resistance to human virus induced by bovine virus.
Science 1979;203:548-550.

20. Vesikari T, Isolauri E, Delem A, D'Hondt E, Andre FE, Zissis G. Immunogenicity and safety of live oral attenuated bovine rotavirus vaccine strain RIT 4237 in adults and young children. *Lancet* 1983;2:807-811.
21. Vesikari T, Isolauri E, D'Hondt E, Delem A, Andre FE, Zissis G. Protection of infants against rotavirus diarrhoea by RIT 4237 attenuated bovine rotavirus strain vaccine. *Lancet* 1984;1:977-981.
22. Clark HF, Borian FE, Bell LM, Modesto K, Gouvea V, Plotkin SA. Protective effects of WC3 vaccine against rotavirus diarrhoea in infants during a predominantly serotype 1 rotavirus season. *J Infect Dis* 1988;158:570-587.
23. Vesikari T, Kapikian AZ, Delem A, Zissis G. A comparative trial of rhesus monkey (RRV-1) and bovine (RIT 4237) oral rotavirus vaccines in young children. *J Infect Dis* 1986;153:832-839.
24. Midthun K, Greenberg HB, Hoshino Y, Kapikian AZ, Wyatt RG, Chanock RM. Reassortant rotaviruses as potential live rotavirus vaccine candidates. *J Virol* 1985;53:949-954.
25. CDC. Rotavirus vaccine for the prevention of rotavirus gastroenteritis among children – recommendations of the Advisory Committee on Immunization Practices. *MMWR* 1999;48 (no.RR-2).
26. CDC. Intussusception among recipients of rotavirus vaccine – United States, 1998-1999. *MMWR* 1999;48(no.27).
27. Kapikian AZ, Chanock RM. Rotaviruses. In: Fields BN, Knipe DM, Howley PM, et al, eds. *Fields Virology*. 3rd ed. Philadelphia: Lippincott-Raven, 1996:1657-1708.
28. Adams WR, Kraft LM. Epizootic diarrhoea of infant mice: identification of the etiologic agent. *Science* 1963;141:359-360.

29. Malherbe HH, Harwin R, Ulrich M.
The cytopathic effect of vervet monkey viruses.
S Afr Med J 1963;37:407-411.
30. Banfield WG, Kasnie G, Blackwell JH.
Further observations on the virus of epizootic diarrhea of infant mice.
An electron microscopic study.
Virology 1968;36:411-421.
31. Malherbe HH, Strickland-Cholmley M.
Simian virus SA-11 and the related "O" agent.
Arch Ges Virusforsch 1967;22::235-245.
32. Mebus CA, Kono M, Underdahl NR, Twiehaus MJ.
Cell culture propagation of neonatal calf diarrhea (scours) virus.
Can Vet J 1971;12:69-72.
33. Femelius AL, Ritchie AE, Classick LG, Norman JO, Mebus CA.
Cell culture adaptation and propagation of a reovirus-like agent of calf
diarrhea from a field outbreak in Nebraska.
Arch Ges Virusforsch 1972;37:114-130(227).
34. Kapikian AZ.
Norwalk and Norwalk-like viruses.
*In: Kapikian, ed. Viral infections of the gastrointestinal tract, 2nd ed. New
York: Marcel Dekker, 1994;471-518(434).*
35. Bishop RF, Davidson GP, Holmes IH, Ruck BJ.
Detection of a new virus by electron microscopy of fecal extracts from
children with acute gastroenteritis.
Lancet 1974;2:1281-1283(66).
36. Flewett TH, Bryden AS, Davies H.
Virus particles in gastroenteritis.
Lancet 1973;2:1497(232).
37. Kapikian AZ, Kim HW, Wyatt RG, et al.
Reovirus-like agent in stools: association with infantile diarrhea and
development of serologic tests.
Science 1974;185:1049-1053(453).
38. Middleton PJ, Syzmanski MT, Abbott GD, Bortolussi R, Hamilton JR.
Orbivirus acute gastroenteritis of infancy.
Lancet 1974;1:1941-1944(583).

39. Kapikian AZ.
Viral gastroenteritis.
J Am Med Assoc 1993;269:627-630(432).
40. Huilan S, Zhen LG, Mathan MM, *et al*.
Etiology of acute diarrhoea among children in developing countries: a multicentre study in five countries.
Bull WHO 1991;69(5):549-555).
41. Flewett TH, Davies H, Bryden AS, Robertson MJ.
Diagnostic electron microscopy of feces. II. Acute gastroenteritis associated with reovirus-like particles.
J Clin Pathol 1974;27:603-614(235).
42. Much D, Zajac I.
Purification and characterization of epizootic diarrhea of infant mice virus.
Infect Immun 1972;6:1019-1024(600).
43. Flewett TH, Bryden AS, Davies H, Woode GN, Bridger JC, Derrick JM.
Relation between viruses from acute gastroenteritis of children and newborn calves.
Lancet 1974;2:61-63(101*).
44. Prasad BW, Wang GJ, Clerx JP, Chiu W.
Three-dimensional structure of rotavirus.
J Mol Biol 1988;199:269-275(253).
45. Shaw AL, Rothnagel R, Chen D, Ramig RF, Chiu W, Venkataraman Prasad BV.
Three-dimensional visualization of the rotavirus hemagglutinin structure.
Cell 1993;74:693-701(285).
46. Yeager M, Berriman JA, Baker TS, Bellamy AR.
Three-dimensional structure of the rotavirus hemagglutinin VP4 by cryo-electron microscopy and difference map analysis.
EMBO J 1994;13:1011-1018(337*).
47. Flewett TH, Davies H, Bryden AAS, Robertson MJ.
Diagnostic electron microscopy of feces. II. Acute gastroenteritis associated with reovirus-like particles.
J Clin Pathol 1974;27:603-614(236).

48. Zissis G, Lambert JP.
Different serotypes of human rotaviruses.
Lancet 1978;1:39
49. Yolken RH, Wyatt RG, Zississ G, *et al.*
Epidemiology of human rotavirus types 1 and 2 as studied by enzyme linked immune sorbent assay.
N Engl J Med 1978;299:1156-1161.
50. Hoshino Y, Wyatt RG, Greenberg HB, Flores J, Kapikian AZ.
Serotypic similarity and diversity of rotaviruses of mammalian and avian origin as studied by plaque-reduction neutralization.
J Infect Dis 1984;149:694-702.
51. W.H.O. Steering Committee of the Scientific Working Group on Viral Diarrheas.
Nomenclature of human rotaviruses: designation of sub-groups and serotypes.
Bull WHO 1984;62:501-503.
52. Kapikian AZ, Cline WL, Greenberg HB, *et al.*
Antigenic characterization of human and animal rotaviruses by immune adherence hemagglutination assay (IAHA).
Infect Immun 1981;33:415-425.
53. Woode GN, Bridger JC, Jones JM, *et al.*
Morphological and antigenic relationships between viruses (rotaviruses) from acute gastroenteritis of children, calves, piglets, mice and foals.
Infect Immun 1976;14:804-810.
54. Kalica AR, Greenberg HB, Wyatt RG, *et al.*
Genes of human (strain Wa) and bovine (strain UK) rotaviruses that code for neutralization and subgroup antigens.
Virology 1981;112:385-390.
55. Rodger SM, Holmes IH.
Comparison of the genomes of simian, bovine, and human rotaviruses by gel electrophoresis and detection of genomic variation among bovine isolates.
J Virol 1979;30:839-846.
56. Obijeski JF, Palmer EL, Martin ML.
Biochemical characterization of infantile gastroenteritis virus (IGV).
J Gen Virol 1977;34:485-497.

57. Rodger SN, Schnagl RD, Holmes IH.
Biochemical and biophysical characterization of diarrhea viruses of human and calf origin.
J Virol 1975;16:1229-1235.
58. Todd D, McNulty MS.
Biochemical studies on a reovirus-like agent (rotavirus) from lambs.
J Virol 1977;21:1215-1218.
59. Kalica AR, Greenberg HB, Espejo RT *et al.*
Distinctive ribonucleic acid patterns of human rotavirus subgroups 1 and 2.
Infect Immun 1981;33:958-961.
60. Kutsuzawa T, Konno T, Suzuki H, Ebina T, Ishida N.
Two distinct electrophoretic migration patterns of RNA segments of human rotaviruses prevalent in Japan in relation to their serotypes.
Microbiol Immunol 1982;16:727-730.
61. Albert MJ, Unicomb LB, Bishop RE.
Cultivation and characterization of human rotaviruses with "super short" RNA patterns.
J Clin Microbiol 1987;25:183-185.
62. Pocock DH.
Isolation and characterization of two group A rotavirus with unusual genome profiles.
J Gen Virol 1987;68:653-660.
63. Kalica AR, Wyatt RG, Kapikian AZ.
Detection of differences among human and animal rotaviruses, using analysis of viral RNA.
J Am Vet Med Assoc 1978;173:531-537.
64. Beards GM.
Polymorphism of genomic RNAs within rotavirus serotypes and subgroups.
Arch Virol 1982;74:65-70.
65. Flores J, Perez I, White L, *et al.*
Genetic relatedness among human rotaviruses as determined by RNA hybridization.
Infect Immun 1982;37:648-655.

66. Gerna G, Arista S, Passarani N, Sarasini A, Battaglia M.
Electropherotype heterogeneity within serotypes of human rotavirus strains circulating in Italy.
Arch Virol 1987;95:129-135.
67. Albert MJ, Bishop RF.
Cultivation of human rotaviruses in cell culture.
J Med Virol 1984;3:377-383.
68. Albrey MB, Murphy AM.
Rotavirus growth in bovine monolayers.
Lancet 1976;1:753.
69. Wyatt RG, James WD, Bohl EH, *et al.*
Human rotavirus type 2: cultivation *in vitro*.
Science 1980;207:189-191.
70. Clark SM, Roth JR, Clark ML, Barnett BB, Spendlove RS.
Trypsin enhancement of rotavirus infectivity: mechanism of enhancement.
J Virol 1981;39(3):816-822.
71. Sato K, Inaba Y, Shinozaki T, Fujii R, Matumoto M.
Isolation of human rotavirus in cell cultures.
Arch Virol 1981;69:155-160.
72. Wyatt RG, James Jr. HD, Pittman AL, *et al.*
Direct isolation in cell culture of human rotaviruses and their characterization into four serotypes.
J Clin Microbiol 1983;18(2):310-317.
73. Kutsuzawa T, Konno T, Suzuki H, Kapikian AZ, Ebina T, Ishida N.
Isolation of human rotavirus subgroups 1 and 2 in cell culture.
J Clin Microbiol 1982;16(4):727-730.
74. Superti F, Tinari A, Baldassarri L, Donelli G.
HT-29 cells: A new substrate for rotavirus growth.
Arch Virol 1991;116:159-173.
75. Shinozaki K, Yamanaka T, Tokieda M, Shirasawa H, Simizu B.
Isolation and serial propagation of human group C rotaviruses in a cell line (CaCo-2).
J Med Virol 1996;48(1):48-52.

76. Dryden KA, Wag G, Yeager M, *et al.*
Early step in reovirus infection are associated with dramatic changes in supramolecular structure and protein conformation: analysis of virions and subviral particles by cryoelectron microscopy and image reconstruction.
J Cell Biol 1993;122:1023-1041.
77. Bridger JC, Woode GN.
Characterization of two particle types of calf rotavirus.
J Gen Virol 1976;31:245-250.
78. Estes MK, Palmer EL, Obijeski JF.
Rotaviruses: a review.
Curr Top Microbiol Immunol 1983;105:123-184.
79. Weiss C, Clark HF.
Rapid inactivation of rotaviruses by exposure to acid buffer or acidic gastric juice.
J Gen Virol 1985;66:2725-2730.
80. Welch AB, Thompson TL.
Physicochemical characterization of a neonatal calf diarrhea virus.
Can J Compr Med 1973;37:295-301.
81. Shirley MJ, Beards GM, Thouless ME, Flewett TH.
The influence of divalent cations on the stability of human rotavirus.
Arch Virol 1981;67:1-9.
82. Bastardo JW, Holmes IH.
Attachment of SA-11 rotavirus to erythrocyte receptors.
Infect Immun 1980;29:1134-1140.
83. Estes MK, Graham DY, Smith EM, Gerba CP.
Rotavirus stability and inactivation.
J Gen Virol 1979;43:403-409.
84. Sattar SA, Raphael RA, Lochnan H, Springthorpe VS. Rotavirus inactivation by chemical disinfectants and antiseptics used in hospitals.
Can J Microbiol 1983;29:1464-1469.
85. Holmes IH, Ruck BJ, Bishop RF, Davidson GP.
Infantile enteritis viruses morphogenesis and morphology.
J Virol 1975;16:937-943.

86. Moon HW.
Pathophysiology of viral diarrhea.
In: AZ Kapikian, ed. Viral infections of the gastrointestinal tract. New York: Marcel Dekker Inc., 1994;24-52.
87. Mebus CA, Wyatt RG, Kapikian AZ.
Intestinal lesions induced in gnotobiotic calves by the virus of human infantile gastroenteritis.
Vet Pathol 1977;14:273-282.
88. Uhnnoo IS, Freiherst J, Riepenhoff-Talty M, Fisher JE, Ogra PL.
Effect of rotavirus infection and malnutrition on uptake of a dietary antigen in the intestine.
Pediatr Res 1990;27:153-160.
89. Davidson GP, Bishop RF, Townlee RR, Holmes IH, Ruck BJ.
Importance of a new virus in acute sporadic enteritis in children.
Lancet 1975;1:242-245.
90. Crouch CF, Woode GN.
Serial studies of virus multiplication and intestinal damage in gnotobiotic piglets infected with rotavirus.
J Med Microbiol 1978;11:325-334.
91. Cameron DJS, Bishop RF, Davidson GP, Townley RRW, Holmes IH, Ruck BJ.
New virus associated with diarrhea in neonates.
Med J Aust 1976;1(4):85-86.
92. Cameron DJS, Bishop RF, Veenstra AA, Barnes GL, Holmes IH, Ruck BJ.
Pattern of shedding of two non-cultivable viruses in stools of newborn babies.
Lancet 1978;2:7-13.
93. Rodriguez WJ, Kim HW, Arrobio JO, *et al.*
Clinical features of acute gastroenteritis associated with human reovirus-like agent in infants and young children.
J Pediatr 1977;91:188-193.
94. Rodriguez WJ, Kim HW, Arrobio JO, *et al.*
Clinical features of acute gastroenteritis associated with human reovirus-like agent in infants and young children.
J Pediatr 1977;91:188-193.

95. Tallett S, Mackenzie RN, Middleton P, Kerzner B, Hamilton R.
Clinical, laboratory and epidemiological features of viral gastroenteritis in infants and children.
Pediatrics 1977;60:217-222.
96. Mueller FM, Oender G, Kamin W, Gutjahr P, Schmitt HJ.
Role of salmonellae and rotaviruses in 1337 cases of diarrhoea in the pediatric department of Mainz University.
Klin Paediatr 1993;205(1):9-13.
97. Rotavirus surveillance - United States, 1989-1990.
MMWR 1991;40(5):80-81.
98. Laboratory-based surveillance for rotavirus – United States, July 1997-June 1998.
MMWR 1998;47(45):978-980.
99. Bern C, Glass RI.
Impact of diarrheal diseases worldwide.
In: Kapikian AZ, ed. Viral infections of the gastrointestinal tract, 2nd ed. New York: Marcel Dekker, Inc., 1994;1:-26.
100. Ho MS, Glass RI, Pinsky PF, Anderson LL.
Rotavirus as a cause of diarrheal morbidity and mortality in the United States.
J Infect Dis 1988;158:1112-1116.
101. Ho MS, Glass RI, Pinsky PF, *et al.*
Diarrheal deaths in American children. Are they preventable?
JAMA 1988;260:3281-3285.
102. Institute of Medicine.
Prospects for immunizing against rotavirus.
In: New vaccine development. Establishing priorities. Diseases of importance in the United States, vol 1. Washington, DC: National Academy Press, 1985;410-423.
103. Shukry S, Zaki AM, Shoukry I, Tagi ME, Hamed Z.
Detection of enteropathogens in fatal and potentially fatal diarrheas in Cairo, Egypt.
J Clin Microbiol 1986;24:959-962.
104. Saidi SM, Iijima Y, Sang WK, *et al.*
Epidemiological study on infectious diarrhoeal diseases in children in a coastal rural area of Kenya.
Microbiol Immunol 1997;41:773-778.

105. The microbial aetiology of summer paediatric gastroenteritis at Ga-Rankuwa hospital in South Africa.
East Afr Med J 1993;70(2):78-81.
106. El-Assauli SM, Banjar ZM, Mohammed KA, Zamakhchari FT.
Rotavirus infection in children in Saudi Arabia.
Am J Trop Med Hyg 1992;46(2):272-277.
107. Guerrant RL, Kirchoff LV, Schields DS *et al*.
Prospective study of diarrheal illnesses in Northeastern Brazil: patterns of disease, nutritional impact, etiologies, and risk factors.
J Infect Dis 1983;148:986-997.
108. Jinming Z, Boqin Q, Tongxing Z.
Detection of diarrhoea viruses in children with acute gastroenteritis.
Clin J Exp Clin Virol 1995;9(1):45-49.
109. Hendricks MK, Cuevas LE, Hart CA.
Rotavirus diarrhoea in Thai infants and children.
Ann Trop Paediatr 1995;15(2):147-152.
110. Black RE, Merson MH, Mizanur Rahman ASM, *et al*.
A 2 year study of bacterial, viral and parasitic agents associated with diarrhea in rural Bangladesh.
J Infect Dis 1980;142:660-664.
111. Bhan MK, Bhandari N, Sazawal S, *et al*.
Descriptive epidemiology of persistent diarrhoea among young children in rural northern India.
Bull WHO 1989;67(3):281-288.
112. Yaccha SK, Singh V, Kanwar SS, Mehta S.
Epidemiology, subgroups and serotypes of rotavirus diarrhoea in north Indian communities.
Indian Pediatr 1994;31(1):27-33.
113. Gangopadhyay NN, Naik TN, Sengupta PG, Deb BC, Pal SC.
Genomic diversity and low prevalence of rotavirus infection in a rural Indian community.
Ind J Virol 1991;7(1):16-21
114. Patwari AK, Srinivasan A, Diwan N, Aneja S, Anand VK, Peshin S.
Rotavirus as an aetiological organism in acute watery diarrhoea in Delhi children: reappraisal of clinical and epidemiological characteristics.
J Trop Pediatr 1994;40(4):214-218.

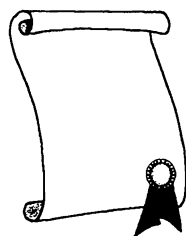
115. Chakravarti A, Kumar S, Mittal SK, Broor S.
Clinical and epidemiological features of acute gastroenteritis caused by human rotavirus subgroups.
J Diarrh Dis Res 1992;10(1):21-24.
116. Singh V, Broor S, Mehta S, Mehta SK.
Clinical and epidemiological features of acute gastroenteritis associated with human rotavirus subgroups 1 and 2 in northern India.
Ind J Gastroenterol 1989;8(1):23-25.
117. Nath G, Singh SP, Sanyal SC.
Childhood diarrhoea due to rotavirus in a community.
Ind J Med Res 1992;95:259-262.
118. Brown DW, Mathan MM, Mathew M, Martin R, Beards GM, Mathan VI.
Rotavirus epidemiology in Vellore, south India: group, subgroup, serotype, and electropherotype.
J Clin Microbiol 1988;26(11):2410-2414.
119. Singh PB, Sreenivasan MA, Pavri KM.
Viruses in gastroenteritis in children in Pune, India.
Epidemiol Infect 1989;102(2):345-353.
120. Desai HS, Banker DD.
Rotavirus infection among children in Bombay.
Ind J Med Sci 1993;47(2):27-33.
121. Santosham M, Yolken RH, Wyatt RG, *et al.*
Epidemiology of rotavirus diarrhea in a prospectively monitored American Indian population.
J Infect Dis 1985;152:778-783.
122. Kapikian AZ, Wyatt RG, Levine MM, *et al.*
Oral administration of human rotavirus to volunteers: induction of illness and correlates of resistance.
J Infect Dis 1983;147:95-106.
123. Blacklow NR, Echeverria P, Smith DA.
Serological studies with reovirus-like enteritis agent.
Infect Immun 1976;13:1563-1566.
124. Prince DS, Astry C, Vonderfecht S, Jakab G, Shen FM, Yolken RH.
Aerosol transmission of experimental rotavirus infection.
Pediatr Infect Dis 1986;5(2):218-222.

125. LeBaron CW, Furutan NP, Lew JF, *et al.*
Viral agents of gastroenteritis: public health importance and outbreak management.
MMWR 1990;39(RR-5):1-24.
126. Ward RL, Bernstein DI, Young EC, Sherwood JR, Knowlton DR, Schiff GM.
Human rotavirus studies in volunteers: determination of infectious dose and serological response to infection.
J Infect Dis 1986;154:871-880.
127. Engleberg NC, Holburt EN, Barrett TJ, *et al.*
Epidemiology of diarrhea due to rotavirus on an Indian reservation: risk factors in the home environment.
J Infect Dis 1982;145:894-898.
128. Grimwood K, Abbott GP, Ferguson DM, Jennings LC, Allan JM.
Spread of rotavirus within families: a community based study.
Br Med J 1983;287:575-577.
129. Pickering LK, Bartlett AV 3d, Reeves RR, Morrow A.
Asymptomatic excretion of rotavirus before and after rotavirus diarrhea in children in day care centers.
J Pediatr 1988;112:361-365.
130. Albrey MB, Murphy AM.
Rotavirus and acute gastroenteritis of infants and children.
Med J Aust 1976;1:82-85.
131. Birch CJ, Lewis FA, Kennet ML, Homola M, Pritchard H, Gust I.
A study of the prevalence of rotavirus infection in children with gastroenteritis admitted to an infectious diseases hospital.
J Med Virol 1977;1:69-77.
132. Cook SM, Glass RI, LeBaron CW, Ho MS.
Global seasonality of rotavirus infections.
Bull WHO 1990;68:171-177.
133. Stoll BJ, Glass RI, Huq MI, Holt JE, Banu H.
Surveillance of patients attending a diarrheal disease hospital in Bangladesh.
Br Med J 1982;285:1185-1188.

143. Matsuno S, Inouye S, Kono R.
Quantitative estimation of infantile gastroenteritis virus antigens in stools by immune adherence hemagglutination test.
J Clin Microbiol 1978;7:273-280.
144. Theil KW, Reynolds DL, Saif YM.
Comparison of immune electron microscopy and genome electropherotyping techniques for detection of turkey rotaviruses and rotavirus-like viruses in intestinal contents.
J Clin Microbiol 1986;23:695-699.
145. Cukor G, Blacklow NR.
Human viral gastroenteritis.
Microbiol Rev 1984;48:find-179.
146. Dennehy PH, Gauntlett DR, Tente WE.
Comparison of nine commercial immunoassays for the detection of rotavirus in fecal samples.
J Clin Microbiol 1988;26:1630-1634.
147. Thomas EE, Puterman ML, Kawano E, Curran M.
Evaluation of seven immunoassays for detection of rotavirus in pediatric stool samples.
J Clin Microbiol 1988;26:1189-1193.
148. Rodger SM, Bishop RF, Bitch C, Mel can B, Holmes IH.
Molecular epidemiology of human rotaviruses in Melbourne, Australia, from 1973 to 1979, as determined by electrophoresis of ribonucleic acid.
J Clin Microbiol 1981;13:272-278.
149. Pyndiah N, Beguin R, Richard J, Charles M, Rey A, Bonifas V.
Accuracy of rotavirus diagnosis: modified genome electrophoresis versus electron microscopy.
J Virol Methods 1988;20:39-44.
150. Allen JR, Gouvea V, Moe C, Monroe SS.
Viral agents of gastroenteritis: public health importance and outbreak management.
MMWR 1990;39(RR-5):1-25.
151. Editorial (Agarwal A).
A cure for a killer – but how to deliver it?
Nature 1979;278:389-391.

152. Santosham M, Burns B, Nadkarni , *et al.*
Oral rehydration therapy for acute diarrhea in ambulatory children in the United States: a double blind comparison of four different solutions.
Pediatrics 1985;76:1159-166.
153. Sack DA, Chowdhury AMAK, Eusof A, *et al.*
Oral hydration in rotavirus diarrhea: a double blind comparison of sucrose with glucose eletrolyte solution.
Lancet 1978;2:280-283.
154. Sack RB, Rabbani GH.
Treatment of diarrheal diseases.
In: Kapikian AZ, ed. Viral infections of the gastrointestinal tract. New York: Marcel Dekker, Inc, 1994;758-775.
155. Sazawal S, Bhatnagar S, Bhan MK, *et al.*
Alanine-based oral rehydration solution: assessment of efficacy in acute noncholera diarrhea among children.
J Pediatr Gastroenterol Nutr 1991;12:461-468.
156. Carpenter CCJ, Greenough WB, Pierce NB.
Oral rehydration therapy – the role of polymeric substrates.
N Engl J Med 1988;319:1346-1348.
157. Vesikari T.
Rotavirus vaccine studies in Europe.
Acta Paediatr Suppl 1999;88(426):9-13.
158. Bishop R.
The present status of rotavirus vaccine development.
Southeast Asian J Trop Med Public Health 1988;19(3):429-435.
159. Vesikari T, Joensuu J.
Review of rotavirus vaccine trials in Finland.
J Infect Dis 1996;174(Suppl 1):S81-87
160. Characters of Gram-negative bacteria.
In: Barrow GI, Feltham RKA, eds. Cowan and Steel's manual for the identification of medical bacteria. 3rd ed. Cambridge University Press, 1993;94-164.
161. Herring AJ, Inglis NF, Ojeh OK, Snodgrass DR, Menzies JD.
Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels.
J Clin Microbiol 1982;16:473-477.

162. Glass RI, Lew JF, Gangarosa RE, LeBaron CW, Ho MS.
Estimates of morbidity and mortality rates for diarrheal diseases in American children.
J. Pediatr 1991;118:S27-33.
163. Coello-Ramirez P, Lifshitz F.
Enteric microflora and carbohydrate intolerance in infants with diarrhea.
Pediatrics 1972;49:233-40.
164. Konno T, Suzuki H, Imai A, *et al.*
A long term survey of rotavirus infection in Japanese children with acute gastroenteritis.
J Infect Dis 1978;138:569-576.
165. Bryden AS, Davies HA, Hadley RE, Flewett TH, Morris CA, Oliver P.
Rotavirus enteritis in the West Midlands during 1974.
Lancet 1975;2:241-243.
166. Desikan P, Daniel JD, Kamalarathnam CN, Mathar MM.
Molecular epidemiology of nosocomial rotavirus infection.
J Diarrh Dis Res 1996;14(1):12-15.
167. Chakravarti A, Kumar S, Mittal SK, Broor S.
Comparison of latex agglutination and polyacrylamide gel electrophoresis with enzyme linked immunosorbent assay for detecting human rotavirus in stool specimens.
Indian Pediatr 1991;28(5):507-510.
168. Birch CJ, Rodgers SM, Marshall JA, Gust ID.
Replication of human rotavirus in cell culture.
J Med Virol;1983;11:241-250.
169. Hasegawa A, Matsuo S, Inouye S, *et al.*
Isolation of human rotaviruses in primary cultures of monkey kidney cells.
J Clin Microbiol;1982;16:387-390.



SYNOPSIS

**A SYNOPSIS OF THE THESIS
TO BE SUBMITTED
TO
THE UNIVERSITY OF MUMBAI
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
MICROBIOLOGY**

TITLE OF THE THESIS : Study of rotaviral diarrhoea in paediatric patients

NAME OF THE CANDIDATE : Anuradha Kirtikar

**NAME AND DESIGNATION
OF THE GUIDE** : Dr.(Mrs.) R. A. Deshmukh,
Officer-in-Charge, Dept. Of Virology,
Haffkine Institute for Training, Research and
Testing, Parel, Mumbai – 400 012.

PLACE OF RESEARCH : Dept. Of Virology,
Haffkine Institute for Training, Research and
Testing, Parel, Mumbai – 400 012.

NUMBER OF REGISTRATION : 31

DATE OF REGISTRATION : December 4th, 1997


SIGNATURE OF STUDENT
Anuradha Kirtikar


SIGNATURE OF THE GUIDE
(Dr. (Mrs.) R. A. Deshmukh)

INTRODUCTION

In 1973, Bishop and colleagues observed by electron microscopy, in the duodenal epithelium of children with diarrhoea, a 70 nm virus, subsequently designated rotavirus (1). Before this discovery, a bacterial, viral, or parasitic agent could be detected in only 10% to 30% of children with diarrhoea. Within five years, rotavirus was recognized as the most common cause of severe vomiting and diarrhoea in infants and young children worldwide, accounting for approximately one third of cases of severe diarrhoea requiring hospitalization (2-4). Rotavirus infects virtually all children by five years of age. Some of these infections are severe and many children are infected more than once, although severity of disease decreases with each infection (5).

In every population studied, rotaviruses account for 20-50 per cent of hospitalizations for gastroenteritis (6). In developing countries, rotavirus leads to an estimated 600,000 to 870,000 deaths each year, accounting for 20-25 per cent of all deaths due to diarrhoea and 6 per cent of all deaths among children under 5 years of age (7).

Thus, the public health burden of infectious diarrhoea is substantial, world-wide and especially in developing countries. However, systematic surveillance for rotavirus diarrhoea has not yet been established in developing countries.

Rotaviruses are shed in high concentrations in the stools of infected children and are transmitted by the oral-fecal route, both through close person-to-person contact and through fomites (8). Rotaviruses also might be transmitted by other modes, such as respiratory droplets (9). Because the clinical features of rotavirus gastroenteritis are non-specific, confirmation of rotavirus infection in children with gastroenteritis laboratory testing of fecal specimens is necessary for reliable rotavirus surveillance (10).

Rotaviruses affecting humans were once thought to be limited to one antigenic family termed Group A, whereas other antigenic groups (B-G) were thought to be strictly zoonotic. In 1982, however, an epidemic of Group B rotavirus affected millions of

persons in China (including adults, children, and neonates) (11), and since then outbreaks have occurred, although affecting fewer persons. Studies of immunoglobulin pools from Shanghai suggest that the Chinese population had been exposed to this pathogen in the past (12). Since Group B rotavirus is a common diarrhoeal pathogen for swine, and since all rotaviruses have a segmented genome capable of antigenic changes through reassortment of genes, investigators have hypothesized that this human Group B epidemic arose by a reassortment that allowed the swine virus to propagate in the human gut (13). The group B epidemic was not known to have spread beyond China until Krishnan T *et al* reported the detection of this virus in Calcutta in 1999 (14). The detection of this strain in Calcutta calls for systematic surveillance of rotavirus in India (15). Group C rotaviruses are also primarily swine pathogens but have been detected among humans in all parts of the world. Outbreaks have occurred in Japan and England, but the importance of Group C rotaviruses in endemic disease is unknown (16, 17).

Because rotavirus infection is the leading cause of diarrhoeal mortality in the world and because natural infection appears to induce immunity, rotavirus has been the main focus of efforts at vaccine development. Within 10 years of its discovery in 1973, trials were already under way. But the results have been inconsistent (18,19). These inconsistencies may be due to differences between vaccine type and the strain of rotavirus in the community. The present study was undertaken to report the prevalence of rotavirus gastroenteritis among hospitalized children and to provide basic epidemiological data like the association of the disease with risk factors such as age, sex and seasons. It also aims to detect any novel or unusual rotaviruses and to determine the prevalent rotavirus electropherotype in the children of Mumbai.

AIMS AND OBJECTIVES

1. To determine the prevalence of rotavirus infection in paediatric cases of diarrhoea.
2. To study the prevalence of enteric bacteria in paediatric cases of diarrhoea.
3. Comparison of three methods for detection of rotaviruses, namely latex agglutination, polyacrylamide gel electrophoresis and isolation of virus in cell culture.

MATERIALS AND METHODS

Specimen collection :

Stool specimens were collected from children under 5 years of age and admitted for diarrhoea in the paediatric wards of Lokmanya Tilak Hospital, Sion, K.E.M. Hospital, Parel, and Kasturba Hospital for Infectious Diseases, Mumbai Central, on the day of admittance. Control patients were children who were admitted to these hospitals with other illnesses and who showed no symptoms of gastroenteritis. Stools from these children were collected on any day after admittance to the hospitals. Information regarding sex, age, date of admission, locality and family income was collected from all subjects. The specimens were collected on the day of admission of the patients in sterile 15.0 ml containers with wide mouths and screw caps. The specimens were stored at 4°C until processed.

Detection of enteric bacteria:

The specimens were plated on selective and differential media within an hour of sample collection. The bacteria were identified by standard biochemical tests (20).

Detection and electropherotyping of rotavirus:

Detection of rotavirus was done using two methods, viz. latex agglutination (LA) and polyacrylamide gel electrophoresis (PAGE). A commercially available latex agglutination kit was used to detect rotaviruses from fresh samples as per the instructions enclosed with the kit.

PAGE was used to detect as well as electropherotype the virus. RNA was extracted from stool and run along with positive controls on a 10% polyacrylamide gel kept at a constant current of 25 mA for 16 hrs. using a modification of the original protocol (21). The gels were silver stained and visualized against white light. Based on the pattern of the bands obtained, the detected virus was grouped

Proposed plan of work:

Isolation of virus in cell culture: Cell line MA- 104 will be used for this purpose. It will be maintained in Minimum Essential Medium with 10% foetal calf serum. Virus will be inoculated on the cell line and isolated according to protocol standardized by Wyatt *et al* (22).

RESULTS AND DISCUSSION

This study covers the period from March 1998, to January 2000. 225 diarrhoea stool samples and 25 control stool samples were collected from paediatric patients admitted to the three mentioned hospitals.

Rotavirus was detected in 36 samples by PAGE and in 33 samples by LA. Of the 25 controls, none tested positive for rotavirus by either method. PAGE was regarded as the standard test for detection as its sensitivity is more than 90% and specificity is 100% (23). In addition, atypical rotaviruses (Groups B and C) can be detected. LA gave 4 false positive results and 7 false negative results showing a sensitivity of 87.87%.

The prevalence rate of rotavirus infection in paediatric cases was found to be 16%. Of the 36 rotavirus positive patients, 63.88% were males and 36.11% were females which shows that male babies are more prone to rotavirus infection than female babies (24). However, bearing in mind the excess of male births over females, the greater general resistance of the female, and the tendency in some communities to lavish more care and attention on, males than females, it might be likely that these figures do not reflect any genuine difference between the susceptibility of the two sexes. All the detected rotavirus belonged to Group A. No atypical virus was observed proving that rotavirus infection of children in Mumbai as of yet is by the conventional strain (Table 1).

Table 1. Prevalence of Rotavirus Diarrhoea Cases

Sex	Total no. of Diarrhoea cases	Percentage (n=225)	No. of rotavirus diarrhoea cases	Percentage (n=36)
	225		36	
Male	133	59.11	23	63.88
Female	92	40.88	13	36.11

All 36 cases of rotavirus belonged to Group A.

The age group of 6-12 months was found to be most susceptible to infection with 55.55% of the rotavirus cases occurring in this age group. This finding is in accordance with those from other developing countries (25,26). Over all, the age group up to 24 months was more susceptible (88.88%) than the age group from 24 months to 5 years (11. 11%). (Table II). This is because of decreased incidence of disease with increasing age (10).

Table II. Age-wise distribution of rotavirus diarrhoea cases diarrhoea cases

<i>Age group (mths)</i>	<i>Diarrhoea cases</i>	<i>Rotavirus Diarrhoea Cases</i>	<i>Percentage (n=36)</i>
<6	28	3	8.33
6 – 12	107	20	55.55
12 – 18	36	7	19.44
18 – 24	21	2	5.55
> 24	33	4	11.11
Total No. of patients	225	36	

Although rotavirus disease was recorded around the year, a distinct seasonal variation was observed. 47.22% of the observed infections took place during the months of November to February. This confirms that rotavirus is endemic throughout the year with a peak in the winter months. (Table III).

Table 111. Season-wise distribution of rotavirus diarrhoea casts

<i>Seasons</i>	<i>Total no. of cases</i>	<i>Rotavirus cases</i>	<i>Percentage (n=36)</i>
Rainy (July – October)	89	9	25
Winter (November – February)	74	17	47.22
Summer (March – June)	62	10	27.77
Total	225	36	

E.coli was isolated from a majority of the 225 samples (38.22%). Other bacteria identified belonged to the *Vibrio* and *Proteus* species. An astonishing 49.77% of the test samples showed no growth. (Table IV). This may be due to treatment of the patients with antibiotics, which would destroy even the normal flora along with the pathogens in the gastrointestinal tract. All the detected rotavirus cases were those from which no bacteria could be isolated i.e. no co-infection of rotavirus with bacterial pathogens was observed.

Table IV. Isolation of enteric bacteria from stools

<i>Bacteria isolated</i>	<i>Diarrhoea cases</i>	<i>Percentage (n=225)</i>
<i>E.coli</i>	86	38.22
<i>Vibrio</i> sp.	18	8.0
<i>Proteus</i> sp.	9	4.0
No growth	112	49.77

All the data are being statistically analyzed.

The study thus conclusively associates the risk factors like age, sex and seasons with increased incidence of rotavirus infection. Also, socio-economic deprivation and poor environmental sanitation are known to increase the incidence of this disease in developing countries (27). However, since all the study subjects belonged to similar socioeconomic strata, this factor could not be assessed.

Continuous surveillance of this infection is necessary in order to determine the prevalent strains and patterns of infection in the community.

REFERENCES

1. Bishop RF, Davidson GP, Holmes IH, Ruck BJ.
Virus particles in epithelial cells of duodenal mucosa from children with viral gastroenteritis
Lancet 1973 ; 2:1281-1283.
2. Kapikian AZ, Cline WL, Mebus CA, Wyatt RG, Kalica AR, James HD Jr., VanKirk D, Chanock RM.
New complement-fixation test for the human reovirus-like agent of infantile gastroenteritis. Nebraska calf diarrhoea virus used as antigen.
Lancet 1975 ; 1: 1056-1061
3. Kapikian AZ,, Cline WL, Kim HW, Kalica AR, Wyatt RG, VanKirk DH, Chanock RM, James HD Jr., Vaughn AL.
Antigenic relationships among five reovirus - like (RVL) agents by complement fixation (CF) and development of new substitute CF antigens for the human RVL agent of infantile gastroenteritis.
Proc Soc Exp Biol Med 1976 ; 152:535-539.
4. Yolken RH, Kim HW, Clem T, Wyatt RG, Kalica AR, Chanock RM, Kapikian AZ
Enzyme-linked immunosorbent assay (ELISA) for detection of human reovirus-like agent of infantile gastroenteritis.
Lancet 1977 ; 2:263-267.
5. Mata L, Simhon A, Urrutia JJ, Kronmal RA, Fernandez R, Garcia B.
Epidemiology of rotaviruses in a cohort of 45 Guatemalan Mayan Indian children observed from birth to the age of three years.
J Infect Dis 1983 ; 148:452-461.
6. Kapikian AZ, Flores J, Hoshino Y, *et al.*
Rationale for the development of a rotavirus vaccine for infants and young children.
In: Progress in Vaccinology, Talwar GP, ed. New York: Springer-Verlag, 1989, pp 151- 180.
7. Institute of Medicine.
The burden of disease resulting from various diarrhoeal pathogens.
In : New vaccine development: Establishing priorities. Washington, DC National Academy Press; 1986, pp 113.
8. Butz Am, Fosarelli P, Dick J, Cusack T, Yolken R.
Prevalence of rotavirus on high-risk fomites in day-care facilities.
Pediatrics 1993 ; 92:202-205

9. Santosham M, Yolken RH, Wyatt RG, Bertrando R, Black RE, Spira WM, Sack RB.
Epidemiology of rotavirus diarrhoea in a prospectively monitored American Indian population.
J Infect Dis 1985 ; 152:778-783.
10. Kapikian AZ-, Chanock RM.
Rotaviruses.
In: Fields BN, Knipe DM, Howley PM *et al.*, eds. *Fields virology*. 3'd ed. Philadelphia Lippincott-Raven, 1996:1657-1708.
11. Hung T, Chen O, Wang C, *et al.*
Waterborne outbreak of rotavirus diarrhea in adults in China caused by a novel rotavirus.
Lancet 1984 ; i 1139-1142.
12. Hung T, Chen G, Wang C, *et al.*
Seroepidemiology and molecular epidemiology of the Chinese rotavirus.
In : Bock G, Whelan J, eds. *Novel diarrhoea viruses*. (Ciba Foundation Symposium., 128). Chichester, UK: John Wiley & Sons, Ltd, 1987 ; 49-62
13. Bridger JC,.
Non-group-A rotaviruses.
In: *Viruses and the gut : proceedings of the Ninth BSGSk & F International Workshop* Welwyn Garden City, UK: Smith Kline & French Laboratories, Ltd, 1988 ; 79-81.
14. Krishnan T, Sen A, Choudhury JS, Das S, Naik TN, Bhattacharya SK.
Emergence of adult diarrhoea rotavirus in Calcutta.
Lancet 1999 ; 353 (9150) :380-381.
15. Jain K.
Countries need constant surveillance to detect the new cholera bug: experts,
The Times of India, Mumbai. March 19, 1999, pp 11.
16. Bridger JC, Pedley S, McCrae MA.
Group C rotaviruses in humans.
J Clin Microbiol 1986;23:760-763.
17. Penaranda ME, Cubitt WD, Sinarachatanant P, Taylor DN, Likansakul S, Saif L, Glass RI.
Group C rotavirus infections in patients with diarrhoea in Thailand, Nepal, and England.
J Infect Dis 1989;160:392-397.
18. Bishop R.
The present status of rotavirus vaccine development.
Southeast Asian ,J Trop Med Public Health 1998;19(3):429-435

19. Vesikari T.
Rotavirus vaccine studies in Europe.
Acta Paediatr Suppl 1999;88(426):9-13
20. Characters of Gram-negative bacteria.
In: Barrow GI, Feltham RKA, eds. *Cowan and Steel's manual for the identification of medical bacteria*. 3d ed. Cambridge University Press, 1993 ; 94-164.
21. Espejo RT, Calderon E, Gonzalez N, Salomon A, Martuscelli A, Romero P.
Presence of two distinct types of rotavirus in infants and young children hospitalized with acute gastroenteritis in Mexico City, 1977.
J Infect Dis 1979;139(4):474-477.
22. Wyatt RG, James Jr. J, Pittman A, Hoshino Y, Greenberg H, Kalica A, Flores J, Kapikian A.
Direct isolation in cell culture of human rotaviruses and their characterization into four serotypes.
J Clin Microbiol 1983.18:310-317.
23. Allen JR, Gouvea V, Moe C, Monroe SS.
Viral agents of gastroenteritis: Public health importance and outbreak management
MMWR 1990; 39(RR-5): 1-24.
24. Kelkar SD, Purohit SG, Simha KV.
Prevalence of rotavirus diarrhoea among hospitalized children in Pune, India
Indian J Med Res 1999; 109:131-135.
25. Saha MR, Sen D, Datta P, Datta D, Pal SC.
Role of rotaviruses as the cause of acute paediatric diarrhoea in Calcutta.
Trans R Soc Trop Med Hyg 1984;78:818-820.
26. Steele AD, Alexander JJ, Hay IT.
Rotavirus-associated gastroenteritis in black infants in South Africa
J Clin Microbiol 1986-23:992-994.
27. Engleberg NC, Holburt EN, Barrett TJ, Gary GW Jr., Trujillo MH, Feldnan RA, *et al.*
Epidemiology of diarrhoea due to rotavirus on an Indian reservation. risk factors in the home environment.
J Infect Dis 1982;145:894-898.

