INFECTION AND IMMUNITY, Feb. 1983, p. 569–574
0019-9567/83/020569-06$02.00/0

Vol. 39, No. 2

Analysis of Human Rotavirus Mixed Electropherotypes

EUGENIO G. SPENCER,[1]* LUIS F. AVENDAÑO,[2] AND BERNARDO I. GARCÍA[1]

Laboratorio de Virología, División de Ciencias Básicas, Instituto de Nutrición y Tecnología de los Alimentos,[1] and Sección Virología, Departamento de Microbiología y Parasitología, Facultad de Medicina, División Ciencias Médicas Norte,[2] Universidad de Chile, Santiago 11, Chile

Received 25 May 1982/Accepted 14 October 1982

Mixed human rotavirus electropherotypes were detected in stool samples from patients with acute gastroenteritis in Santiago, Chile. These electropherotypes accounted for 10% of 149 samples studied. The finding of extra RNA fragments with respect to the regular 11 genome segments suggests the possibility of simultaneous or sequential infection by more than one electropherotype in a single diarrhea event or occurrence of modification in the length of the RNA segments during an infection. These possibilities arose from gel electrophoretic analysis of unique and sequential samples of human rotavirus genome RNA.

Human rotavirus has been recognized as a major cause of acute gastroenteritis in infants and young children (4, 11, 14). The incidence of the virus as a diarrheal agent has been established in several epidemiological studies with different diagnostic procedures (8, 18). The structure of rotavirus particles has been well defined in terms of morphology, protein, and nucleic acid content (14, 15). However, immunological characterization of the virus is so far incomplete, due to a number of reasons such as the relatedness with other animal rotaviruses. The recent development of a reliable system for in vitro growth of human rotavirus will provide a valuable tool for clarifying this point (21).

Besides electron microscopy, one of the most widely used methods for rotavirus characterization is gel electrophoresis of viral genome RNA (2, 3, 11). This method takes advantage of the fact that the rotavirus particle contains 11 segments of double-stranded RNA as the genome. The electrophoretic mobility of each segment is very characteristic and allows unequivocal determination of the presence of rotavirus in stool samples (2, 4, 11). By means of this technique, several investigators (2–4, 11, 14) have shown that human rotavirus does not show a unique electropherotype or pattern of genome RNA migration (i.e., of electrophoretic mobility of individual RNA segments) (5, 17).

On the contrary, gel electrophoresis of genome RNA obtained from individual stool samples always shows a well-defined and reproducible pattern of migration for each one of the 11 RNA segments, suggesting that an electropherotype may define an homogeneous population of viral particles in terms of their genome composition.

Several reports have shown that an electropherotype may reflect immunological properties of the virus. For instance, a relationship has been reported between the so called short and long rotavirus electropherotypes and serotypes 1 and 2, respectively (1, 17). Relationships between changes in viral genome size and changes of immunological properties have been determined in other viral systems, such as influenza virus (7, 16).

In the present report we have analyzed stool samples obtained from single diarrhea episodes, finding human rotavirus electropherotypes which contain extra RNA segments. Some infrequent cases of mixed electropherotype have been previously reported (11). The most interesting feature of these extra RNA segments is the fact that they seem to correspond to segments present in human rotavirus genome RNA. Also, the possibility that the presence of additional RNA fragments may correspond to sequential infection with two or more rotavirus electropherotypes was studied in three patients hospitalized at the same time, from whom daily samples were collected.

MATERIALS AND METHODS

Purification of virus from stools. Stool samples (1 to 2 g) were obtained from June 1979 to November 1981 from ambulatory and hospitalized patients aged 0 to 2 years with acute diarrhea. The sampling covered the children's hospitals Roberto del Rio and Luis Calvo Mackenna of the city of Santiago, Chile.

From a follow-up of one ward for infants with acute diarrhea, stools from three patients shedding rotavirus were studied. Patient A (see Fig. 3A) arrived at the hospital first, and rotavirus was detected from the first stool sample. Patients B and C (see Fig. 3B and C) were admitted 2 to 4 days later, and they started shedding rotavirus on day 8 of hospitalization.

Virus was partially purified as previously described

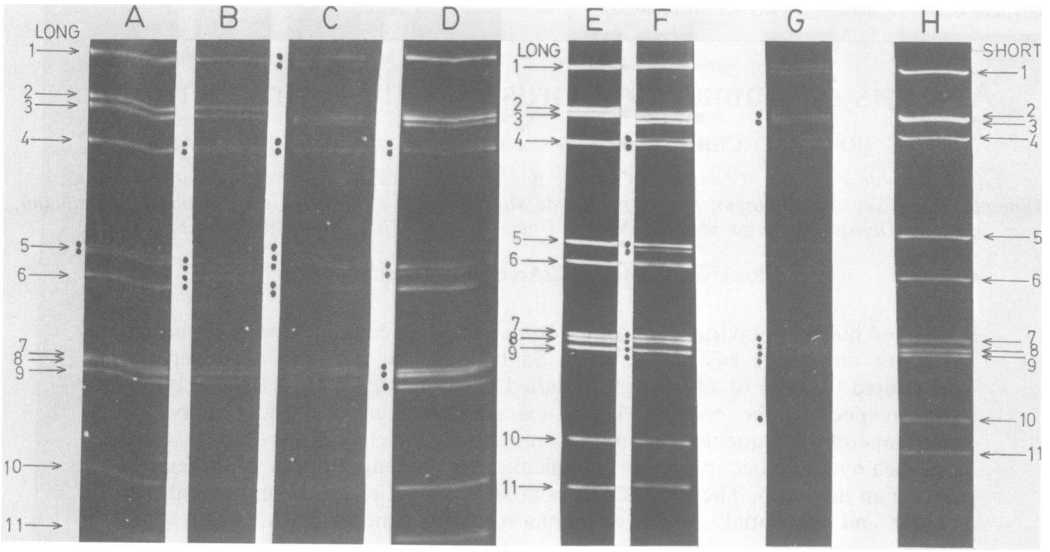

FIG. 1. Comparison of migration patterns of human rotavirus electropherotypes in 8% polyacrylamide gels. Migration was from top to bottom, and genome segments are numbered in decreasing order of size. Black circles indicate the presence of extra RNA fragments, as compared with the regular 11 that make up viral genome. LONG (lanes A and E) and SHORT (lane H) show the migration pattern of standard long and short electropherotypes, respectively. Samples A, B, C, and D were run in the same gel, and samples E, F, G and H were run in another gel.

(20). Briefly, stool samples were diluted with 5 volumes of distilled water, and the suspension was mixed with 1 volume of triflurotrichloroethane and centrifuged at 2,000 × *g* for 10 min. The supernatant was then precipitated overnight with polyethylene glycol at 4°C and centrifuged at 10,000 × *g* for 30 min. The supernatant was discarded, and the pellet was suspended in 2 mM Tris-hydrochloride buffer (pH 7.8). These fractions were stored at −20°C and used for RNA gel electrophoresis.

Viral RNA gel electrophoresis. Fractions (25 ul) of the resuspended polyethylene glycol pellet were mixed with 100 μl of 200 mM Tris-hydrochloride buffer (pH 8.4) containing 40 mM NaCl, 0.5 mM EDTA, and 1% sodium dodecyl sulfate. This solution was then deproteinized by extraction with 1 volume of phenol and chloroform and ethanol precipitated overnight at −20°C as previously described (20).

After centrifugation the RNA pellet was suspended in Laemmli sample buffer and subjected to gel electrophoresis. Gel electrophoresis was carried out in 8% polyacrylamide slab gels with 3% polyacrylamide stacking gels with the discontinuous buffer system of Laemmli (10), except that sodium dodecyl sulfate was omitted in all solutions. The gels were run 14 h at room temperature at 100 V. After the run the gels were stained for 3 to 4 h with ethidium bromide (1 μg/ml) and then photographed under UV light. Comparisons between different virus electropherotypes were done by mixing equal volumes of viral RNA samples and subjecting the mixture to gel electrophoresis.

RESULTS

Analysis of mixed electropherotypes. The electropherotypes of 149 samples were determined from human rotavirus isolates collected during the years 1979 through 1981. In 10% of them, the presence of extra RNA segments, as compared with the regular electropherotypes, was observed. As shown in Fig. 1, sample B, the electropherotype contains besides the regular 11 RNA segments, a doublet close to segment 4, and in the region of segments 5 and 6 there are two extra RNA segments that seem to correspond to combinations of the electropherotypes previously described for that particular region (5, 11). In sample C, there is also a mixture of segments close to segment 1 as well as close to segment 4. In the region of segments 5 and 6, the mixture of RNA segments shows differences in electrophoretic mobility in relation to sample B: close to fragment 5 there are two extra segments, and near 6 there is apparently only one. Samples A and D also have a mixture of RNA segments in region 5 and 6, but the situation seems to differ from those observed in samples B and C, because the mixture seems to correspond only to fragment 5. Samples A, B, and C do not show extra RNA fragments in the region of segments 7, 8, and 9 or segments 10 and 11. Only sample D seems to contain an extra RNA segment of higher electrophoretic mobility than that of segment 9. Sample F appears to be very similar to sample D, except that there is a doublet in the region of fragment 2 that is not clearly present in sample D. The most surprising result is shown in sample G, in which mixtures

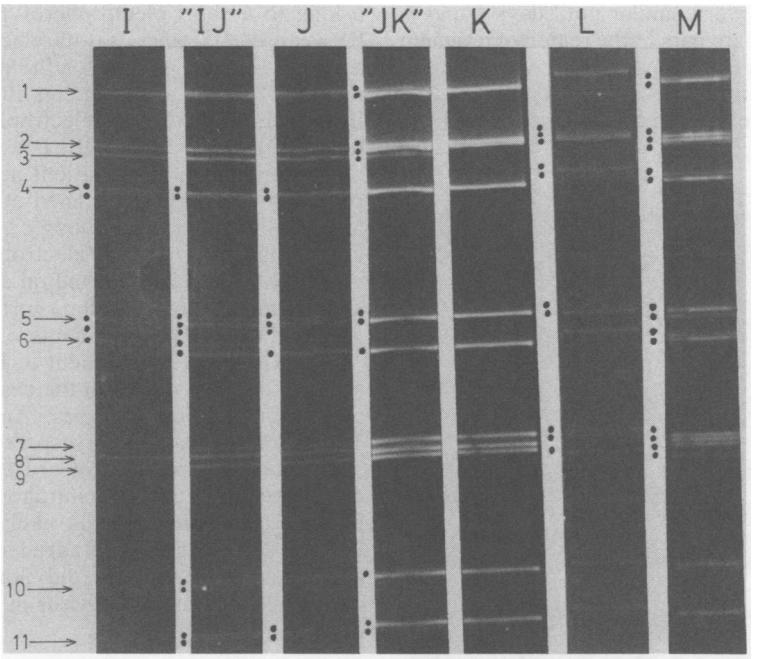

FIG. 2. Comparison of electrophoretic patterns of human rotaviruses. Conditions and symbols are as in Fig. 1. Samples I, IJ, J, JK, and K were run in the same gel, and samples L and M were run in another gel. Lane IJ corresponds to the coelectrophoresis of samples I and J, and lane JK corresponds to the same procedure applied to samples J and K.

of RNA segments seem to be present in all of the segments, including the region of fragments 10 and 11. This sample shows that the difference between the short and long electropherotypes may not correspond to changes in a single segment, as has been previously suggested (1).

Furthermore, evidence suggesting the occurrence of several, not a unique, long electropherotypes was obtained by coelectrophoresis of viral RNA samples (Fig. 2, lanes I and J). Sample I contains extra RNA segments in the regions of segments 4, 5, and 6, and sample J contains extra RNA segments in the regions of segments 4, 5, and 6 in the region of segments 10 and 11. These differences are also shown in the coelectrophoresis of both samples (lane IJ) in which a new doublet in fragment 1 is apparent. The presence of extra RNA segments in region 5 and 6 is also clarified; sample J seems to contain a triplet in the region of fragment 5, but only one type of fragment 6. No difference seems to exist between fragments 7, 8, and 9 in both samples. The characteristics of fragments 10 and 11 make sample J different from all cases described up to date for a long electropherotype. In fact, sample J exhibits a doublet in segment 11, as confirmed in the mixture of I and J as well as in the coelectrophoresis of samples J and K. Therefore sample J seems to contain a mixture of two long electropherotypes. The mixtures of electropherotypes described in Fig. 1 differ from those present in Fig. 2, lanes L and M, in which besides the mixture in segments 2, 3, 4, 5, and 6 there are mixtures in the region of fragments 7, 8, and 9. Sample L, for example, shows an extra segment between fragments 8 and 9, and sample M shows four RNA segments instead of the regular three.

The analysis of all of these samples shows that segments having a very close electrophoretic mobility seem to be present in different amounts in some cases, but in very similar amounts in others (e.g., sample G), as judged by their fluorescence intensity under UV light. Due to the characteristics of the nucleic acid stain used, molar ratios between RNA fragments which differ largely in molecular weight cannot be compared directly.

Changes of electropherotypes during diarrheal events. To detect possible changes in virus electropherotype during a diarrheal event, daily stool samples were obtained from three infants hospitalized at the same ward. Figure 3A, lanes 1 to 6, shows electropherotypes present in daily samples from one patient. Shedding of rotavirus was detected between days 1 and 6. All samples show a mixture of long and short electropherotypes in the region of segments 10 and 11, as can be observed by comparison with a standard short electropherotype (lane ST). These electro-

pherotypes are very similar until days 5 and 6, when a doublet appears in the region of fragment 5. Figure 3B, corresponding to a second case, represents a situation similar to the one described above, except that a sudden change from a long to a short electropherotype takes place between days 1 and 2. Both electropherotypes are very similar, except for the regions of segments 7, 8, and 9 and segments 10 and 11. Apart from this, no mixture of electropherotypes was detected by day 2 (Fig. 3B, lane 2). Figure 3C corresponds to a third patient in whose stools rotavirus could be determined at days 2 and 3 after hospitalization as a long electropherotype and at day 7 as a short electropherotype. No viral RNA could be detected on days 1, 4, or 6, and neither the long nor the short electropherotype had extra RNA fragments.

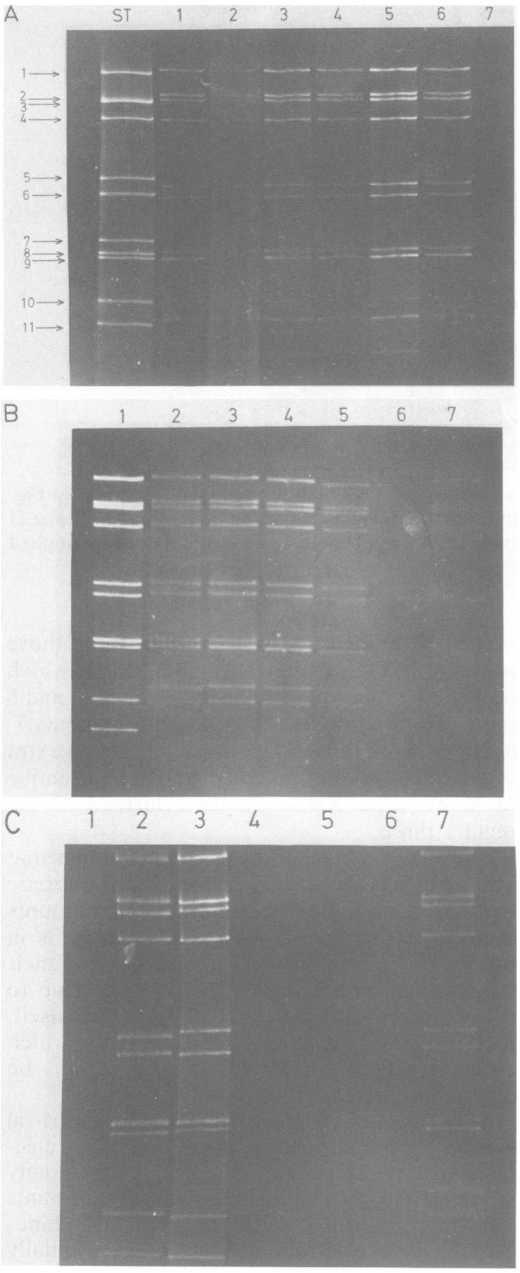

FIG. 3. Comparison of human rotavirus electropherotypes. Each panel shows serial determinations performed on samples obtained from a single patient. The numbers at top of the lanes indicate sequential days in which samples were taken, 1 corresponding to the day 1 of diarrheal illness in each case.

The RNA patterns present in Fig. 3B and C seem to be very similar in the case of both long and short electropherotypes. Analysis of Fig. 3A, B, and C leads us to suspect that the three short-electropherotype rotaviruses were identical, suggesting a probably intrahospital contamination. This conclusion is likely because patients B and C were hospitalized after patient A, and their stool samples did not contain any detectable amount of rotavirus in the first 3 or 4 days.

DISCUSSION

In recent years gel electrophoresis of RNA has been used not only to determine the presence of rotavirus, but also to classify the virus according to the electrophoretic mobility of the genome RNA segments (17, 18). The mobility pattern, called electropherotype, has been correlated with some immunological properties of the virus, e.g., the so-called short and long electropherotypes have been ascribed to serotypes 1 and 2, respectively (1, 17).

In this report we have studied the presence of extra RNA segments in some isolates of human rotavirus by gel electrophoresis.

An inspection of the electrophoretograms in Fig. 1 and 2 reveals that extra RNA segments can be found in rotavirus isolates covering the whole molecular weight range of standard RNA segments and varying in number from one to several for a single sample. Also, Fig. 1 and 2 show that these additional fragments may be usually correlated with segments that make up particular standard electropherotypes (11). Furthermore, the results suggest that extra RNA pieces do not arise from enzymatic cuts, for they could not explain the occurrence of extra segments related by size with diverse genome RNA groups in various samples. Moreover, the finding of supernumerary electropherotypes is a rather uncommon event among the number of cases so far analyzed in this laboratory. Alternatively, if nonspecific degradation of genome RNA segments were taking place, it would be expected to produce a family of fragments with a wide molecular weight range, rather than a few

well-defined segments. Apart from this, it has been shown elsewhere (1, 12, 13, 19) that each one of the 11 viral RNA segments codes for a particular polypeptide. Thus, any segment decay, either enzymatic or nonspecific, would be likely to prevent efficient virus infectivity or replication (or both).

There are several possible explanations for the appearance of mixed electropherotypes. Among them we can cite (i) variations in the number of RNA genome segments within the viral particle, (ii) modifications of the size of RNA segments during infection, (iii) sequential or simultaneous infection with two or more similar electropherotypes, and (iv) production of reassortant viruses.

The first possibility does not seem very likely if we take into account the morphology of virus particles (14, 15), which hints at the existence of a close relation between the amount of protein and the amount of nucleic acid. Thus, the presence of more than 11 RNA fragments within rotavirions is improbable. Large, aberrant viral particles of cylindrical shape have been reported (9), in which various arrangements of protein and nucleic acid may be possible, but these forms are scarce and they are probably uninfectious.

If modifications of the size of RNA segments happened during infection, one could expect finding some evidence thereof when electropherotypes are determined daily during diarrhea episodes. In contrast, the results in Fig. 3A, B, and C show that the appearance of extra RNA segments takes place suddenly, and that they do not undergo further modification, as detected by electrophoretic mobility, until they vanish or the illness is overcome. Nevertheless, the possibility exists that any unusual mutagenic event, such as insertion or deletion, may bring about an altered segment which, in turn, could coexist with the standard one, giving rise to an heterogeneous viral progeny. If altered segments do not preclude virus infectivity or replication, finally an heterogenous viral population could arise, with both electropherotypes being present in significant amounts and a sample of them showing one extra RNA fragment by gel electrophoresis assay.

The third possibility is suggested by the sequential infection by two very similar electropherotypes (Fig. 3C) in which the mixed electropherotype may correspond to the moment in which both viruses are being shed. In the case of Fig. 3C no virus was detected in between. However, the other results do not agree completely with this hypothesis. In fact, Fig. 3B shows a sudden change from one electropherotype (long) to another (short), whereas no mixed electropherotype could be detected. Furthermore, the results in Fig. 3A show an initial mixture of long and short electropherotypes to which an extra RNA fragment is added later. Clearly, a sequential or simultaneous infection model does not fit these findings thoroughly.

In the last possibility, two viruses may coinfect the same cell yielding a reassortant particle, as has been described for in vitro systems (6). Different reassortants may be obtained when reassortants or similar combinations of viruses reinfect other cells, but this does not account for the results in Fig. 3A. In that case, the late-appearing extra band near fragment 5 does not seem to originate in any of the previous existing segments of the mixed electropherotype. In addition, Fig. 3B shows a case of sudden change in the electrophoretic pattern, from which no reassortant electropherotype could be detected.

From the possibilities analyzed here, it seems likely that mixed electropherotypes may arise from sequential or simultaneous infections in which a more virulent virus having a particular electropherotype is able to direct the generation of reassortant viruses. However, the results in Fig. 3A (lanes 5 and 6) suggest that the appearance of mixed electropherotypes should be due to an amplification—by a still unknown mechanism—of base sequences in an individual RNA segment. Hence, variations in segment length may not be ruled out as an explanation for extra RNA segments. As a consequence, it should be kept in mind that particular electropherotypes with extra RNA segments may come from a set of causes rather than from a single one. The consequence of this event may be the appearance of defective interfering particles, especially from events that produce deletions in the viral genome.

The results in Figs. 1 and 2 suggest that electropherotypes may not serve as a valid approach for human rotavirus classification from which serological properties may be inferred (1, 17). Suitable characterization of human rotavirus strains will be attained in the future by means of virus propagation by in vitro tissue culture techniques.

ACKNOWLEDGMENTS

We are grateful of the expert technical assistance of Inés Orellana and Patricio Ortiz. We also appreciate the secretarial assistance of Felicita Rodríguez.

This work was supported in part by University of Chile grants B-15078213 to E.G.S. and M-8768.233 to L.F.A.

LITERATURE CITED

1. **Dyall-Smith, M. L., and I. Holmes.** 1981. Gene coding assignments of rotavirus double-stranded RNA segments 10 and 11. J. Virol. **38**:1099–1103.
2. **Espejo, R. T., L. F. Avendaño, O. Muñoz, P. Romero, J. Games, S. López, and J. Moncaya.** 1980. Comparison of human rotaviruses isolated in Mexico City and in Santiago, Chile, by electrophoretic migration of their double-

stranded ribonucleic acid genome segments. Infect. Immun. **30**:342–348.
3. **Espejo, R. T., E. Calderón, and N. González.** 1977. Distinct reovirus-like agents associated with acute infantile gastroenteritis. J. Clin. Microbiol. **6**:502–506.
4. **Espejo, R. T., E. Calderón, N. González, A. Salomón, A. Martuscelli, and P. Romero.** 1979. Presence of two distinct types of rotavirus in infants and young children hospitalized with acute gastroenteritis in México City, 1977. J. Infect. Dis. **139**:474–477.
5. **Espejo, R. T., O. Muñoz, F. Serafin, and P. Romero.** 1980. Shift in the prevalent human rotavirus detected by ribonucleic acid segment differences. Infect. Immun. **27**:351–354.
6. **Greenberg, H. G., A. R. Kalica, R. G. Wyatt, R. W. Jones, A. Z. Kapikian, and R. M. Chanock.** 1981. Rescue of noncultivatable human rotavirus by gene reassortment during mixed infection with its mutants of a cultivatable bovine rotavirus. Proc. Natl. Acad. Sci. U.S.A. **78**:420–424.
7. **Hinshaw, V. S., W. J. Bean, R. G. Webster, and B. C. Easterday.** 1978. The prevalence of influenza viruses in swine and antigenic relatedness of influenza viruses from man and swine. Virology **84**:51–62.
8. **Kalica, A. R., M. M. Sereno, R. G. Wyatt, C. A. Mebus, R. M. Chanock, and A. Z. Kapikian.** 1978. Comparison of human and animal rotavirus strains by gel electrophoresis of viral RNA. Virology **87**:247–255.
9. **Kimura, T., and T. Murakami.** 1977. Tubular structures associated with acute non-bacterial gastroenteritis in young children. Infect. Immun. **17**:157–160.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
11. **Lourenco, M. H., J. C. Nicolas, J. Cohen, R. Scherrer, and F. Bricout.** 1981. Study of human rotavirus genome by electrophoresis: attempt of classification among strains isolated in France. Ann. Virol. Inst. Pasteur **132**:161–173.
12. **Mason, B. B., D. Y. Graham, and M. K. Estes.** 1980. In vitro transcription and translation of simian rotavirus SA11 gene products. J. Virol. **33**:1111–1121.
13. **McCrae, M. A., and G. P. Faulkner-Valle.** 1981. Molecular biology of rotaviruses. I. Characterization of basic growth parameters and pattern of macromolecular synthesis. J. Virol. **39**:490–496.
14. **McNulty, M. S.** 1978. Rotaviruses. J. Gen. Virol. **40**:1–18.
15. **Newman, J. F., F. Brown, J. C. Bridger, and G. N. Woode.** 1975. Characterisation of a rotavirus. Nature (London) **258**:631–633.
16. **Palese, P.** 1977. The genes of Influenza virus. Cell **10**:1–10.
17. **Rodger, S. M., R. F. Bishop, C. Birch, B. McLean, and I. H. Holmes.** 1981. Molecular epidemiology of human rotaviruses in Melbourne, Australia, from 1973 to 1979 as determined by electrophoresis of genome ribonucleic acid. J. Clin. Microibol. **13**:272–278.
18. **Schnagl, R. D., S. M. Rodger, and I. H. Holmes.** 1981. Variations in human rotavirus electropherotypes occurring between rotaviruses gastroenteritis epidemics in Central Australia. Infect. Immun. **33**:17–21.
19. **Smith, M. L., I. Lazdins, and I. Holmes.** 1980. Coding assignments of double-stranded RNA segments of SA-11 rotavirus established by in vitro translation. J. Virol. **33**:976–982.
20. **Spencer, E., and M. L. Arias.** 1981. In vitro transcription catalyzed by heat-treated human rotavirus. J. Virol. **40**:1–10.
21. **Urasawa, T., S. Urasawa, and K. Taniaguchi.** 1981. Sequential passages of human rotavirus in MA-104 cells. Microbiol. Immunol. **25**:1025–1035.