UNIVERSITAS SCIENTIARUM

REVISTA DE LA FACULTAD DE CIENCIAS

Volumen 4 N° 1, Enero-Junio de 1997

PONTIFICIA UNIVERSIDAD JAVERIANA

UNIVERSITAS SCIENTIARUM
Revista de la Facultad de Ciencias
PONTIFICIA UNIVERSIDAD JAVERIANA
Vol 4(1): 71-81, 1997

MOLECULAR CHARACTERIZATION OF ROTAVIRUS STRAINS OBTAINED FROM HUMAN DIARRHEIC SAMPLES AND THEIR EPIDEMIOLOGICAL IMPLICATIONS.

Liliana Bermeo[1], Darío Mogollón[2-1], Francisco Ariza[3], José Barrera[3] Lois Jerabek[1], y María F. Gutierrez[1]

1 Departamento de Microbiología, Facultad de Ciencias, Universidad Javeriana, Cra. 7ª # 43-82, Bogotá, COLOMBIA;
2 Instituto Colombiano Agropecuario (ICA), Bogotá, COLOMBIA
3 Corporación Colombiana de Investigación Agropecuaria (CORPOICA), Bogotá Colombia.

RESUMEN

Con el fin de determinar los patrones electroforéticos del RNA de rotavirus aislados de muestras diarreicas, se analizaron 328 heces de las cuales 56 fueron positivas para rotavirus. Por análisis electroferotípico, las muestras positivas fueron agrupadas en cuatro diferentes patrones de acuerdo a la movilidad de los segmentos del RNA. Las diferencias entre los grupos radicaron principalmente en cambios en la movilidad de los fragmentos 7, 8 y 9, que conforman las bandas del grupo III. Todos los rotavirus presentaron características compatibles con rotavirus del grupo A, y todos presentaron patrón corto. Los resultados sugieren una moderada variación entre las cepas de rotavirus que afectan a la población infantil.

ABSTRACT

An analysis of RNA electropherotypes was carried out on rotavirus isolates from diarrheic samples. Of the 328 samples analyzed, 56 were positive for rotaviral RNA and these could be classified into four different electropherotypes, according to the electrophoretic mobilities of their various RNA segments. The differences among the various electrophoretic patterns were basically due to changes in the mobilities of the genomic segments 7, 8 and 9 which make up group III. However, all electropherotypes were compatible with the patterns considered to be characteristic for group A rotavirus, and were likewise classified as short patterns. The findings suggest a moderate genetic variation among the rotavirus strains affecting the infant population studied.

PALABRAS CLAVE: Diarrea, Electroferotipos de RNA, Humanos, Rotavirus grupo A

INTRODUCTION

Viral gastroenteritis is one of the principal causes of morbo-mortality for infants in developing countries (Blacklow & Cukor 1981). According to WHO, diarrhea is responsible for a large percentage of deaths in this age group, and rotavirus is considered to be the major viral etiological agent. It is interesting to point out that similar findings have been reported for neonates in many mammalian and avian species (Bridger *et al.* 1982, Estes *et al.* 1984, Kang *et al.* 1988, Nicolas *et al.* 1984).

Taxonomically, rotaviruses are classified as members of the Reoviridae family. Under electron microscopy they show a characteristic spoked-wheel morphology with a diameter of approximately 70 nm. They contain 11 segments of double stranded RNA packed inside an inner layer of protein, which is in turn packed inside the inner and outer capsids which both display a

T= 13 icosahedral lattice symmetry. The innermost protein layer, which is sometimes called the core (Espejo *et al.* 1980, Gorziglia *et al.* 1990, Labbe *et al.* 1991, Nicolas et al. 1984, Yeager *et al.* 1994), consists mostly of the protein VP2, with some VP1 and VP3. The inner icosahedral capsid has the protein VP6, which is the site of the "group" antigens (and sub-group antigens, where that is relevant). The outer capsid contains the proteins VP7 and VP4, which are referred to as the neutralizing antigens, since they both induce neutralizing antibodies which protect at least partially against disease (Matsui *et al.* 1989).

The rotaviruses are also classified in groups according to the cross-reactivity of the group antigen located on VP6. Since most of the rotaviruses found in humans belong to group A, they have often been referred to as "typical" rotaviruses, and they are often found in animals as well. Rotaviruses that do not share this antigen have been called "atypical" and belong to other groups (B,C,D,E,F,G). The last four only infect animals, as far as is known (Di Matteo *et al.* 1989, Eiden *et al.* 1994, Flewett 1978).

The rotavirus genome of eleven segments of double-stranded RNA (dsRNA) can be separated by electrophoresis in polyacrylamide gels, thus giving reproducible patterns called electropherotypes (Kapikian & Chanock 1990, Paul & Stevenson 1992), which can be used in the characterization of specific viral isolates.

The study of the genomic dsRNA patterns has shown that "typical" Group A strains, which affect humans more frequently, have a characteristic migration pattern of the 11 segments in four distinct regions: segments 1,2,3 and 4 in region I; segments 5 and 6 in region II, segments 7,8, and 9 in region III; and 10 and 11 in IV. Also, according to migration differences in the viral genome segments 10 and 11, electrophoretic patterns have been described as long, short and super-short. (Bingan *et al.* 1991, Estes *et al.* 1984, Fijtman *et al.* 1987). In "atypical" rotaviruses belonging to different groups, the assignment of segments to migration regions is different.

Changes in electropherotype distribution are often seen to occur in the course of a season or epidemic (Konno *et al.* 1984) often with an increase of pattern diversity. Electrophoretic diversity among rotavirus has been assumed to be caused by sequential point mutations and other genetic changes due to replication errors, with possible selection of neutralization "escape" mutants. These can cause slight changes in size as well as in the secondary and tertiary structures of the segments. Although there is a lack of carefully documented support for the process, it has been shown (Dunn *et al.* 1993) that a single nucleotide substitution can cause changes in the migration of a gene segment, but does not necessarily do so, and there can be two substitutions without a change in mobility; the main issue is probably whether these cause changes in secondary structure.

Thus, this approach, using RNA isolation and electropherotype analysis, has often been used in studies to detect rotavirus as an etiologic agent of acute gastroenteritis and to monitor its epidemiologic behavior. In this study, variations in the electrophoretic profiles of the rotavirus isolates found in the population of infants with diarrhea in different pediatric centers in Bogotá were determined.

METHODS

Samples and reference strains

A total of 328 diarrhea samples from children between the ages of six months and five years were obtained during the seven month period, May-November, 1994. Samples came from four pediatric centers in Bogotá (Hospital Lorencita Villegas de Santos - 168 samples, Clínica David Restrepo- 60, Clínica del Niño del Seguro Social - 45, Hospital de Soacha- 30) and from one in Pereira (Clínica del Seguro social - 25). This last group of samples also included some taken from adults. Samples were stored at -20°C until processed in the laboratory.

Two reference strains were used as electrophoretic standards: 1) The Control bovine strain obtained from the Central Veterinary Laboratory of

Weibridge, England, and 2) the Bohl type porcine strain obtained from the Central Veterinary Laboratory, Ames, Iowa, U.S.A. Both strains were grown in the cell line MA-104 (Urasawa *et al.* 1981) obtained from the National Institute of Health, Bogotá.

Virus purification

The monolayer cell cultures were infected according to standard procedures. First, the viral innoculum was pre-treated with trypsin (one ml of 20 ug/ml trypsin in MEM combined with 1 ml of virus preparation; i.e., clarified, frozen-thawed tissue culture fluid, and incubated for 30 minutes in a 37°C water bath (Albert *et al.* 1987). Then the typsin-treated innoculum was added to the cell monolayer and incubated at 37°C for one hour; next the inoculum was discarded, and 10 ml of cell growth medium MEM- Eagle suplemented with 10% fetal calf serum, 1% HEPES medium, 2% L-glutamine, 1% non essential amino acids, penicillin (100 units/ml), and streptomycyn (100 ug/ml), was added and the cell cultures were again incubated at 37°C in a CO_2 incubator and observed at 24 hour intervals until the appearance of cytopathic effects. (Estes *et al.* 1984).

Once the cytopathic effects were observed, the cells were freeze-thawed three times in order to lyse the cells and liberate the virus.

Electrophoresis of ds RNA

The Chomczynski method (Chomczynski & Sacchi 1987) was used, with the following modifications: 2 g of a fecal sample was homogenized with 1 ml of denaturing solution (25 g of Guanidine thiocyanate dissolved in 29.3 ml of water with DEPC, 1.76 ml of 25 mM Sodium Citrate pH 7.0, 2.64 ml of 10% Sarcosyl and 0.24 ml of 0.1M 2-mercaptoethanol). Next, 0.5 ml of a solution containing 1M lithium chloride, 20 mM EDTA pH 7.8 was added and the combination mixed with a vortex for 20 seconds.

Then, 1.5 ml of a mixture of phenol-chloroform-isoamyl alcohol (24:26:1) was added, then mixed by vortex for 20 seconds. The final suspension was placed on ice for 15 minutes. It was centrifuged at 10,000 x g for 20 minutes at 4°C. The phenol phase was discarded and the aqueous phase, containing the RNA, was transferred to a Corex tube. It was mixed with 1 ml isopropanol and allowed to stand at -20°C for one hour. The RNA precipitate was pelleted by centrifugation at 10,000 x g. The supernatant was discarded and the pellet was resuspendend in 0.5 ml of denaturing solution, transferred to an Eppendorf tube with one volume of isopropanol and allowed to stand for one hour at -20°C. The suspension was then centrifuged at 14,000 x g for 15 minutes at 4°C, the supernatant was discarded and the pellet was washed with 70% ethanol. The pellet was then dried at 37°C and solubilized in 50 ul distilled water treated with DEPC (Diethyl pyrcarbonate). (Estes *et al.* 1984, Wallace 1987).

For electrophoretic analysis of the viral RNA, unidimensional polyacrylamide gel electrophoresis (gel size 25 x 14 cm) was used. The discontinuous Laemmli system was used under non-denaturing conditions (without SDS) (Laemmli 1970). After the run, the gel was stained with silver nitrate according to Herring *et al.* (1982) and it was then photographed with black and white film, ASA 400 (Caetano & Gresshoff 1993).

RESULTS

Of the 328 samples examined, 56 (17%) were found to be positive for rotavirus RNA. In our comparison of the electrophoretic patterns of the isolates obtained from the samples analyzed, those collected from each medical center were compared with each other, and then with isolates taken from other centers. This was to determine whether the degree of genome variation would be fairly uniform throughout, or whether there seemed to be a "clustering" of different electropherotypes, according to the various geographic areas represented by the clinics.

When the electrophoretic patterns of the positive samples were examined and compared to one another, it was possible to identify four different

electropherotypes, all of which were compatible with the types of patterns described as characteristic of group A. These were also found to be positive when tested by an ELISA specific for group A antigen. Furthermore, they were all classified as short patterns, in accordance with the mobilities of the genomic segments 10 and 11 in region IV. These electropherotypes were differentiated solely by the genomic variations found among the segments that made up group III. On the basis of these differences, we arbitrarily designated these electropherotypes as W,X,Y, and Z. (Figures 1 to 5).

Electropherotype W (59% of our isolates), which was characterized by a migration of the triplet of segments where segment number 8 migrated slowly and thus was located closer to segment 7 than to segment 9. This electropherotype was found in all the pediatric centers investigated. (Figure 2, Lane 2).

In the electrophoretic profile of X (30.3% of our isolates) , the segments of group III migrated as a duplex, where segment 8 was apparently very close to 7, so 7 and 8 constituted the duplex. This profile was also found in all of the pediatric centers investigated. (Figure 3, Lane 1).

In Electropherotype Y (9% of our isolates) , although the segments of group III also migrated as a duplex, their migration was slower than that described above where segment 8 was probably nearer to 9, rather to 7, so that 8 and 9 constituted the duplex. Moreover, it was found only in samples from Clinica David Restrepo. (Figure 3, Lane 3).

The electropherotype profile Z (1.7% of our isolates) was characterized by the migration of the segments of group III as a triplet, but with the difference that segment number 8 migrated with a greater velocity and was located closer to segment 9 than to segment 7. This pattern was found only in samples from Clínica del Niño del Seguro Social. (Figura 4, Lane 1). No rotavirus genomic segments were found in any of the samples collected at the Social Security Clinic in Pereira.

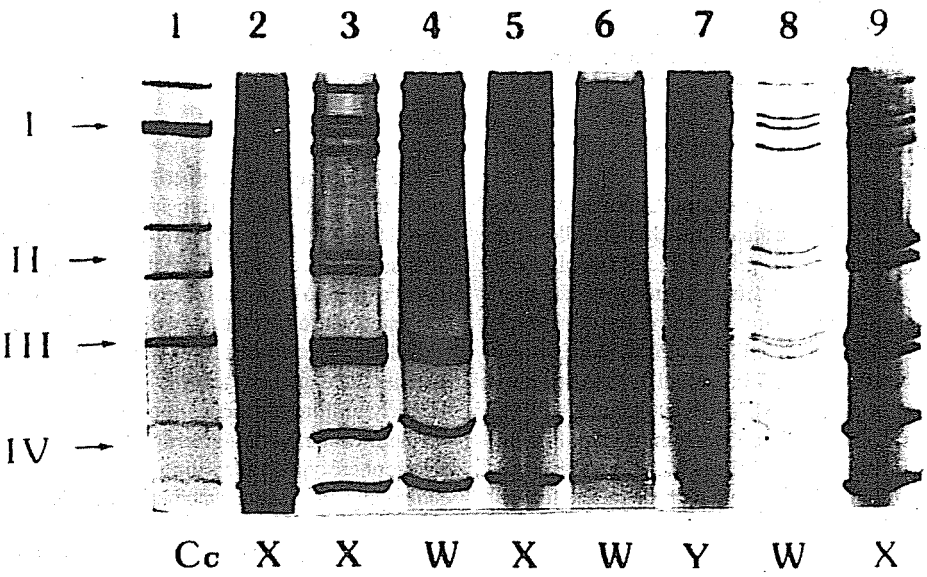

Figure 1. Comparison of the electropherotypes W, X and Y. Cc: Control bovine strain.

DISCUSSION

RNA electropherotyping has often been used in epidemiologic studies of rotavirus infection, since this method makes it possible to determine genetic differences at the molecular level among the viral isolates, and gives criteria for classifying them into their respective groups, and thus is useful for obtaining epidemiologic information concerning the origin of the isolates, since each strain has its characteristic pattern.

The present study demonstrates that four distinct electropherotypes were circulating at the same time, all of which had patterns consistent with that described as characteristic of group A (Noel *et al.* 1991, Unicom & Bishop 1989, Yap et al. 1992) . These electropherotypes remained constant during the entire period of seven months while the study was being carried out. This finding appears to be similar to the observations of Konno *et al.* (1984) who concluded that it is possible that a dominant strain of rotavirus is introduced to a susceptible community, causing infectious outbreaks, and persisting with apparent genomic stability for long periods of time (Konno et al. 1984).

All electropherotypes found were classified as short patterns, in accordance with the mobilities of segments 10 and 11, and their variations in electrophoretic mobility were strictly confined to the genomic segments that make up group III. In constrast, Rasool *et al.* (1989), during a seven year study in Malaysia, found long patterns predominating, with only occasional samples showing a short pattern (*Ibid.*). Our results also differ from some previous studies in Latin America done in Mexico and Chile where long forms were more predominant . However, since these studies are 15 years old, time might be as important as geography (Lebaron *et al.* 1990).

According to Bishop´s proposal (Bishop 1994), rotaviruses with short patterns belong to serotypes G2 and G8, and since G2 has the greater

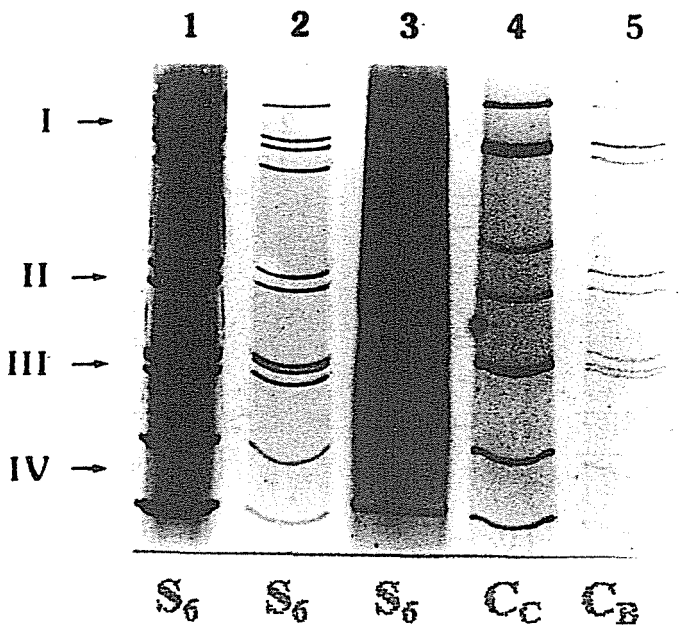

Figure 2. Lanes 1,2 Electropherotype W. Segment 8 is near to segment 7. Cc: Control bovine strain. CB: Bohl type porcine strain.

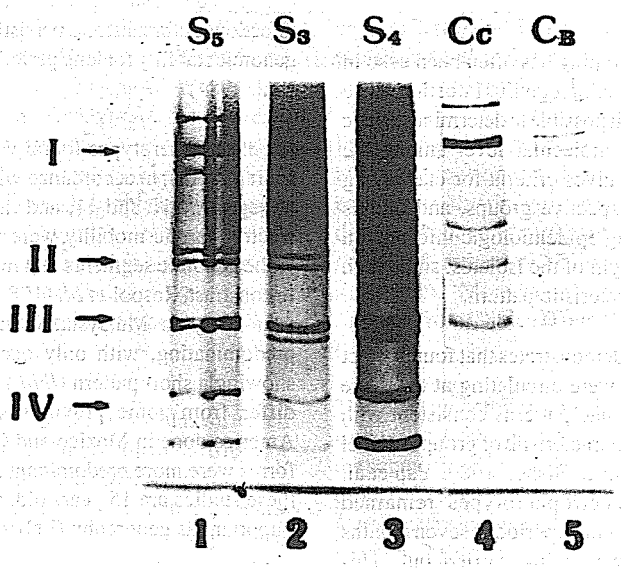

Figure 3. Lanes 1,2 Electropherotype X. The segments of group III migrate as a duplex. Electropherotype Y. (Lane 3) The segments of group III also migrate as a duplex but their migration is slower than that of Electropherotype X.

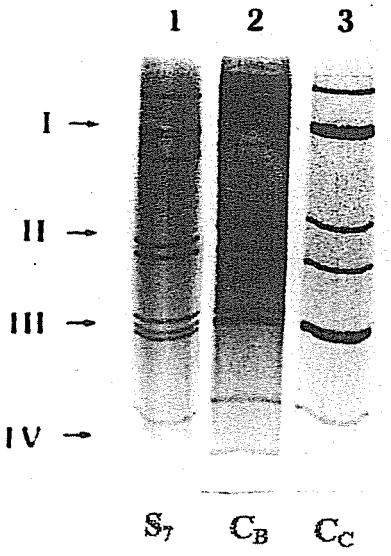

Figure 4. Lane 1 Electropherotype Z. The segments of group III migrate as a triplet. Segment number 8 migrates with a greater velocity and is closer to segment 9 than to segment 7.

prevalence in human diarrhea, we can suppose that the rotaviruses found here are G2. Since this is a preliminary study in which serotyping was not done, the above situation makes it very clear why it is necessary to include this type of classification in our subsequent studies. When the gel patterns of our samples are compared with those of known G2 rotaviruses, such as DS-1, a great resemblance is found, especially with our Y pattern due to the location of bands 8 and 9 (Gerna *et al.* 1992, Goueva *et al.* 1990).

However, further support of our rather unexpected findings was provided by Guerrero *et al.* (1994) who reported that their samples, which were collected in Bogotá during part of the same time period as ours, also showed only short patterns, with one super-short pattern, though their results showed electrophoretic variations in the migration of all genomic segments in all of the regions (I, II, III, IV), all of which suggest a great deal of genomic variability (Gerna *et al.* 1992) (more than our findings do). The three hundred samples that they analyzed yielded an electropherotype isolation incidence of slightly less than 15%. These samples were collected only during the months of April and May in 1994, from children with diarrhea in three hospitals, two of which, Clinica del Niño del Seguro Social and Lorencita Villegas, were the same as in our study.

Since there was a considerable overlap of both time and place in this study with ours, a closer comparison of our respective results could be useful to enhance the significance of both studies and give a broader scope through which we could view the rotavirus electropherotype distribution in the city of Bogotá in 1994. Both studies collected a similar number of samples; ours was over a longer period of time and a broader geographic area, theirs was more in-depth, in a shorter period and smaller area. Since both studies used the Laemmli system for electropherotype gels, the pH conditions were identical and the acrylamide concentrations were very close, (4.5 vs 5% for the stacking gel and 10 vs 8 % for the separating gel), it is possible to compare the results of the two studies to the point of seeing whether the electropherotypes are similar or not. They used minigels which were shorter than ours, and since longer gels resolve smaller differences than shorter, their finding of greater genetic diversity seems to be genuine.

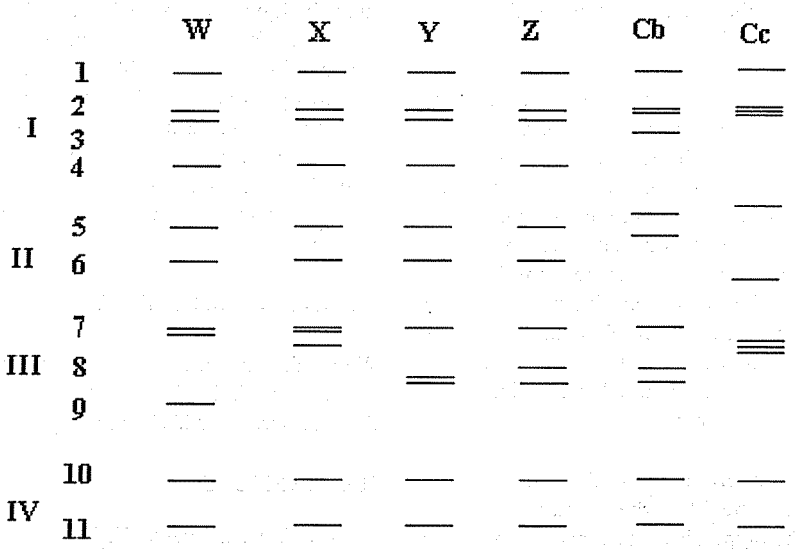

Figure 5. Schematic representation of electropherotypes W, X, Y and Z and controls (Cb and Cc).

In comparing the results, we can indeed see a great resemblance, inasmuch as our incidence of positive isolates was 17% here, and theirs was 15%. In addition, the electrophoretic patterns resembled each other in 86.6% of the total samples, since their electropherotype, designated S1, with a prevalence of 33.3%, could be our W (prevalence 59%). Their S2 with a prevalence of 43% resembles our Z, except that in region III the bands of the triplet are equidistant from each other, instead of segment 8 migrating closer to 9, making it a sort of transition between our patterns W (with 8 closer to 7) and Z. These both could be modifications produced by relatively few sequence changes in the clearly widespread "W-S1" electropherotype. Also their S4, with 10%, could easily be our electropherotype, X or Y since both have a doublet in region III. Since the only difference between X and Y is the relative migration speeds of the doublet, the only way to determine whether S4 is X or Y would be co-electrophoresis, which has not been done. About 13.4% of their samples are not similar to ours, since they had variations in regions I, II and IV. Also, they were all low incidence eletropherotypes, which our study would be statistically less likely to catch. These also could be emerging strains, candidates for replacement ot W-S1 as the dominant strain in the future (Konno *et al.* 1984).

Our results would tend to give an indication of broad trends over time, whereas theirs would be more likely to detect the less common electropherotypes circulating at the same time the samples were taken, and therefore might well show greater genetic diversity. Perhaps if both groups had processed more samples, the differences between their findings would be smaller. In any case, the results of the two studies combined do suggest a great deal of genetic variability with an unusual predominance of short electropherotypes.

Another important point that needs to be discussed, besides the behavior of the electropherotypes, is the fact that only 56 of the 328 diarrhea samples were positive for rotavirus by this technique. This gives an incidence of 17%, which is low compared to other studies (Matsui *et al.* 1989), where it reached 40% in winter and dropped to 8% in the summer. There *E. coli* was the principal cause of diarrhea, and rotavirus the second. However, the very high percentages (56-66%) ocurred for the winter months in the north, with summer isolation rates of 0-5.6% for the same years. Therefore, an isolation rate of 17% is not that unexpected for the tropics, where the viruses circulate all year round without all that much seasonal variation (Kapikian & Chanock 1990).

In addition, the composition of the populations sampled can affect the isolation rates considerably. For instance, since the rate of infection drops rapidly after the age of two, until the age of five, after which it usually becomes negligible, studies which sampled only children and babies two years old and under would also show higher isolation rates. Therefore, the samples from adults included would artificially drop the percentage, and doing a separate percentage for children two and under would probably raise it. The percentages are also affected when only fatal, or potentially fatal cases are analyzed, since rotavirus is up to 14 times more likely to be involved in severely dehydrating diarrhea (Wyatt *et al.* 1979). For example, one study in Egypt which analyzed only fatal or potentially fatal illnesses in infants under 18 months of age found that 34% of the cases were rotavirus positive, with the enterogenic *E. coli* ranking second (Shukry *et al.* 1986). In conclusion, many factors are involved in the activation and dissemination of this virus, and the experimental design can affect the apparent findings.

ACKNOWLEDGMENTS

We thank COLCIENCIAS for supporting this research and Carlos Corredor for his revision of the manuscript and his helpful suggestions.

REFERENCES

ALBERT, M.J., LEANNE, E.U. & BISHOP, R.F. 1987. Cultivation and characterization of human rotavirus with "super short" RNA patterns. *J. Clin. Microbiol.* **25**: 183-185.

BINGAN, F., UNICOMB, L.E., GUANGLI, T., ALI, A., RAHIM, M.Z. & TZIPORI, S. 1991. Cultivation and characterization of novel human group A rotavirus with long RNA electropherotype, subgroup II specificities, and serotype 2 VP7 genes. *J. Clin. Microbiol.* **29**: 2224-2227.

BLACKLOW, N.R. & CUKOR, G. 1981. Viral gastroenteritis. *N. Engl. J. Med.* **304**: 397-406.

BRIDGER, J.C., CLARKE, I.N. & McCRAE, M.A. 1982. Characterization of an antigenically distinct porcine rotavirus. *Infect. Immun.* **35**:1058-1062.

CAETANO, G.A. & GRESSHOFF, P.M. 1993. Staining nucleic acids with silver an alternative to radioisotopic and fluorescent labeling. *Promega Notes* **45**: 13-18.

CHOMCZYNSKI, P. & SACCHI, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Annal. Biochem.* **162**: 156-159.

DI MATTEO, A., SARASINI, A., SCOTTA, M.S., PAREA, M., LICARDI, G. & GERNA, G. 1989. Nosocomial outbreak of infant rotavirus diarrhea due to the appearance of a new serotype 4 strain. *J. Med. Virol.* **27**: 100-104.

DUNN, S.J., WARD, R.L., McNEAL, M., CROSS, T.J. & GREENBERG, H.B. 1993. Identification of a new neutralization epitope on VP7 of human serotype 2 rotavirus and evidence for electropherotype differences caused by single nucleotide substitutions. *Virology* **197**:397-404.

EIDEN, J.J., MOUZINHO, A., LINDSAY, D.A., GLASS, R.I., FANG, Z.Y. & TAYLOR, J.L. 1994. Serum antibody response to recombinant major inner capsid protein following human infection with group B rotavirus. *J. Clin. Microbiol.* **32**: 1599-1603.

ESPEJO, R.T., MARTINEZ, E., LOPEZ, S. & MUÑOZ, O. 1980. Different polypeptide composition of two human rotavirus types. *Infect. Immun.* **28**: 230-237.

ESTES, M.K., GRAHAM, D.Y. & DIMITROV, D.M. 1984. The molecular epidemiology of rotavirus gastroenteritis. *Prog. Med. Virol.* **29**: 1-22.

FIJTMAN, N.L., BARRANDEGUY, M.E., CORNAGLIA, E.M. & SCHUDEL, A.A. 1987. Variations and persistency of electropherotypes of bovine rotavirus field isolates. *Arch. Virol.* **96**: 275-281.

FLEWETT, T.H. 1978. Electron microscopy in the diagnosis of infectious diarrhea. *JAMA.* **173**: 538-543.

GERNA, G., SARASINI, A., PAREA, M., ARISTA, S., MIRANDA, P., BRUSSOW, H., HOSHINO, Y. & FLORES, J. 1992. Isolation and characterization of two distinct human rotavirus strains with G6 specificity. *J. Clin. Microbiol.* **30**: 9-16

GOMWALK, N.E., UMOH, U.J., GOSHAM, L.T. & AHMAD, A.A. 1993. Influence of climatic factors on rotavirus infection among children with acute gastroenteritis in Zaria northen Nigeria. *J. Trop. Pediatrics.* **39**: 293-297.

GORZIGLIA, M., NISHIKWA, K., HOSHINO, Y. & TANIGUCHI, K. 1990. Similarity of the outer capsid protein VP4 of the Gottfried-strain of porcine rotavirus to that of asymtomatic human rotavirus strain. *J. Virol.* **64**: 414-418

GUERRERO, C.A., MORENO, L.P., PENARANDA, J. & ACOSTA, O. 1994. Caracterización electroferotípica de rotavirus aislados de infantes con gastroenteritis en la ciudad de Bogotá. *Rev. Facultad de Medicina Univ. Nal.* **42**: 203-208.

GOUEVA, V., GLASS, R.I., WOODS, P., TANIGUCHI, K., CLARK, H.F., FORRESTER, B. & FANG, Z.Y. 1990. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. *J. Clin. Microbiol.* **28** (2): 276- 282.

HERRING, A.J., INGLIS, N.F., OJEH, C.K., SNODGRASS, D.R. & MENZIES, J.D. 1982.

Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. *J. Clin. Microbiol.* **16**:473-477.

KANG, S.Y., NAGARAJA, K.B. & NEWMAN, J.A. 1988. Physical, chemical, and serological characterization of avian rotaviruses. *Avian diseases* **32**:195-203.

KAPIKIAN, A.Z. & CHANOCK, R.M. 1990. Rotaviruses. In: Virology 2a edition. Edited by B.M. Fields. D.M. Knipe et al Raven Press. New York 353-387 pp.

KONNO, T., SATO, T., SUZUKI, H., KITAOKA, S., KATSUSHIMA, N., SAKAMOTO, M., YASAKI, N. & ISHIDA, N. 1984. Changing RNA patterns in rotavirus of human origin: Demonstration of a single dominant pattern at the start of an epidemic and various patterns thereafter. *J. Infec. Dis.* **149**: 683-687.

LABBE, M., CHARPILIENNE, A., CRAWFORD, S.E., ESTES, M.R. & COHEN, J. 1991. Expression of rotavirus VP2 produces empty corelike particles. *J. Virol.* **65**: 2946-2952.

LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

LeBARON, C.W., LEW, J., GLASS, R.I., WEBER, J.N. & RUIZ-PALACIOS, J.M. 1990. Annual rotavirus epidemic patterns in North America. Results of a 5-year retrospective survey of 88 centers in Canada, Mexico and United States. *JAMA* **264**: 983-988.

MATSUI, S.M., MACKOW, E.R. &GREENBERG, H.B. 1989. Molecular determinant of rotavirus neutralization and protection. *Adv. Virus Res.* **36**: 181-214

NICOLAS, J.C., POTHIER, P., COHEN, J., LOURENCO, M.H., THOMPSON, R., GUIMBAUD, P., CHENON, A., DAUVERGNE, M. & BRICOUT, F. 1984. Survey of human rotavirus propagation as study by electrophoresis in genomic RNA. *J. Infec. Dis.* **149**: 688-693.

NOEL, J.S., BEARDS, G.M. & CUBITT, W.D. 1991. Epidemiology survey of human rotavirus serotypes and electropherotypes in young children admitted to two children's hospitals in Northeast London from 1984 to 1990. *J. Clin. Microb.* **29**: 2213-2219.

PAUL, P.S. & STEVENSON, G.W. 1992. Rotavirus and Reovirus.In: Diseases of swine 7a edition Iowa State University Press, Ames U.S.A. 331-348 pp.

RASSOL, N., OTHMAN, R.Y., ADENAN, M.I. & HAMZAH, M. 1989. Temporal variation of Malasyan rotavirus electropherotypes. *Am. Soc. Microbiol.* **27**: 785-787.

SHIF, Y., SILBERSTAIN, Y., ABOUDY, Y., MENDELSON, E., MATTES, A. & GOTLIEB-STEMATSKY, T. 1992. Uniformity of serotype and electropherotype in local human rotavirus isolates during each of three successive cold seasons. *Int. J. Epidemiol.* **21**: 399-405.

SHUKRY, S., ZAKI, A.M., SHOUKRY, Y., TAGI, M.E. & HAMED, Z. 1986. Detection of enterophatogens in fatal and potentially fatal diarrheas in Cairo, Egypt. *J. Clin. Microbiol.* **24**:959-962.

STEELE, A.D., MINISE, Y.N., WILLIAMS, M.N., BOS, P. & ASPINALL, S. 1993. Electropherotyping of nosocomial rotavirus infection in a general pediatric unit showing the continual introduction of community strains. *J. Med. Viro.* **40**: 126-132.

SUKUMARAN, M., GOWDA, K., MAIYA, P.P., SRINIVAS, T.P., KUMAR, M.S., AIJAZ, S., REDDY, R.R., PADILLA, L., GREENBERG, H.B. & RAO, C.D. 1992. Exclusive asymtomatic neonatal infections by human rotavirus strains having subgrup I specificity and "Long" RNA electropherotype. *Arch. Virol.* **126**: 239-251.

UNICOMB, L.E. & BISHOP, R.F. 1989, Epidemiology of, rotavirus strains infecting children throghtout Australia during 1986-1987. A study of serotype and RNA electropherotype. *Arch. Virol.* **106**:23-34.

URASAWA, T., URASAWA, S. & TANIGUCHI, K. 1981. Sequential passages of human rotavirus in MA-104 cells. *Microbiol. Immunol.* **25**: 1025-1035.

WALLACE, D.M., 1987. Large and small-scale phenol extractions. *Methods in enzimology* **152**:33-83.

WYATT, R.G., YOLKEN , R.H. & URRUTIA, J.J. 1979. Diarrhea associated with rotavirus in rural Guatemala: a longitudinal study of 24 infants and young children. *Am J. Trop. Med Hyg.* **28**:325-328.

YAP, K.L., WONG, Y.H., KHOR, C.M. & OOI, Y.E. 1992. Rotavirus electropherotypes in Malasyan children. *Can. J. Microbiol.* **38**: 996-999.

YEAGER, M., BERRIMAN, J.A., BAKER, T.S. & BELLAMY, A.R. 1994. Three-dimensional structure of the rotavirus haemagglutinin VP4 by crio-electron microscopy and difference map analysis. *The EMBO Journal* **13**:1011-1018.