

GENOMIC RNA ELECTROPHEROTYPES OF GROUP ROTAVIRUSES DETECTED IN CHILDREN WITH DIARRHOEA IN PRAMSO, GHANA.

Jones Gyamfi (Msc)

University of Health and Allied Sciences, Department of Medical Laboratory Science, Ho–Ghana.

Eric Awuah (Bsc)

Kwame Nkrumah University of Science and Technology, Department of Theoretical and Applied Biology, Kumasi – Ghana.

George Armah (PhD)

University of Ghana, Noguchi Memorial Institute for Medical Research, Department of Electron Microscopy and Histopathology, Legon – Ghana.

John Aseidu Larbi (PhD)

Kwame Nkrumah University of Science and Technology, Department of Theoretical and Applied Biology, Kumasi – Ghana.

ABSTRACT: *In childhood diarrhoea infections worldwide, rotaviruses have been identified as the major cause, leading to hospitalizations and about 20% of all associated diarrhoea death is related to rotaviruses. The rotavirion has a non-enveloped, isocohedrail complex; triple layered capsid structure that surrounds a genome consisting of 11 segments of double-stranded RNA which can be separated by Polyacrylamide Gel Electrophoresis (PAGE). In this study we determined the presence of group A rotavirus by ELISA, and the genomic RNA electrophoretic pattern of 84 children below 5 years visiting the St. Michael's Hospital in Pramso, Ghana with symptoms of diarrhoea. Of the entire specimen examined 24 (28.6%) tested positive for group A rotavirus by Enzyme Linked Immunosorbent Assay (ELISA) and subsequently subjected to PAGE to determine the electropherotypes. Rotavirus electropherotypes were characterized into long 'L' electropherotype (40.9%) and short 'S' electropherotype (59.1%). Detailed analysis of the two electropherotypes revealed four minor variants in both the long and short electropherotypes and were classified as L_A , L_B , L_C , L_D , S_A , S_B , S_C and S_D . The S_D electropherotype was the most common electropherotype in circulation (27.3%). This study is the first to present information on electropherotypic distribution of rotavirus strains in Ghana.*

KEYWORDS: Diarrhoea, Rotavirus, Enzyme Linked Immunosorbent Assay, Polyacrylamide Gel Electrophoresis, Serotypes.

INTRODUCTION

Diarrhoea is the leading cause of childhood morbidity and mortality worldwide, causing about three million deaths worldwide (*Tropical Medicine and International Health, Volume 8 number 9, pp 840-846, September 2003*). Persistent diarrhoea is dangerous to health because it may be an indication of an underlying infection and means that the body is unable to absorb some nutrients and water due to a defective or inflamed colon, which when unchecked can lead to dehydration and even death (*Blacklow et al., 1972*). It may be due to a myriad of

infections or non-infectious cause; due to bacteria such as *Vibrio cholerae*, *Shigella sp*, *Samonella typhi*; Protozoan parasites such as *Cryptosporidium parvum*, *Giardia lamblia* and viruses such as caused by noroviruses, adenoviruses, astroviruses, and rotaviruses (*Blacklow et al.*, 1972).

Of the viral infections leading to diarrhoea, rotaviruses have been identified as the major cause, leading to hospitalizations and death. Rotavirus was first identified by electron microscope in 1972 from duodenal biopsies of children with diarrhoea. Rotaviruses belong to a genus in the family Reoviridae (*Fields et al.*, 2001). The rotavirus structure shows a 60-80nm wheel-like structure with radiating spikes (the name is derived from the Latin word Rota meaning Wheel) (*Kapikian et al.*, 1990; *Prasad et al.*, 1988). The rotavirion has a non-enveloped, isocahedrail complex; triple layered capsid structure that surrounds a genome consisting of 11 segments of double- stranded RNA which can be separated by Polyacrylamide Gel Electrophoresis (PAGE) (*Clark et al.*, 1982; *Desselberger et al.*, 1986). There are six (6) structural proteins and six (6) non-structural proteins that form its complex capsid structure. There are several structural proteins that make up the viral capsid [4, 5]. The outer structural proteins are made up of the viral protein seven (VP7) and viral protein four (VP4). The inner core proteins are the viral protein 1, 2, 3 and 6 thus (vp1, vp2, vp3 and vp6) (*Kapikian et al.*, 1990; *Prasad et al.*, 1988; *Desselberger et al.*, 1986).

Electrophoresis of the rotavirus RNA genome allows detection and classification of the virus into two major groups; the long (L) and the short (S) electrophoretic profiles based on the migration patterns of gene segments 10 and 11 on polyacrylamide gel (*Kalica et al.*, 1981). Variations in the electrophoretic mobility of the rotavirus RNA allow different rotavirus strains to be assigned to distinct electrophoretic pattern (*Schangal et al.*, 1981; *Brown et al.*, 1988; *Ghosh et al.*, 1989). This often provides useful information pertaining to the genomic diversity of rotavirus isolates in a population over a certain period.

This study determined the occurrence of rotavirus infection among young children and the RNA electropherotype characterization of the rotavirus isolates, to help broaden the understanding of the molecular epidemiology of the rotavirus infection in Ghana.

MATERIALS AND METHOD:

All samples for the study were faecal specimen collected from diarrhoea children below five years visiting the St. Michael's Hospital at Pramso, in the Ashanti Region of Ghana, from October 2007 to March 2008.

Sample Collection

Stools specimens were obtained from children below five years who sought medical attention at the hospital with symptoms of diarrhoea. Diarrhoea for the study was defined as the passage of more than 3 looser than normal stool within an hour. Basic demographic and clinical information were collected by administered questionnaire after informed consent had been obtained from mothers or care givers of children. Faecal specimens was collected into sterile containers and glycerol, and transported on ice to Noguchi Memorial Institute for Medical Research (NMIMR), Accra – Ghana (a WHO Regional Reference Laboratory for Rotavirus), where they were stored at -20°C until testing.

Detection of Group A rotavirus antigen by Enzyme Linked Immunosorbent Assay (ELISA)

Detection of group A rotaviruses was done using a commercially available Dako Rotavirus ELISA Kit following the manufacturer's instructions (*Dakopatts, Denmark*). Briefly 10% suspension of each faecal specimen was made in supplied sample diluent buffer; 100µl of the prepared suspension was then dispensed into each well of the break-apart 96 microliter plate pre-coated with rotavirus specific rabbit polyclonal antibody. The first three wells were occupied by blank, negative and positive controls and two drops (100µl) of the conjugate was added to each micro-well. The plate was incubated at room temperature for 60 minutes and the wells were washed five times using freshly prepared working strength washing buffer to remove excess specimen and any unbounded enzyme labelled antibody. Two drops (100µl) of substrate was added to each micro-well and the plates incubated at room temperature for 10 minutes. Wells were visually read after the second incubation. A stopping buffer solution was added to each microwell to stop the substrate reaction. The absorbance of each well was read spectrophotometrically at a wavelength of 450nm.

RNA Electropherotypes of rotavirus by polyacrylamide gel electrophoresis (PAGE)

The specimens that tested positive for group A rotavirus during the ELISA, were subjected to further analysis by PAGE to determine the electropherotypes, confirm the results from ELISA and determine non-group A rotavirus.

Viral RNA was extracted by Bender Buffer Extraction Method described by Flook et al., (1992), and the electrophoresis of rotaviral RNAs was performed by method described by Herring et al., (1982) with slight modification for PAGE analysis. Briefly, a 10% suspension of each stool sample was made by adding 0.1g (the size of peanut) of stool to 1 ml of Phosphate Buffered Saline (PBS). The suspension was vortexed for at least 1 minute and centrifuged at 5,000rpm for 3 minutes. Two hundred micro litres (200µl) of the supernatant was pipetted into a sterile eppendorf tube containing 200µl of Bender buffer and incubated at 65°C for 30 minutes in a water bath. 60µl of 8M potassium acetate (KAC) solution was then added, mixed and incubated on ice for 45 minutes to coagulate the viral RNA from other particles in solution.

The mixture was then centrifuged at 10,000 rpm for 20 minutes and the supernatant was then transferred into new eppendorf tubes and the pellet discarded. Twice the volume of cold absolute ethanol (920µl) was added to the supernatant and the mixture was allowed to stand on ice for 10 minutes. It was then spun at 10,000rpm for 20 minutes and the alcohol decanted. Two hundred micro litres (200µl) of Tris EDTA (TE) buffer was added and then left for 30 minutes at room temperature. 10µl of 5M sodium chloride (NaCl) and 420µl absolute ethanol were added to purify and precipitate the viral genomic RNA and incubated at -20° C for one hour. The mixture was then spun at 10,000rpm for 20 minutes and the supernatant decanted. The pellets were dried under vacuum for 2 hours and re-suspended in 15µl of loading buffer.

The extracted dsRNA was applied to separate lanes of a 10% polyacrylamide gel slab and electrophoresed for 18 hours at 100V using the discontinuous buffer system as described by Laemmli et al., (1970)]. Gels were visualized by silver-nitrate staining technique as described by Herring et al., (1982)].

Classification of rotavirus electropherotypes

The 11 genomic RNA of rotaviruses when separated on PAGE shows a characteristic 4-2-3-2 distribution pattern. The RNA segments 1 to 4, are classified as Group I; 5 and 6 as Group II; 7 to 9 as Group III and 10 and 11 as Group IV in the 4-2-3-2 migration pattern (Figure 3) (*Gatheru et. al., 1993; Kapikian et al., 2001*).

Rotavirus electropherotypes were classified into two major distinctive groups, i.e. long patterns and short patterns, based on the rate of migration of the 10 and 11 segment (*Kobayashi et al., 1989; Gatheru et. al., 1993*). Rotaviruses with 'long' RNA profiles due to the fast migrating RNA segments 10 and 11 and 'short' RNA profiles due to the slower migrating segments 10 and 11 were labelled 'L' and 'S' respectively (*Kobayashi et al., 1989; Gatheru et. al., 1993*).

The long and short electropherotypes can be further subdivided using Tam's classification method (*Tam et al., 1986*), into A when all the 11 segments were resolved, B when there is co-migration of segment 2 and 3, C when there is co-migration of segment 7 and 8 and D when there is co-migration of segments 8 and 9 (*Tam et al., 1986; Zuridah et al., 2004*).

When the RNA segments do not conform to any of the migration pattern described above it is referred to as mixed electropherotypes.

RESULTS AND DISCUSSION

Results:

Sample Collection

During the ELISA process, the presence of specially bound enzyme labelled antibody in the micro-wells resulted in a colour change, an indicative of a positive result. A total of 84 children were recruited for the study, 24 (28.6%) of the specimen tested positive for group A rotavirus antigen by ELISA (Table 1). The highest percentage of positive samples was found in age group 6 to 12 months (54.2%), followed by age group 0 to 6 months (29.2%) and the least, being the children between 1 to 5 years (16.6%).

Table 1: Distribution of rotavirus by age groups and the number of positives by ELISA and PAGE.

Age Groups	Number of samples	Number of Positives by ELISA (%)	Number of positives by PAGE (%)
0 – 6 months	37	7 (29.2)	7 (31.8)
6 – 12 months	29	13 (54.2)	11 (50.0)
1 – 5 years	18	4 (16.6)	4 (18.2)
Total	84	24 (28.6)	22 (26.2)

The incidence of rotavirus was highest in the dry months of Ghana (December and January) and all the other months within the study period had relatively low incidence rates on both years (Table 2; Fig. 1).

Table 2: Distribution of rotavirus infection by months and the number of positives obtained by ELISA and PAGE.

Months	Number of samples	Number of Positives by ELISA (%)	Number of Positives by PAGE (%)	Electropherotype
October	9	2 (8.4)	2 (9.1)	1 L _C , 1 S _D
November	12	3 (12.5)	3 (13.6)	1 L _C , 1 L _A
December	17	8 (33.3)	7 (31.8)	1 L _C , 1 L _B , 5 S _D
January	18	5 (20.8)	5 (22.8)	2 L _D , 1 L _B , 1 S _A , 1 S _C
February	13	3 (12.5)	3 (13.6)	1 L _D , 1 S _A , 1 S _B
March	15	3 (12.5)	2 (9.1)	1 S _B , 1 S _C
Total	84	24 (28.6)	22 (26.2)	

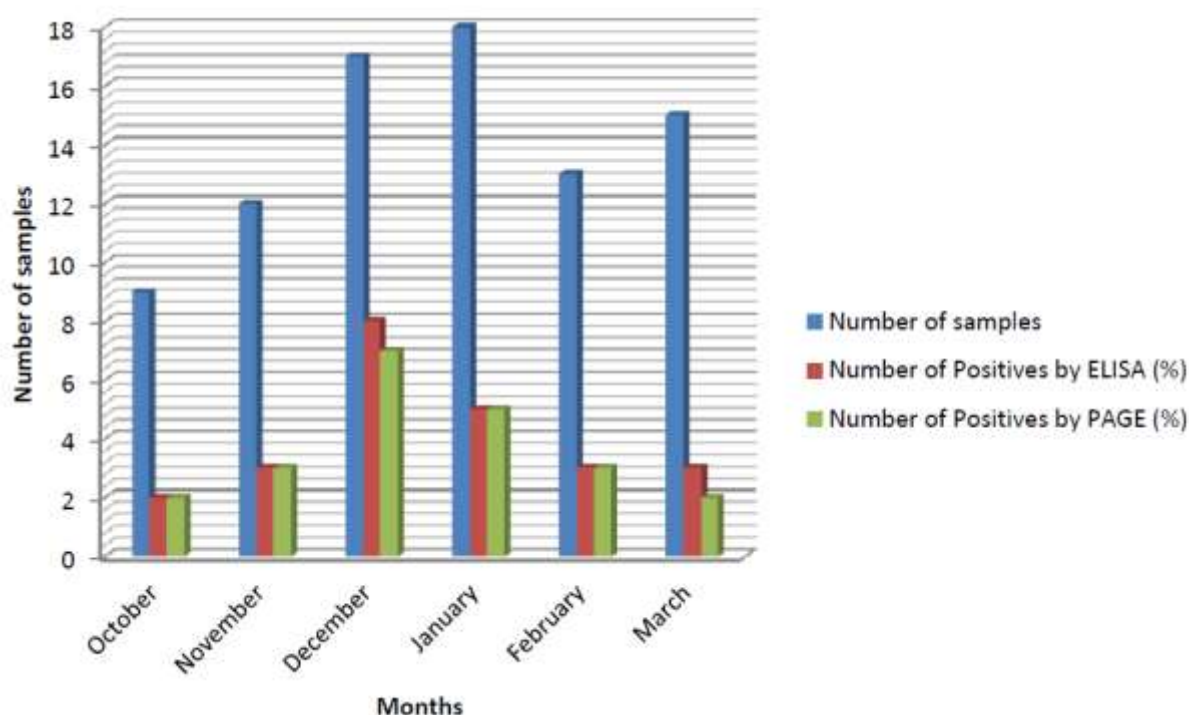


Figure 1: Distribution of total numbers of fecal specimen and numbers of rotavirus-positive (by ELISA and PAGE) strains by month of samples collection.

Rotavirus Electropherotypes

All specimen testing positive by ELISA, were further analyzed by PAGE to allow for determination of genomic diversity of the dsRNA of the strains and to identify non-group A rotaviruses. Among the 24 stool specimens that tested positive with ELISA, when examined by PAGE, 22 yielded typical group A rotavirus electrophoretic migration profiles while the

remaining 2 isolates showed no profile indication the false positive result. Only group A rotavirus were detected and classified as short and long electropherotypes. Using the Tim's classification method, there were four variations of long RNA electropherotype occurring in 9 cases and four variations in the short RNA electropherotype occurring in 13 cases (Tables 2 and 3). In general, 8 distinct electrophoretic migration variants were noted. In this hospital-based study, the short electrophoretic profile was the most prevalent rotaviruses in circulation (59.1%).

The variants for the long and short electropherotypes obtained were L_A, L_B, L_C, L_D, S_A, S_B, S_C and S_D (Figure 2), with S_D being the most predominate subdivision accounting for 27.3% of all the electropherotypes observed (Table 3). No mixed electropherotypes was observed.

Table 3; Frequency of rotavirus electropherotypes in children from Pramso, Ghana.

Pattern of RNA mobility based on segments 10,11 No.of patients (%)	Electropherotype	Age groupings			Total (%)
		0 – 6 months	6 – 12 months	1 – 5 years	
Long 9 (40.9%)	L _A	0	1	0	1 (4.6%)
	L _B	2	0	0	2 (9.1%)
	L _C	1	2	0	3 (13.6%)
	L _D	1	1	1	3 (13.6%)
Short 13(59.1%)	S _A	0	1	1	2 (9.1%)
	S _B	0	2	0	2 (9.1%)
	S _C	1	1	1	3 (13.6%)
	S _D	2	3	1	6 (27.3%)
Mixed 0 (0%)	0	0	0	0	0

studies in Ghana (*Armah et al., 1994*), India (*Das et al., 1994*) and other parts of the world (*Velazquez et al., 1996; Rowlands et al., 1985*).

Comparing the distribution of rotavirus infection with the various months of the study, the results point to an all year round incidence of rotavirus infection. The highest incidence of infection was in December (31.8%) and January (22.8%). This observation is consistent with various studies that reports high incidence of rotavirus infection during the dry season (*Armah et al. 2001; Bok et al., 2001; Luz et al., 2005; Biritwum et al., 1984*).

Comparing the sensitivity and specificity of ELISA to PAGE in the detection of rotavirus infections, the study shows that ELISA is the most rapid and efficient method for detecting rotaviruses shed in stools, accurately detecting approximately 22 positives cases. Nevertheless, it may not serve as the most accurate method of rotavirus detection since it presented two specimens as false positives. Further analysis by PAGE confirmed those specimens as negative for rotavirus. Hence, PAGE serves as a confirmatory test for positives detected by ELISA, and offers a means of detecting other classes of rotaviruses (Group B, C and D). Analysis by PAGE confirms that all the rotaviruses isolated during the study were group A viruses since it presented the typical 4-2-3-2 pattern associated with group A rotaviruses.

Polyacrylamide Gel Electrophoresis offer a rapid and efficient means of detecting non group A rotaviruses and reliable means of characterizing rotaviruses. Based on the pattern of separation of the genomic dsRNA it can be used to classify rotaviruses into different groups if the pattern deviates from the typical 4-2-3-2. However, serotyping of rotaviruses cannot be achieved by PAGE. The information obtained from analyzing electrophoretic pattern observed by PAGE can assist in epidemiological studies. It gives information the evolution and spread of rotavirus strains. Information obtained from PAGE analysis by different laboratories and communities is only meaningful when the same conditions of gel electrophoresis are used. This is because; different conditions can result in different RNA migration patterns from the same virus.

The study observed a wide variety of electropherotypes in circulation in the study area. The commonest electropherotype was the short type (59.1%). This contradicts previous studies conducted in a different communities in Ghana, that reported the long electropherotype to be the most common one in circulation (*Armah et al., 1994; Armah et al., 2001*).

This study further classified the long and short electropherotype into subdivisions by looking at minor variations in the migrations pattern of the RNA segments (segment 2 and 3, 7 and 8, and 8 and 9). This method gives a better understanding of the variants (short and long) in circulation within a given community and helps to classify the variants properly. Using Tam's classification method, the study revealed variants of the major electropherotypes (L and S) in circulation. The study revealed that both the short and long electropherotypes had 4 variants ($L_A, L_B, L_C, L_D, S_A, S_B, S_C$ and S_D), with the predominant sub-classification being S_D electropherotype (27.3%). The different electropherotypes do not seem to have seasonal variations. Thus, there was a mixture of both long and short electropherotypes occurring within the various months. Since this is the first study that focuses on electrophoretic variation of rotavirus strains in Ghana, reference could not be made to pattern of distribution of these electropherotypes in Ghana.

CONCLUSION

This study reports for the first time that both the long and short electropherotypes for rotaviruses is in circulation in Ghana, with several variants of the long and the short electropherotypes. The short electropherotype was the predominant electropherotype and the S_D variant being the most common variant in circulation in Pramso, Ghana. However, several studies are required to provide a better understanding of the various electropherotypes in circulation among different communities in Ghana.

REFERENCES

- Armah GE, Mingle JA, Dodoo AK, Anyanful A, Antwi R, Commey J, Nkrumah FK (1994) Seasonality of rotavirus infection in Ghana. *Ann Trop Paediatr* 14: 223-229.
- Armah, GE, Pager CT, Asmah RH, Anto, FR, Oduro AR, Binka F, Steele D. Prevalence of unusual human rotavirus strains in Ghanaian children. *J Men Virol* 2001; 63(1): 67-71.
- Bernard N. Fields et al., *Fields Virology*, 4th edition-August 2001, Chapter 25- Reoviruses and their replication, Chapter 54- Rotaviruses and their replication.
- Biritwum RB, Isomura S, Yamaguchi H, Toba M, Mingle JA. Seroepidemiological study of rotavirus infection in rural Ghana. *Ann Trop Paediatr* 1984;4:237-40.
- Blacklow NR, Dolin R, Fedson DL, Du Pont H, Northup RS, Hornick RB and Chanock RM (1972). Acute infectious non-bacterial gastroenteritis aetiology and pathogenesis. *Ann Intern Med.* 76: 993-1008.
- Bok, K.; Castagnaro, N.; Borsa, A.; Nates S.; Espul, C.; Fay, O.; Fabri, A.; Grinstein, S.; Miceli, I.; Matson, D.O; Gómez, J.A. (2001). Surveillance for rotavirus in Argentina. *J. Med. Virol.*, 65, 190-198.
- 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: 2410-14.
- Clark IN, McCrae MA . Structural analysis of electrophoretic variation in the genome profiles of rotavirus field isolates. *Infect Immun* 1982; 36: 492-7.
- Das BK, Gentsch JR, Cicirello HG, Woods PA, Gupta A, Ramachandran M, Kumar R, Bhan MK, Glass RI (1994) Characterization of rotavirus strains from newborns in New Delhi, India. *J Clin Microbiol* 32:1820–1822.
- Desselberger U, Hung T, Follett EAC. Genome analysis of human rotaviruses by oligonucleotide mapping Of isolated RNA segments. *Virus Res* 1986; 4:357-68.
- Flook PK, Wilson MD and Post RJ (1992). The use of repetitive DNA probes in the analysis of natural populations of insects and parasites. In: Berry RJ, Crawford TJ and Hewitt GM (eds). *Genes in ecology* British Ecological Society/Blackwell Scientific Publication, Oxford. pp 484-486.
- Gatheru Z, Kobayashi N, Adachi N, Chiba S, Muli J, Ogaja P, Nyangao J, Kiplagatv E and Tulkei PM (1993). Characterization of human rotavirus strains causing gastroenteritis in Kenya. *Epidemiol. Infet.* (1993). 110. 419-423.
- Ghosh SK, Naik TN. Detection of a large number of subgroup 1 human rotaviruses with a "long" RNA electropherotype. *Arch Viro* 1989; 105: 119-27.
- Herring AJ, Inglis NF, Ojeh CK, Snodgrass DR and Menzies JD (1982). Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. *J. Clin. Microbiol.* 16: 473-477.

- Kalica AR, Greenberg HB, Espejo RT, Flores J, Wyatt RG, Kapikian AA, Chanock RM. Distinct ribonucleic acid patterns of human rotavirus subgroups 1 and 2. *Infect. Immun* 1981; 33: 958-61.
- Kapikian, AZ, Chanock RM. Rotaviruses. In: Fields, BN, Knipe DM, Chanock RM, Hirsch MS, Melnick JL, Monath TP, Rizman B (eds) *Virology*, 2nd ed. Raven Press, New York, 1990; 1353-404.
- Kapikian AZ, Hoshino Y, Chanock RM. Rotaviruses. In: Knipe DM, Howley PM, eds. *Fields Virology*. 4th ed. Vol. 2. Philadelphia: Lippincott Williams and Wilkins; 2001: 1787 – 1833.
- Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 227:680-685.
- Luz, C.R.N.E.; Mascarenhas, J.D.P.; Gabbay, Y.B.; Motta, A.R.B.; Lima, R.T.V.; Soares, L.S.; Linhares, A.C. (2005). Rotavirus serotypes and electropherotypes identified among hospitalized children in São Luis, Maranhão, Brazil. *Rev. Inst. Med. Trop. São Paulo*, 47(5), 287-293.
- Prasad BVV, Wang GJ, Clerx JPM, Chiu W. Three dimensional structure of rotavirus. *J Mol Biol* 1988; 199: 269-75.
- Rowlands, M. G. M., S. G. J. Goh, K. Williams, A. D. Campbell, G. M. Beards, R. C. Sanders, and T. H. Flewett. 1985. Epidemiological aspects of rotavirus infection in young Gambian children. *Ann. Trop. Paediatr.* 5:23-28.
- Schangal RD, Rodger SM, Holmes IH. Variation in human rotavirus electropherotypes occurring between rotavirus gastroenteritis epidemics in Central Australia. *Infect Immun* 1981; 53: 17-21.
- Tam JS, Kum WWS, Lam B, Yeung CY, Ng MH. Molecular epidemiology of human rotavirus infection in children in Hong Kong. *J Clin Micro* 1986; 23: 660-4.
- Tropical Medicine and International Health*, Volume 8 number 9, pp 840-846, September, 2003.
- Velazquez FR, Matson DO, Calva JJ, Gurrero ML, Morrow AL, Carter-Campbell S, Glass RI, Estes MK, Pickering LK and Ruiz-Palacios GM (1996). Rotavirus infection in infants as protection against subsequent infections. *New England Journal of Medicine*. 335: 1022-1028.
- World Health Organization (WHO). Estimated rotavirus deaths for children under 5 years of age, 2004. http://www.who.int/immunization_monitoring/burden/rotavirus_estimates/en/. Last accessed March 2006.
- Zuridah H, Bahaman AR, Mohd Azmi ML, Mutalib AR (2004). Rotavirus RNA Electropherotype in Different States in Malaysia for the Year 2000 and 2001 *Med J Malaysia* Vol 59 No 2.