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THE PREVALENCE AND THE MOLECULAR PATTERNS OF ROTAVIRUSES IN CALVES

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BY

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Praise the Lord!

Amen!!

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LIST OF ABBREVIATIONS

Most of the abbreviations used in this thesis are standard; however, attention is drawn to the following :

A	-	Adenine (in DNA base sequence)
Ap	-	Ampicillin
bp	-	base pairs
BRV	-	Bovine rotavirus
C	-	Cytosine
Ca	-	Capacity
ccc	-	Covalently closed circular
Cm	-	Chloramphenicol
CsCl	-	Caesium chloride
dCTP	-	Deoxycytidine triphosphate
DNA	-	Deoxyribonucleic acid
ds	-	Double-stranded
EDTA	-	Ethylenediamine-tetracetic acid
EM	-	Electron microscopy
ELISA	-	Enzyme-linked-immunosorbent assay
EtBr	-	Ethidium bromide
IEM	-	Immune Electron Microscopy
G	-	Guanine (in DNA base sequences)
HCl	-	Hydrochloric acid
IU	-	International unit
Kb	-	Kilobase pair
L	-	Litre
LB	-	Luria Bertani
M	-	Molar

mM	-	Millimolar
mol	-	Molecule
NaOH	-	Sodium hydroxide
OC	-	Open circular
OD	-	Optical density
PAGE	-	Polyacrylamide gel electrophoresis
PEG 6000	-	Polyethylene glycol 6000
psi	-	Pounds per square inch
RNA	-	Ribonucleic acid
rpm	-	Revolution per minute
SDS	-	Sodium dodecyl sulphate
SET	-	Sucrose-EDTA-Tris
SSC	-	Sodium saline citrate
ST DNA	-	Salmon testis DNA
T	-	Thymine (in DNA base sequences)
TE	-	Tris-HCl-EDTA
TES	-	Tris-HCl-EDTA-Nacl
Tm	-	Temperature of melting
Tris	-	Tris (hydroxymethyl) methylamine
ug	-	Microgrammes
ul	-	Microlitre
UK	-	United Kingdom
UV	-	Ultra violet
V/V	-	Volume per Volume
>	-	More or equal
<	-	Less or equal

Abstract of thesis submitted to the Senate of Universiti Pertanian Malaysia in fulfilment of the requirements for the degree of Master of Science.

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A study was conducted to determine the prevalence of rotavirus in calves in Malaysia. A total of 977 faecal specimens were collected over a four-month period from calves starting from 48 hours. The faeces were collected from diarrhoeic as well as non-diarrhoeic calves. Four hundred samples were collected from calves in Kluang, Johore Bahru and Selangor whilst another 577 were from 23 calves from the Beef Unit, Universiti Pertanian Malaysia. The faecal specimens were analysed for the presence of rotavirus by several methods, that is, electron microscopy (EM), polyacrylamide gel electrophoresis (PAGE) and a commercial enzyme-linked immunosorbent assay (ELISA) kit - Rotazyme II. Bacteriological examination was also carried out on these specimens.

Electron microscopy studies demonstrated rotaviruses in 69 (7.1%) of the total faecal specimens of 977, 50 from loose and 19 from non-diarrhoeic faeces. Seventy two percent of these were detected in calves less than three months old. Parvovirus or enteroviruses were also observed in 32 other diarrhoeic faecal samples. However, no coronavirus or pathogenic bacteria were detected.

Studies by PAGE showed that 97 (9.9%) specimens contained rotaviruses. Thirty two percent (72%) of the positive specimens were from non-diarrhoeic and the remaining percentage (28%) were from loose specimens. All of the rotavirus isolates belonged to the group A "long" electrophoretic pattern. None had unusual segment rearrangement or possessed extra segments. Majority (62%) of the genome electropherotypes belonged to the class of Ib IIa IIIb IVa. Two isolates were found to have a variant of the 11th segment. A total of 82 specimens (8.4%) were found to be positive for rotavirus by ELISA. Fifty five percent of the loose calves gave strong reactions whereas 10% of the non-diarrhoeic animals gave similar reactions. These diagnostic tests revealed that there was an association between diarrhoeic specimens and higher amounts of rotavirus antigen. The detection of rotaviruses in the non-diarrhoeic animals indicated that sub-clinical infections were present.

The analysis carried out in this study indicated that PAGE is clearly the most sensitive method compared with ELISA and EM. The ELISA proved to be more sensitive than the direct method of EM. When the results of PAGE and ELISA were compared to that of EM, the sensitivity and specificity of PAGE and ELISA were 99.0% and 96.8%, and 100% and 98.6% respectively. When ELISA results were compared with that of PAGE, the sensitivity and specificity were 78.4% and 99.3%.

Nucleic acid hybridization technique was also applied to study the prevalence and characteristics of rotavirus. The rotavirus nucleic acid clones were transformed into plasmidless *Escherichia coli (E. coli*), allowed to multiply and were later lysed from the *E. coli* by Nonidet P40. The cDNA were separated from the culture media by gradient density and dialysis. In the preliminary tests performed using selected bovine cDNA clones fo gene segments 2, 8 (group specificity) and 5 (non-structural protein), gene

segments 2 and 8 were shown to be more effective (9.6% and 9.1%) in rotaviral nucleic acid detection. This Sp2 and 8 were also shown to be comparable to the diagnostic method of PAGE for rotaviral detection. Gene segment 5 (8.1%) was comparable to Rotazyme II. These three DNA probes were also found to be more effective than the direct method of EM. In addition, cDNA clones representing different gene segments may be able to pick up in differences in nucleic acids or segments not readily recognisable by other diagnostic methods.

Abstrak tesis yang dikemukakan kepada Senat Universiti Pertanian Malaysia bagi memenuhi keperluan untuk Ijazah Master Sains.

KAJISELIDIK MENGENAI KEJADIAN DAN CORAK MOLEKULAR ROTAVIRUS DALAM ANAK-ANAK LEMBU

oleh

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November 1992

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Satu kajian telah dilakukan untuk menentukan kejadian rotavirus dalam anak-anak lembu di Malaysia. Sejumlah 977 spesimen-spesimen tinja telah dikumpulkan sepanjang empat bulan daripada anak-anak lembu berumur 48 jam. Spesimen-spesimen ini telah dikutipkan daripada anak-anak lembu sihat dan yang ceret-beret. Empat ratus sampel telah dikutip daripada anak-anak lembu di Kluang, Johor Bahru dan Selangor sementara 577 yang lainnya adalah daripada 23 anak- anak lembu daripada Unit Daging, Universiti Pertanian Malaysia. Spesimen-spesimen tinja ini dikaji untuk kehadiran rotavirus dengan beberapa kaedah iaitu, elektron mikroskop (EM), elektroforesis gel poliakrilamid (PAGE) dan satu esei "enzyme- linked immunosorbent" (ELISA) - Rotazyme II.

Kajian dengan elektron mikroskop menunjukkan terdapatnya rotavirus dalam 69(7.1%) daripada sejumlah 977 spesimen-spesimen tinja yang dikaji, 50 daripada tinja

lembek dan 19 daripada tinja sihat. Tujuh puluh dua peratus daripada ini dikesan dalam anak-anak lembu berumur kurang daripada tiga bulan. Parvovirus atau enterovirus juga diperhatikan bersama dalam 32 spesimen-spesimen tinja yang lain, tetapi, tiada coronavirus atau bakteria patogenik dijumpai.

Pengajian dengan PAGE menunjukkan bahawa 97 (9.9%) daripada spesimen mengandungi rotavirus. Tujuh puluh dua peratus (72%) daripada spesimen positif adalah daripada spesimen sihat dan yang bakinya (28%) daripada spesimen lembek. Kesemua elektroferotip tergolong dalam kumpulan A yang bercorak "panjang". Tiada spesimen didapati mempunyai penyusunan segmen yang ganjil dan tiada memiliki segmen yang berlebihan. Kebanyakan (62%) daripada elektroferotip genom digolongkan ke dalam kelas Ib IIa IIIb IVa. Dua isolat didapati mempunyai varian pada segmen kesebelas. Sejumlah 82 spesimen-spesimen (8.4%) didapati positif untuk rotavirus melalui kaedah ELISA. Lima puluh lima peratus daripada tinja lembek memberikan reaksi yang kuat manakala 10% daripada kes-kes sihat memberi reaksi yang sama. Ujian-ujian diagnostik ini menunjukkan bahwa terdapat satu perkaitan di antara spesimen-spesimen cirit-birit dan jumlah antigen yang lebih tinggi. Pengesanan rotavirus dalam binatang-binatang "sihat" menunjukkan keujudan jangkitan sub- klinikal.

Pengajian yang dilakukan menunjukkan bahawa ujian PAGE jelas merupakan kaedah yang paling sensitif dibandingkan dengan ELISA dan EM. Kaedah ELISA dibuktikan lebih sensitif daripada kaedah terus EM. Apabila keputusan-keputusan PAGE dan ELISA dibandingkan dengan EM, kepekaan dan spesifisiti PAGE dan ELISA adalah 99.0% dan 96.8%, dan 100% dan 98.6% masing-masing. Apabila keputusan-keputusan ELISA dibandingkan dengan PAGE, kepekaan dan spesifisiti adalah 78.4% dan 99.3%.

Kaedah penghibridan asid nukleik juga digunakan untuk mengaji kejadian dan sifat-sifat rotavirus. Klon-klon asid nukleik rotavirus dimasukkan ke dalam *Escherichia coli (E. coli)* tanpa plasmid, dibenarkan mengganda dan kemudiannya dipecahkan daripada *E. coli* dengan Nonidet P40. cDNA dipisahkan daripada kultur media melalui ketumpatan kecerunan dan dialisis. Dalam ujian-ujian pendahuluan yang dilakukan dengan menggunakan klon-klon cDNA terpilih, iaitu klon segmen gen 2, 8 (spesifisiti kumpulan) dan 5 (protein bukan-binaan), segmen-segmen gen 2 dan 8 didapati lebih efektif (9.6% dan 9.1%) dalam pengesanan asid nukleik rotavirus Sp2 dan 8 ini juga ditunjukkan setaraf dengan kaedah diagnostik PAGE untuk pengesanan rotavirus. Segmen gen 5 (8.1%) adalah setaraf dengan Rotazyme II. Ketiga-tiga prob DNA ini juga didapati lebih berkesan daripada kaedah terus EM. Tambahan pula, klon- klon cDNA yang mewakili segmen-segmen gen yang berlainan mungkin dapat mencatitkan perbezaan-perbezaan yang kurang jelas dikenali oleh kaedah-kaedah diagnostik yang lain.

CHAPTER I

GENERAL INTRODUCTION

Historical Background

In 1959 Sabin proposed that a group of viruses previously classified as members of the echovirus 10 group be reclassified in a new family. Because these virus were typically isolated from the respiratory and gastrointestinal (GI) tracts and were not associated with any known disease state, he proposed the name `reovirus' (respiratory enteric orphan virus) (Sabin, 1959). In the early 1970s, the orbiviruses were removed from the `unclassified arbovirus' group and were included among the Reoviridae. The initial impetus for this reclassification was the recognition that the orbiviruses differed from other arboviruses in their lack of an envelope and in the presence of a segmented double-stranded RNA genome (Bordon *et al.*, 1971). The reovirus family was enlarged further when it was recognized in the mid and late 1970s that a large group of viruses, named rotaviruses for their distinctive electron microscopic (EM) appearance, also had a segmented dsRNA genome (Flewett and Woode, 1978). Three additional genera of segmented dsRNA viruses which infected insects and plants (cypovirus, phytovirus, fijivirus) are also members of the reovirus family (Joklik, 1983; Matthews, 1979).

The family Reoviridae, as it is currently constituted, contains six genera. Three of the genera-orthoreoviruses, orbiviruses, and rotaviruses-infect animals including man; the remaining three genera include viruses which infect only plants and insects. The virus belonging to these genera are classified together because they have similar structural features, nucleic acid type and composition, and replicative strategies (Table 1).

Table 1

General Characteristics of the Reoviridae

Structure
Size ≈ 70 nm
Shape = nearly spherical icosahedron
Nonenveloped
Double protein capsid shell Genome
Double-stranded RNA
10-12 segments
Replication
Fully cytoplasmic replication
Inclusion body formation
Lack of complete uncoating of virions
Full-length, capped (5'), nonpolyadenylated mRNA transcripts
Possession of all enzymes required for dsRNA transcription

Source

Joklik, 1983

Rotaviruses

Rotaviruses are now widely recognized as the major etiologic agents of gastroenteritis of infants and young children in most areas of the world (Barnett, 1983). The discovery in 1973 of the 70 nm human rotavirus and its association with gastroenteritis of infants and young children represents a major advance in elucidating the cause of acute infectious non-bacterial gastroenteritis (Bishop *et al.*, 1973).

Rotaviruses, which are found in a wide range of animal species, were named from their characteristic wheel-like appearance in the electron microscope (from the Latin `rota' a wheel). As mentioned earlier they belong to the Reoviridae, a group of viruses with double-stranded (ds) ribonucleic acid (RNA) to which reoviruses and orbiviruses belong.

Genome Structure

The viral genome of 11 segments of dsRNA is contained within the virus core capsid. The segments range in size from 667 (segment 11) to 3302 base pairs (segment 1) with the total genome containing approximately 18,522 base pairs (Table 2). This number, compared from sequence data of segments from different virus strains, agree closely with the genome size (18,680 base pairs) determined by EM measurements (Rixon *et al.*, 1984).

Hydrodynamic studies of the flexibility or stiffness of isolated rotavirus RNA segments in solution have indicated that these RNA segments are packaged into the rotavirus capsid by intimate protein-RNA interactions. The proteins directly responsible for segment packaging remain unclear. The structural proteins present in core particles (VP 1, VP 2, and VP 3) are obvious candidate, but nonstructural proteins may also play a scaffolding role.

Table 2

Nucleotide Sequences of Rotavirus RNA Segments

RNA segment	RNA size	Protein
1	3,302	VP1
2	2,687	VP2
3	2,591	VP3
4	2,362	VP4
5	2,581	NS53
6	1,356	VP6
7	1,104	NS34
8	1,059	NS35
9	1,062	VP7
10	751	NS28
11	667	NS26

Source

Rixon *et al.*, 1984.

Viral Structure

The morphologic appearance of rotavirus particles is distinctive. As mentioned before, intact virus particles resemble a wheel, with short spikes and a well-defined rim, when examined by negative-stain EM. Three types of particles (double-shelled, single shelled and core) are often observed by EM (Fig.1). Double-shelled particles are 76.5 nm in diameter, single-shelled particles are 70.5 nm in diameter, and cores are 50 nm in diameter. Single-shelled particles and cores can be produced by chemical disruption of

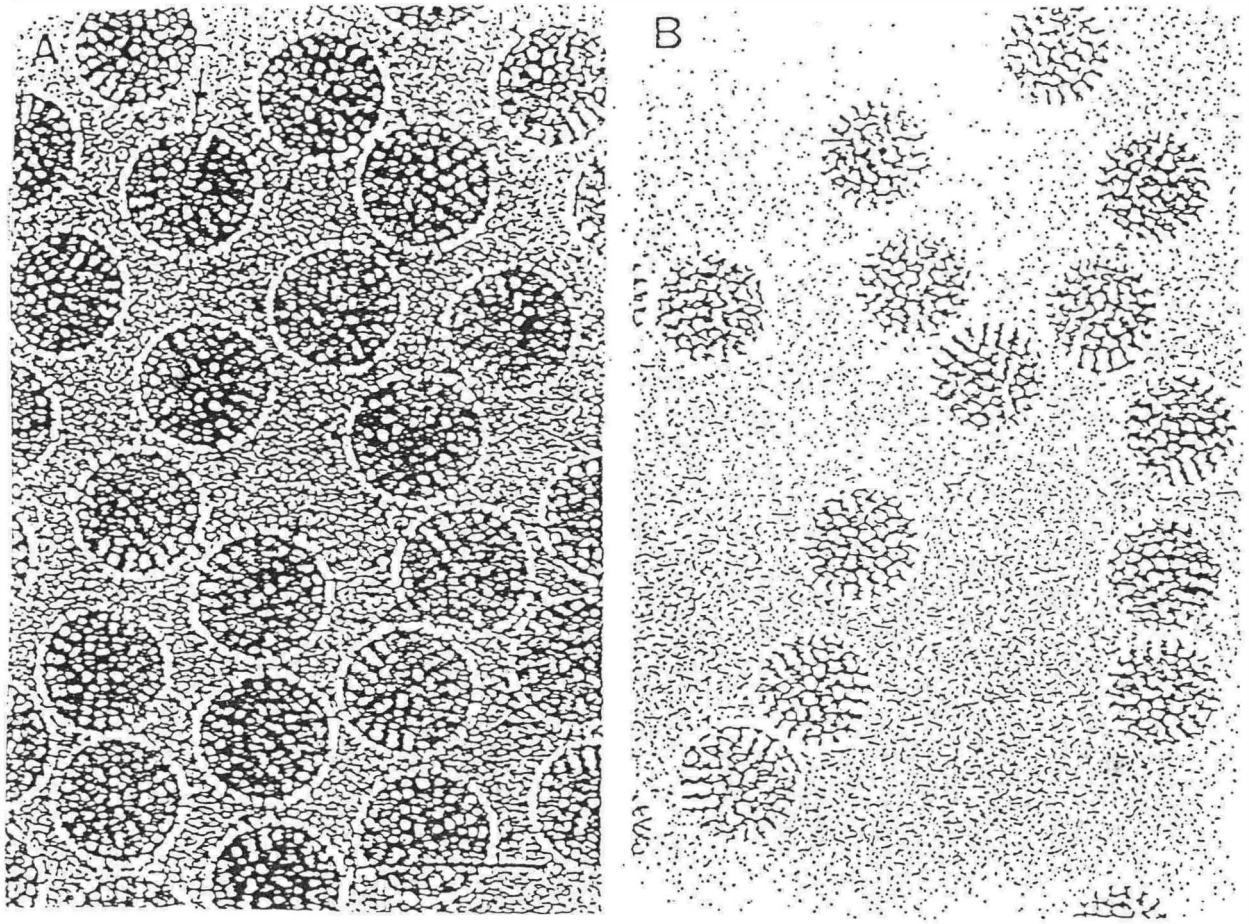

Figure 1 Electron Micrographs of (A) Double-Shelled and (B) Single-Shelled SA-11 Particles Embedded *in vitreous* Ice and Examined by Cryoelectron Microscopy. Arrows indicate the spikes visible on the outer surface of some of the virions. Bar. 100 nm.

double-shelled or single- shelled particles, respectively. It is unknown whether the single-shelled or core particles are identical to subviral particles synthesized during virus replication (Estes and Cohen, 1989).

Early structural studies of rotavirus particles agreed that these particles possessed icosahedral symmetry for the two outer layers. A distinctive feature of the virus structure is the presence of 132 large channels spanning both shells and linking the outer surface with the inner core: 120 channels are along the six-coordinated centers and 12 are along the five- coordinated centers (Estes and Cohen 1989).

Virion Characteristics

Morphologic and biochemical characteristics shared by members of the rotavirus genus include the following: i) mature virus particles are nonenveloped and possess a multilayered icosahedral protein capsid, approximately 75 nm in diameter, composed of an outer layer, an inner layer, and a core; ii) therus genome consists of 11 segments of dsRNA; iii) particles contain an RNA-dependent RNA polymerase and other enzymes capable of producing capped RNA transcripts; iv) virus replication occurs in the cytoplasm of infected cells; v) the viruses are capable of genetic reassortment; vi) virus cultivation *in vitro* is facilitated by treatment with proteolytic enzymes, which enhances infectivity by cleavage of the outer capsid polypeptide VP4; vii) virus particles are formed by budding into the endoplasmic reticulum (ER), and enveloped particles are evident transiently at this stage of morphogenesis; and viii) mature particles are liberated from infected cells by cell lysis. Only a few of the numerous isolates included in the rotavirus genus are known to possess all these characteristics. Instead, most isolates have been included in the genus on the basis of morphology, the presence of 11 segments of dsRNA, or antigenic cross-reactivity (Estes and Cohen, 1989).

Virion Classification

Until 1980, all rotaviruses were thought to have common antigens that were detectable by immunofluorescence, complement fixation, or enzyme-linked immunosorbent assays (ELISAs), (Flewett and Woode, 1978; Woode *et al.*, 1983) and fall into a limited number of species-specific virus serotypes. Recent studies have shown that neither of these early hypotheses is true. Instead, it is now known that (i) many isolates do not share cross-reacting antigens with the rotavirus originally shown to cause gastroenteritis in the young (Bridger, 1987; Pedley *et al.*, 1983a; b), (ii) many (at least six) human serotypes exist (Table 3), (iii) strains of animal and human origin occur within the same serotype (Table 3), and (iv) two genome segments encode neutralization antigens, and these segments can segregate (reassort) independently (Hoshino *et al.*, 1985; Offit *et al.*, 1986).

These developments have emphasized the need for and importance of developing a serologic classification scheme for rotavirus isolates that allows for the presence of multiple groups of rotaviruses and for the existence of serotypes which cross species. This need has been addressed by a number of investigators (Graham and Estes, 1985; Hoshino *et al.*, 1984; Pedley *et al.*, 1983b; Rodger and Holmes, 1979), but a uniform classification system remains to be established.

Although no classification system has been officially adopted, rotaviruses are classified serologically first into groups (or serogroups) containing viruses that share cross- reacting antigens detectable by serologic tests such as immuno- fluorescence, ELISA, and immunoelectron microscopy. Six distinct groups (A to F) of viruses have been described (Bridger, 1987; Nakata *et al.*, 1986; Pedley *et al.*, 1983b) (Fig. 2). Group A, B, and C rotaviruses have been found in both human and animals; group D, E, and F rotaviruses have been found only in animals (Bridger, 1987). Group A

Table 3

Classification of Group A Rotaviruses Based on Outer Capsid Protein VP7

VP7 Serotype	Strain from following species of origin	
	Human	Animal[a]
1	Wa, KU, RV-4, K8, M37D, S12, Mont	
2	DS-1, S2, RV-5, RV-6, HN-126, 1076	
3	Ito, Yo, P, M, Nemoto,	Si/SA11, RRV:1, Si/AU-1, RV-3,W178 RRV:2,Po/CRW-8,Po/MDR-13,Po/AT/76,Ca/K9, Ca/CU-1, La/Ala, La/C-11, La/R-2, Mu/EB, Mu/EW, Eq/H-2, Eq/F1-14, Fe/Taka, Fe/2, Fe/3
4	Hochi, Hosakawa, St. Thomas-3, VA70, 57M	Po/Gottfried, Po/SB-1A, Po/SB-2, Po/BEN144
5		Po/OSU, Po/TFR-41, Po/EE, Eq/H-1
6		Bo/NCDV, Bo/UK. Bo/486, Bo/Rf, Bo/WC3
7		Ch/2, Ty/1
8	69M, B37	
9	WI-61, F45, AU32	
10		Bo/223
11		Po/YM

Source

Hoshino *et al.*, 1985.

[a] Abbreviations: Si, simian: Po, porcine; Bo, bovine; Eq. equine; Fe, feline: La, lapine; Ch, chicken (avian); Ty, turkey (avian); Ca, canine; Mu, murine. The year and country of origin are not indicated because this information is not available for all strains.

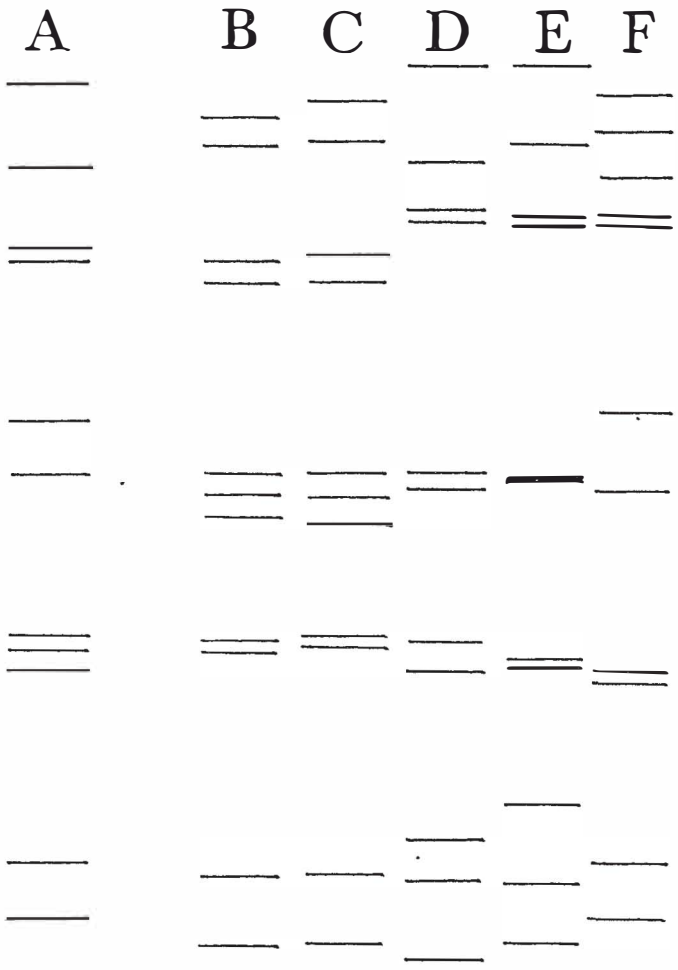

Figure 2 Diagram showing the Different RNA Banding Patterns Observed after Electrophoresis of Rotavirus RNA.