MOLECULAR DETERMINANTS OF ROTAVIRUS VIRULENCE

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DEDICATED TO

MY DAUGHTERS

ADITI & ASTHA

Ho-nge kaamyaab ---------- aek din.

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DECLARATION

All the results presented in this thesis were obtained by the author unless specifically acknowledged and no part of this thesis has been previously presented in application for a degree.

All sources of information and materials are indicated in the text.

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ABBREVIATIONS

Ao	Angstrom
aa	amino acid
Ab	antibody
bp	base pair
cDNA	complementary DNA
Ci	curies
cpe	cytopathic effect
CMI	cell-mediated immunity
CTL	cytotoxic T-lymphocyte
d	day
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ds	double-shelled
ds RNA	double-stranded ribonucleic acid
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
EM	electron microscope
ER	endoplasmic reticulum
FCS	foetal calf serum
g	gravitational acceleration
HA	haemagglutinin
hr	hours
Ig	immunoglobulin
Kb	kilobase
K	kilodalton
LD_{50}	lethal dose 50

mM	millimolar
Mab	monoclonal antibody
MHC	major histocompatibility complex
mg	milligram
min	minutes
ml	millilitre
m.o.i.	multiplicity of infection
mRNA	messenger RNA
mw	molecular weight
NCR	non-coding region
NP40	nonidet P40
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAGE-SS	polyacrylamide gel electrophoresis-silver staining
PBS	phosphate buffer saline
PCR	polymerase chain reaction
pfu	plaque forming units
p.i.	post inoculation
PRN	plaque reduction neutralisation
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
RNP	ribonucleoprotein
rpm	revolutions per minute
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulphate
ss	single-shelled
ss-RNA	single-stranded ribonucleic acid
TEMED	N,N,N'N' tetramethylethylenediamine
Tris	tris(hydroxyl)aminomethane

ts	temperature-sensitive
uv	ultraviolet
v/v	volume for volume
w/v	weight for volume
wt	wild-type
µg	microgram
µl	microlitre

SUMMARY

Rotaviruses are the single most important etiological agent of severe diarrhoea in infants and young children in both developed and developing countries. The World Health Organisation has identified the development of a rotavirus vaccine as a priority area for routine childhood immunisation to control rotavirus infections. However, the candidate vaccine strains have not been very successful. The main aim of this project was to map rotavirus virulence to its gene segments. Such studies can help in developing better vaccines for the control of rotavirus induced diarrhoea.

A three step approach was undertaken (i) development of an animal model, (ii) construction and characterisation of reassortants between rotavirus strains of different virulence, (iii) mapping virulence to rotavirus gene segments.

The mouse model developed revealed that the outcome of rotavirus infection was influenced by viral dose and viral strain as well as by host age and host strain. Homologous murine rotavirus strain was found to be most virulent. Among the heterologous strains studied, the OSU strain was found to be most virulent and UKtc strain the least virulent. The CD-1 strain of mouse was found to be the most susceptible to virus infection and C57/BL the least susceptible.

A very simple and rapid nucleic acid extraction method has been developed that requires only one centrifugation step and circumvents the use of any hazardous organic chemicals, which can be applied to very large numbers of samples saving time and labour.

Rotavirus reassortants were constructed in a variety of ways and their genotype determined from relative mobility of their gene segments on polyacrylamide gels and restriction enzyme digestion of PCR amplified products. Twenty two reassortants (2%) were identified out of more than 1100 progeny clones examined and these reassortants belonged to 15 different genotypes. Possible reasons for obtaining this low number of reassortants are discussed. No reassortant could be identified between a murine rotavirus and other heterologous rotavirus strains. Preliminary sequence of VP7 gene of murine rotavirus strains, EDIM and EBR, was found to be different to the published rotavirus sequences including the recently published five murine rotavirus strains.

The virulence mapping studies conducted in mice with some of the 22 reassortants obtained in the present study showed that gene 4 of the OSU and UKtc strains was involved in virulence. Segment 5 of OSU strain and segments 5, and 8 of UKtc strain may also be involved in virulence.

Chapter 1

Rotaviruses

1.1 Economic importance

Diarrhoea, a multifactorial disease, is the principal cause of mortality in infants and young children in the developing countries in Asia, Africa, and Latin America where the syndrome accounts annually for 3-5 billion cases and approximately 5-10 million deaths (Institute of Medicine, 1985; Walsh and Warren 1979). Another estimate which reviewed data from several longitudinal studies in children revealed that 4.6 million diarrhoea deaths and 744 million to 1 billion episodes of diarrhoea occurred in children less than 5 years of age in developing countries, excluding China (Snyder and Merson, 1982). In developed countries diarrhoeal deaths occur infrequently but morbidity from diarrhoeal illness is high (Institute of Medicine, 1986; Rodriguez *et al.*, 1987). Acute diarrhoeal syndrome is recognised as being second in frequency only to the common cold among the illness affecting US. families under epidemiological surveillance (Cukor and Blacklow, 1984).

Rotavirus induced gastro-enteritis is a global disease problem, which affects primarily infants and young children and also the neonates of a wide variety of mammals and birds including all the major domestic livestock. Rotaviruses cause 35-50% of the severe diarrhoeal episodes in infants and young children in both developed and developing countries (Kapikian and Chanock, 1990). As a consequence they are estimated to cause about a million deaths in the underdeveloped countries (Institute of Medicine, 1986; WHO, 1989) and be responsible for an estimated economic burden of 500 million to 1 billion dollars annually in the developed world (Offit *et al.*, 1991; Glass *et al.*, 1994). In addition to the medical problems they cause, rotaviruses are major veterinary pathogens with one estimate holding them to be responsible for losses to the British dairy industry alone of millions of pounds annually due to animal wasting and death (Johnson and McCrae, 1989).

1.2 Historical Background

Rotavirus genus of the *Reoviridae* family was established as a separate genus by the International Committee on Taxonomy of Virus at the Fourth International Congress for Virology in 1978 (Mathews, 1979a). In early publications, members of the genus were referred to as 'orbivirus-like' (Bishop *et al.,* 1973; Middleton *et al.*, 1974), 'infantile gastro-enteritis virus [orbivirus group]' (Petric *et al.*, 1975), 'reovirus-like agents' (Fernelius *et al.*, 1972; Kapikian *et al.*, 1974), 'Duovirus' (Davidson *et al.*, 1975) besides 'rotavirus' (Flewett *et al.*, 1974).

The earliest reported work on what was probably rotavirus gastro-enteritis was done by Light and Hodes (1943, 1949). They isolated a filterable agent from the stools of infants with gastro-enteritis and showed that it produced diarrhoea in newborn calves. About 30 years later, rotavirus particles were detected by electron microscopy in that lyophilised material (Hodes, 1977). After the work of Light and Hodes, other viruses with characteristics of Reoviridae were isolated from faecal specimens, including the viruses causing epizootic diarrhoea of infant mouse or 'EDIM' (Cheever and Muller, 1947; Kraft, 1957; Banfield *et al.*, 1968), SA11 from vervet monkeys (Malherbe and Harwin, 1963) and 'O' agent from the intestinal washings of cattle and sheep (Malherbe and Strickland-Cholmley, 1967). These viruses were subsequently shown to be rotaviruses.

Mebus *et al.*, (1969) reported experimental production of diarrhoea in colostrum-deprived calves by inoculating them with bacteria-free filtrates of diarrhoeic faeces. Virus particles 65 nm in diameter were found in large numbers in the faeces of infected animals. In 1971, Mebus *et al.*, reported successful cultivation of Nebraska calf diarrhoea virus (NCDV) in primary foetal bovine cell culture. Further characterisation of

the virus showed that it was similar to the reovirus group in its morphology and some of its chemical properties but was serologically unrelated to reovirus types 1 and 3 (Fernelius *et al.*, 1972; Welch and Twiehaus, 1973).

The early work in animals received little attention until electron microscopic examination of biopsy material from children with acute non-bacterial gastro-enteritis revealed the presence of orbivirus-like particles within epithelial cells of the duodenal mucosa (Bishop *et al.*, 1973). This first report was rapidly followed by others in which virus particles morphologically indistinguishable from calf reo-like virus were detected in the faeces of children with gastro-enteritis in England (Flewett *et al.*, 1973), Australia (Bishop *et al.*, 1974), Canada (Middleton *et al.*, 1974), and the U.S.A. (Kapikian *et al.*, 1974).

1.3 Properties of rotavirus

1.3.1 Morphology

Rotaviruses have a distinctive morphological appearance by negative-stain electron microscopy. The term *rotavirus* is derived from the Latin word 'rota', which means wheel, and was suggested because the sharply defined circular outline of the outer capsid gives the appearance of the rim of a wheel placed on short spokes, radiating from a wide hub (Flewett *et al.*, 1974). Three types of particles (double-shelled, single-shelled and core) have been observed by electron microscopy (Estes and Cohen, 1989). The double-shelled particles also called 'complete' or 'smooth' particles, have a diameter of ~70 nm (Figure 1) and exhibit T = 13*l* icosahedral symmetry (Ludert *et al.*, 1986; Roseto *et al.*, 1979; Prasad *et al.*, 1988). Particles lacking the outer shell are called 'incomplete' or 'single-shelled' particles and those lacking both of these shells are called 'core' particles. Some tubular structures and

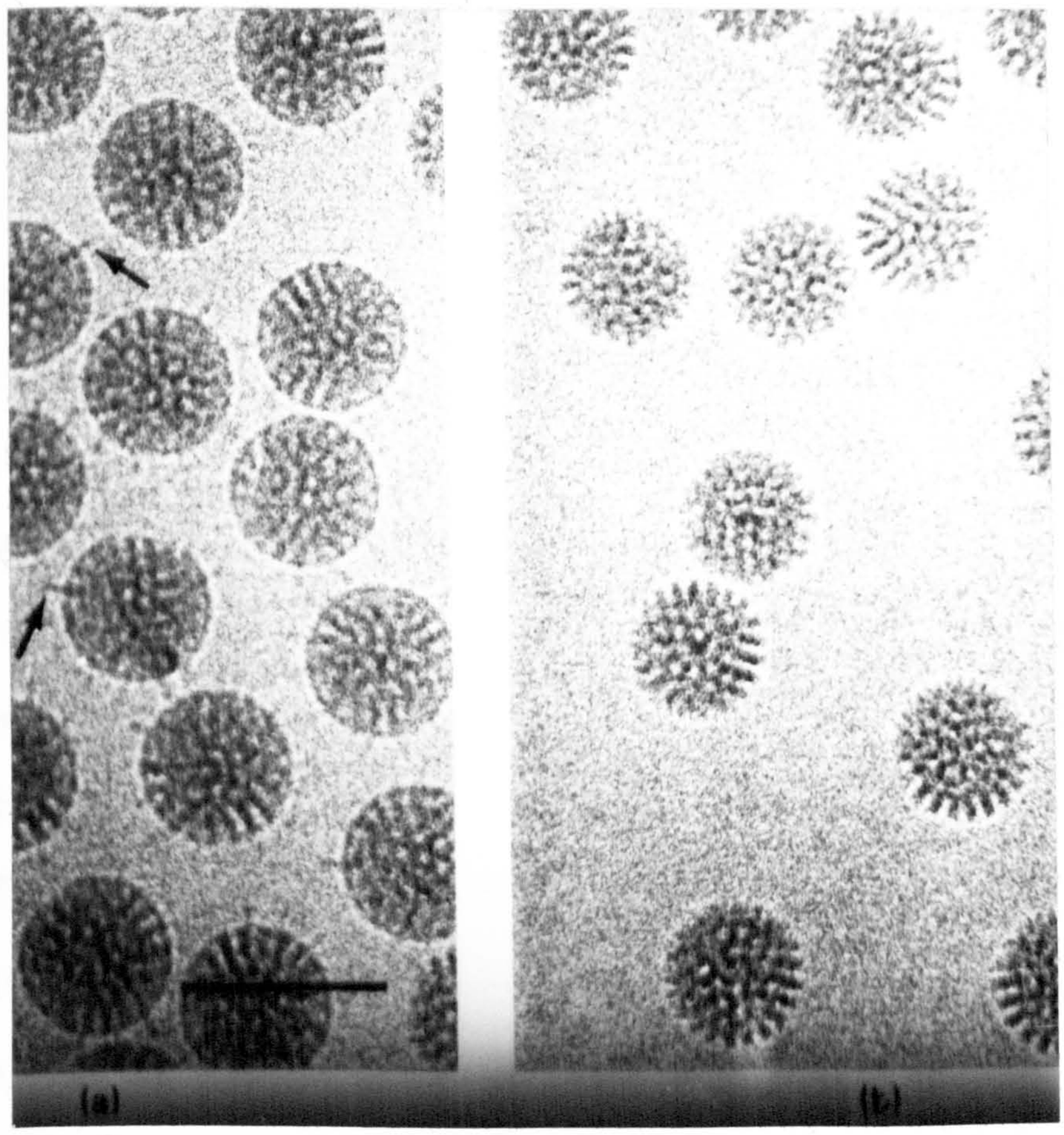


Figure 1 : Electron micrographs of (a) double-shelled and (b) single-shelled rotavirions embedded in vitreous ice. Arrows indicate presence of the spikes on the surface. The scale bar represents 100 nm.

Adapted from Prasad *et al.*, (1988).

disorganised sheets are frequently observed in rotavirus preparations which probably result from the abnormal assembly of viral capsid proteins (Chasey and Labran, 1983). Based on the radial density profile computed from the three dimensional density map of the mature virion, Prasad and Chiu, (1994) have proposed that the infectious virus particle is a triple-shelled structure with an inner VP2 shell, an intermediate VP6 shell, and an outer shell of VP4 and VP7. The VP2 shell lies between the radii of 210 and 265 °A. The existence of this shell has been further substantiated by the self-assembly of baculovirus-expressed VP2 into shells with a radius of ~265 °A (Labbe *et al.*, 1991). However, for the present study virion particles having VP2 and VP6 shells will be considered as single-shelled and virion particles having VP2, VP6, VP4, and VP7 shells will be considered as double-shelled.

The three-dimensional structure of rotavirus was first determined by cryoelectron microscopy and computer image processing techniques (Figure 2) (Prasad *et al.*, 1988). Subsequent studies provided additional evidence for the identification of topographical features of the viral structural proteins (Prasad *et al.*, 1990; Yeager *et al.*, 1990; Shaw *et al.*, 1993). A total of 132 aqueous channels perforate the VP7 outer shell that are in register with VP6 inner shell. Twelve type I channels are located along the icosahedral 5-fold axes. Sixty type II channels are located at the 6-co-ordinated positions immediately surrounding the 5-fold axes. The 60 remaining type III channels are located at the other 6-co-ordinated positions that neighbour the icosahedral 3-fold axes. These channels are thought to be involved in importing the metabolites required for RNA transcription and exporting the nascent RNA transcripts for subsequent virus replication. Studies on non-protease treated particles revealed the presence of sixty spikes situated at an edge of the type II channels surrounding the 5-fold axes (Prasad *et al.*, 1988). These spikes are 4.5-

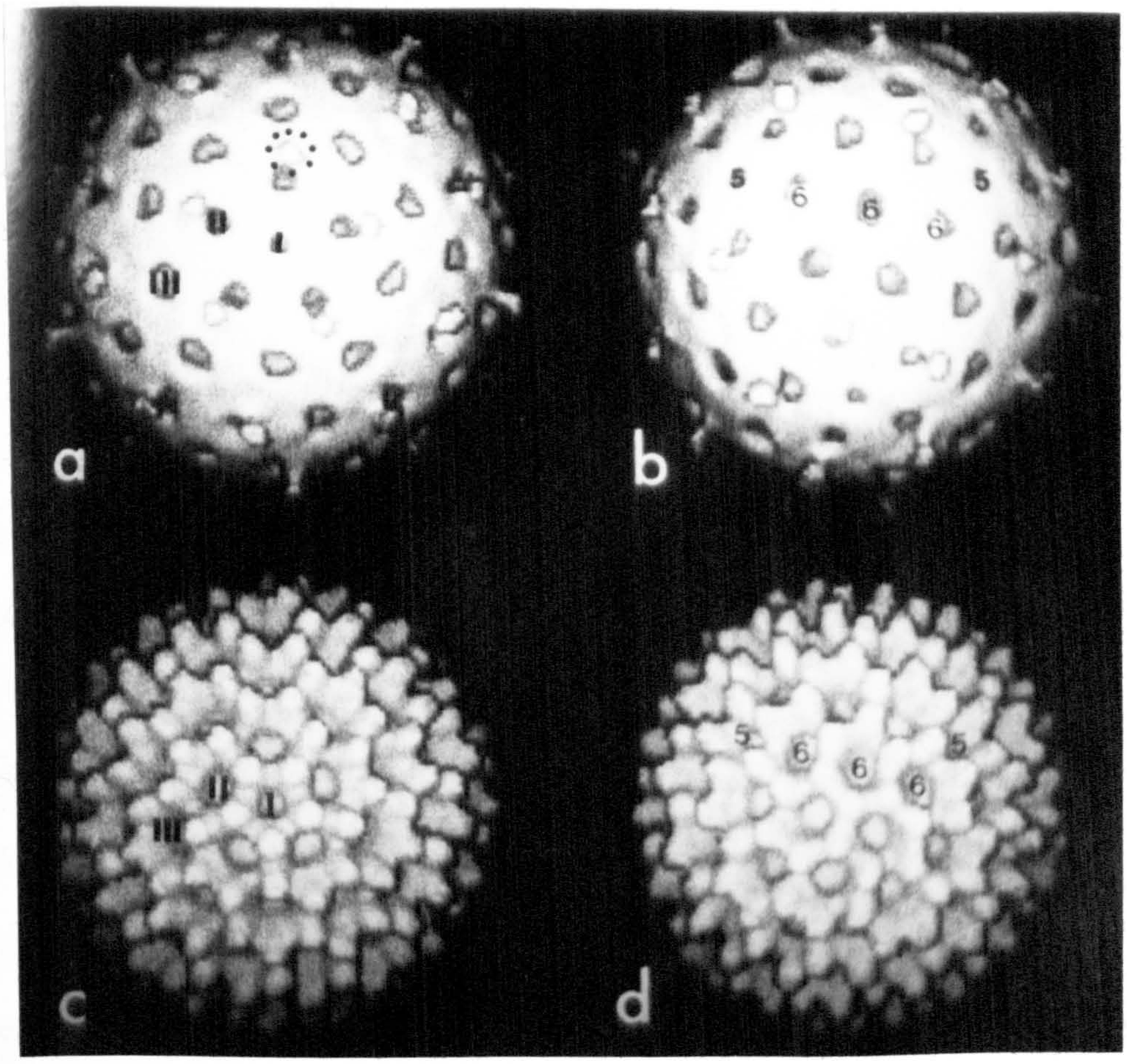


Figure 2 : Surface representations of the 3-dimensional structures of double ((a) and (b)) and single-shelled ((c) and (d)) rotavirions along the icosahedral 5-fold axis ((a) and (c)) and along the 3-fold axis ((b) and (d)). Three type of channels (designated I, II, III in (a) and (c) showing just one of each type) are found at all 5 and 6 co-ordinated positions (designated as 5 and 6, respectively in (b) and (d)) spanning the outer and inner shell proteins. The protein spikes are seen situated at an edge of type II channels surrounding the 5-fold positions in the double-shelled rotavirus.

Taken from Prasad *et al.*, (1988).

6.0 nm in length with a globular knob at the distal end and a bilobed structure at the other end (Yeager *et al.*, 1990). They have now been shown to be made up of VP4 (Prasad *et al.*, 1990).

1.3.2 Genome structure

The rotavirus genome consists of 11 segments of double-stranded RNA that range in molecular weight from approximately 2 X 10^5 to 2.2 X 10^6 daltons (Rodger *et al.*, 1975; Newman *et al.*, 1975; Kapikian and Chanock, 1990). The segments can be divided into four-size classes (groups I- IV) based on contour-length measurements of the RNAs by EM and electrophoretic patterns in polyacrylamide gel electrophoresis (PAGE) (Kalica *et al.*, 1976; Barnett *et al.*, 1978). The usual electropherotypic pattern of the segments in Group A rotaviruses is 4,2,3,2 (Clarke and McCrae, 1981; Pedley *et al.*, 1983). However, in non-group A rotaviruses, or group A rotaviruses that show rearrangements within individual segments, a different migration pattern is seen.

Slight changes in the migrational positions of individual RNA segments have been attributed to genomic variation being generated either by simple point mutations resulting in possible antigenic drift events or through the occurrence of genome segment reassortment events between genetically distinct virus strains. In addition to minor changes in the rotavirus genome profile, two more profound type of changes have been reported. In the first, the tight triplet of RNA segments (genes 7 to 9) is reduced to a doublet with the displaced segment in some cases migrating more slowly on PAGE and in other cases migrating more rapidly (Pedley *et al.*, 1983; 86). In the second type of gross abnormality either additional RNA segments are present on the genome profile or a particular RNA segment has disappeared from its normal migrational

position and been replaced by an RNA migrating much more slowly (Hundley *et al.*, 1987; Scott *et al.*, 1989; Tian *et al.*, 1993).

The complete nucleotide sequence of all 11 RNA segments of both the simian SA11 and bovine UKtc strains of virus have been determined. The segments range in size from 667 (segment 11) to 3302 base pairs (segment 1). Each RNA segment starts with a 5' guanidine and ends with a 3' terminal cytidine. Conserved non-coding sequences occur at both 5' and 3' ends (Clarke and McCrae, 1983; McCrae and McCorquodale, 1983) surrounding the open reading frame and are thought to contain signals important for genome transcription, replication, and possibly assembly of the genome segments. All rotavirus genes are A+T rich and the codon usage is biased against CGN and NCC (Estes and Cohen, 1989).

1.3.3 Physicochemical properties

Different particles possess different biophysical and biochemical properties. Double-shelled rotavirus particles have a density of 1.36 g/ ml in CsCl and a sedimentation coefficient of 520-530S in sucrose, whereas single-shelled particles with a density of 1.38g/ ml will sediment at 380-400S. Core particles have a density of 1.44 g/ ml in CsCl and have a sedimentation coefficient of 280S (Tam *et al.*, 1976; Bican *et al.*, 1982). The infectivity of the virion depends on the integrity of the outer capsid and treatments with chelating agents (EDTA, EGTA) convert double-shelled particles into single-shelled particles resulting in the loss of infectivity. The loss of the outer capsid also activates the endogenous RNA polymerase. Core particles can be produced by disruption of single-shelled particles with chaotropic agents (sodium thiocyanate or calcium chloride). This latter change is associated with a loss of polymerase activity (Bican *et al.*, 1982; Cohen *et al.*, 1979).

Virus infectivity and particle integrity are resistant to fluorocarbon extraction and exposure to ether, chloroform, or deoxycholate, and pH over the range of 3-10 (Tam *et al.*, 1976; Estes *et al.*, 1979). All rotaviruses are stable at -70°C, and retain infectivity at 4°C or even at 20°C, when stabilised by 1.5 mM CaCl (Shirley *et al.*, 1981). Snodgrass and Herring, (1977) found formalin and lysol, which is an iodophore preparation (but not chlorine) to be useful disinfectants. Ethanol, 95% was the most effective disinfectant (Tan and Schnagl, 1981).

1.4 Gene protein assignments

The proteins encoded by each of the 11 genome segments of UKtc and SA11 rotaviruses are shown in Table 1. These assignments have been made by *in vitro* translation using mRNA or denatured dsRNA (Dyall-Smith *et al.*, 1981; Mason *et al.*, 1980; 83; McCrae and McCorquodale, 1982) and by analysis of reassortant viruses (Gombold *et al.*, 1985; Greenberg *et al.*, 1981; Liu *et al.*, 1988; Offit and Blavat, 1986). The absolute order of migration of cognate genes may differ for different rotavirus strains. Consequently the identification of cognate genes must be based on hybridisation with gene-specific probes (Dyall-Smith *et al.*, 1983), reassortant analysis (Gombold *et al.*, 1985; Gombold and Ramig, 1986), or protein identification based on biochemical or immunological identification of the protein synthesised in a cell-free translation system programmed with denatured dsRNA or sequence homology (Green *et al.*, 1987; 88). The virus genome encodes six structural and six non-structural proteins. The conflicting conclusions regarding the number and location have now largely been resolved when it was recognised that post translational modifications such as glycosylation, trimming of carbohydrate residues, and proteolytic cleavages occur following polypeptide synthesis (Estes *et al.*, 1983; Holmes, 1983).

Table 1 : Gene-protein assignments of UKtc strain of bovine rotavirus[a]

Genome segment	Length (base pairs)	Protein product	Deduced molecular weight (daltons)	Approximate % of capsid protein	Location/Comments
1	3302	VP1	125,005	2	Inner core protein RNA polymerase?
2	2687	VP2	102,431	15	Inner core protein, RNA binding, Leucine zipper
3	2591	VP3	88,000	0.5	Inner core protein Guanyl transferase
4	2362	VP4	86,751	1.5	Outer capsid protein, Haemagglutinin, Neutralisation antigen
5	1611	VP5 (NS53/NSP1)[b]	58,654		Non-structural protein Zinc fingers
6	1356	VP6	44,816	51	Inner capsid protein, Group & Subgroup antigen
7[c]	1104	VP8 (NS35/NSP2)[b]	36,700		Non-structural protein
8[c]	1059	VP7	33,919	30	Outer capsid protein, Neutralisation antigen
9[c]	1062	VP9 (NS34/NSP3)[b]	34,600		Non-structural protein RNA binding
10	751	VP10 (NS28/NSP4)[b]	20,290		Non-structural protein, Morphogenesis
11	667	VP11 (NS26/NSP5)[b]	21,725		Non-structural protein
		VP11* (NS11/NSP6?)b	11,000		Non-structural protein

[a]Adapted from Desselberger and McCrae, (1994). [b]The text in parenthesis is the equivalent nomenclature used for SA11 rotavirus. [c]In SA11 strain, the segments 7, 8, and 9 code for NS34/NSP3, NS35/NSP2, and VP7 proteins, respectively.

1.5 Rotavirus proteins

1.5.1 Core proteins

1.5.1.1 VP1

This is a 125K basic protein which forms ~ 2% of the virion mass (Cohen *et al.*, 1989; Estes and Cohen, 1989). It is considered to be the RNA dependent RNA polymerase based on sequence analysis (Cohen *et al.*, 1989; Mitchell and Both, 1990a) and photoaffinity labelling experiments with azido-ATP (Valenzuela *et al.*, 1991). It often fails to react with hyperimmune sera to purified virus particle (Ericson *et al.*, 1982) or convalescent sera (Offit *et al.*, 1983). However, expressed VP1 is both antigenic and immunogenic (Cohen *et al.*, 1989).

1.5.1.2 VP2

This is a 92K protein which is the most abundant component of the core and forms 15% of the mature virion mass (Estes and Cohen, 1989). There has been one report which found it to be myristylated (Clark and Desselberger, 1988). It forms the innermost of the three protein layers described by Shaw *et al.*, (1993). It possesses nucleic acid binding activity in a nucleotide sequence-independent manner, but has higher affinity for ssRNA than dsRNA (Boyle and Holmes, 1986). It has a 'leucine zipper' between amino acids (aa) 517 and 636, which is a feature of nucleic acid-binding proteins (Landschulz *et al.*, 1988; Kumar *et al.*, 1989). VP2 is highly immunogenic, and serum antibodies to this protein are a good indicator of prior infection (Svensson *et al.*, 1987).

1.5.1.3 VP3

This is a 88K basic protein present in the viral core and normally comigrates with the protein product of segment 4 of SA11(Liu *et al.*,

1988). Previous studies have concluded that the gene 3 product was a structural protein (Smith *et al.*, 1980) coding for VP4 in SA11 strain, a non-structural protein (Arias *et al.*, 1982), or a structural protein that was translated poorly *in vitro* and was synthesised and processed rapidly (McCrae and McCorquodale, 1982). It has been shown to be associated with guanylyltransferase activity (Pizarro *et al.*, 1991; Liu *et al.*, 1992).

1.5.2 Outer capsid proteins

1.5.2.1 VP4

VP4 is the 84-88K nonglycosylated protein product of gene segment 4 and was formally known as VP3 in the SA11 strain (Estes *et al.*, 1983; Liu *et al.*, 1988). It is present on the outer capsid as spikes made up of dimers of VP4. There are sixty spikes per virion that constitute 1.5 % of virion protein (Prasad *et al.*, 1988; Anthony *et al.*, 1991).

VP4 is associated with a number of properties such as haemagglutination (Kalica *et al.*, 1983; Prasad *et al.*, 1990; Fiore *et al.*, 1991; Shaw *et al.*, 1993), cell attachment (Ruggeri and Greenberg, 1991), growth restriction in cell culture (Greenberg *et al.*, 1983a; Ramig and Galle, 1990), protease enhancement of viral infectivity (by cleavage of VP4 into VP8* and VP5*) (Chen *et al.*, 1989; Estes *et al.*, 1981), viral virulence (Offit *et al.*, 1986), neutralisation and protective immune responses (Offit and Blavat, 1986; Hoshino *et al.*, 1988).

The neutralisation studies of rotavirus VP4 has revealed eight areas on the linear sequence that are involved in neutralisation. VP8* contains the location of greatest sequence diversity and serotype-specific neutralisation epitopes, whereas cross-reactive epitopes have been mapped to VP5* (Shaw *et al.*, 1986; Burns *et al.*, 1988; Mackow *et al.*, 1988a; Taniguchi *et al.*, 1988a; Larralde *et al.*, 1991). Furthermore,

immunoblot and hybridisation data led to the conclusion that a particular region in VP8* extending from aa 84 through 180 (region B) may be involved in the VP4 serotype and subtype specificities of VP4 (Larralde and Gorziglia, 1992)

1.5.2.2 VP7

This protein is encoded by gene segment 9 of SA11, OSU and EDIM, gene segment 8 of UKtc and B223, and gene segment 7 of rhesus rotavirus (Estes and Cohen, 1989). There are 780 copies of VP7 in the virion which form the smooth rippled outer capsid of the virion (Prasad *et al.*, 1988). It is a glycoprotein that contains only N-linked high-mannose oligosaccharide residues, these are added cotranslationally as the protein is inserted into the membrane of rough endoplasmic reticulum (RER). VP7 is an integral membrane protein with a luminal orientation whose oligosaccharides are modified by trimming which occurs in the RER (Ericson *et al.*, 1983; Kabcenell and Atkinson, 1985). There are 1-3 glycosylation sites depending on virus strains (Estes and Cohen, 1989).

The open reading frame (ORF) codes for 326 amino acids containing two hydrophobic domains (H1 and H2) near the N terminus, each preceded by a methionine (Both *et al.*, 1983a). Signal peptide cleavage occurs between Ala (50) and Gln (51), downstream of the H2 hydrophobic domain (Stirzaker *et al.*, 1987). Removal of residues 47-61 results in the secretion of VP7 into the medium (Poruchynsky *et al.*, 1985; Whitfield *et al.*, 1987). However, another deletion that removes only residues 51-61 is not secreted (Poruchynsky *et al.*, 1985). The H2 signal peptide plays a key role in targeting of VP7 in the RER and the signal peptide and a region which lies within residues 62-111 of VP7 together are sufficient for retention of the protein in RER (Stirzaker and Both, 1989).

Nine discrete variable regions designated VR1- VR9 of amino acid sequence have been identified among the VP7s from different serotypes that are highly conserved within a serotype (Green *et al.*, 1987; 1988; 1989) . Three variable regions viz. VR5, VR7, and VR8 correlate with the neutralisation epitopes identified by Dyall-Smith *et al.*, (1986) (designated regions A, B, and C) in mapping studies of neutralising monoclonal antibody escape mutants. It has been suggested that these VP7 antigenic sites are in close proximity and are highly dependent on the conformational structure of VP7 (Dyall-Smith *et al.*, 1986; Mackow *et al.*, 1988b; Taniguchi *et al.*, 1988b). Cross-reactive epitopes are located only in VR5, whereas serotype-specific epitopes are located in VR5, VR7, or VR8 (Mackow *et al.*, 1988b; Taniguchi *et al.*, 1988b).

There is some evidence that suggests that calcium ions bind to VP7, and this may be important in maintaining the stability of the virion (Cohen *et al.*, 1979; Shahrabadi *et al.*, 1987). VP7 is the major neutralisation antigen and may be involved in cell-binding (Sabara *et al.*, 1985; Fukuhara *et al.*, 1988). However, VP4 (Ruggeri and Greenberg 1991) and NS35 (Bass *et al.*, 1990) have also been documented as being involved in cell binding.

1.5.3 Inner Capsid Proteins

1.5.3.1 VP6

VP6 is the nonglycosylated major inner capsid protein which has a molecular weight of 41-45K and forms 50% of the virion mass (Estes and Cohen, 1989). It was found to be myristylated in one report (Clark and Desselberger, 1988). Biochemical and 3-dimensional structural analysis has shown that it exists as trimeric units on single-shelled particles and in the infected cells (Gorziglia *et al.*, 1985; Sabara *et al.*, 1987; Prasad *et al.*, 1988). Treatment with chaotropic agents selectively removes VP6

from the single-shelled particles producing core particles which lack RNA transcriptase activity. Viral transcriptase activity can be restored by the addition of VP6 to the core particles, indicating that VP6 is structurally required for viral transcriptase activity (Bican *et al.*, 1982; Sandino *et al.*, 1986). The domains for both trimerisation and assembly into single-shelled particles are located within the highly conserved regions of VP6 but may overlap (Clapp and Patton, 1991).

VP6 is highly antigenic and immunogenic and contains rotavirus group and subgroup determinants. Most diagnostic assays employ antibodies directed against VP6 to detect the rotavirus particles. Deduced amino acid sequences of VP6 show that more than 90% of the amino acids are conserved among the four subgroups. However, subgroup I and II epitope-specific monoclonal antibodies reacted only with the trimeric form of the VP6 indicating its conformational nature (Gorziglia *et al.*, 1988a).

1.5.4 Non-structural proteins

1.5.4.1 VP5/NS53/NSP1

This is a basic protein made at low levels early in the infectious (Johnson and McCrae, 1989). Although it has been shown to bind zinc, it has not been demonstrated that this binding activity is due to the putative zinc fingers which remain conserved despite extensive sequence diversity (Mitchell and Both, 1990b).

1.5.4.2 VP8/NS35/NSP2

This is a basic protein encoded by genome segment 8 in the SA11 strain of rotavirus and has been considered by some to be the cellular attachment protein (Bass *et al.*, 1990; Estes and Cohen, 1989). It is localised in the viroplasm (Petrie *et al.*, 1984), is a major component of

the replicase particles and possesses RNA-binding activity (Kattoura *et al.*, 1992).

1.5.4.3 VP9/NS34/NSP3

This is a slightly acidic protein encoded by genome segment 7 of the SA11 rotavirus (Estes and Cohen, 1989). The domain responsible for oligomerisation and interaction with viral RNA has recently been characterised. This protein is speculated to have a role in replication (Mattion *et al.*, 1992).

1.5.4.4 VP10/NS28/NSP4

This is encoded by genome segment 10, and is a protein of 175 amino acids containing three hydrophobic domains, H1, H2, and H3 (Both *et al.*, 1983b; Baybutt and McCrae, 1984). It is an integral membrane glycoprotein of the ER, and is rich in high mannose oligosaccharides (Kabcenell and Atkinson, 1985). The signal peptide remains uncleaved (Both *et al.*, 1983b; Kabcenell and Atkinson, 1985). It acts as the receptor for budding ss-particles (Au *et al.*, 1989; Meyer *et al.*, 1989). Glycosylation of NS28 is required for removal of the transient envelope from the budding particles (Ericson *et al.*, 1983).

1.5.4.5 VP11/NS26/NSP5

The primary translational product of gene 11 has a molecular weight of 26K. It undergoes post-translational modification, phosphorylation (Welch *et al.*, 1989) and O-linked glycosylation (Gonzalez and Burrone, 1991). The O-linked glycosylation is responsible for the observed molecular weight of 28K. Segment 11 also encodes for a second out of frame ORF that can be expressed *in vitro* to produce a 11K polypeptide (Mattion *et al.*, 1991).

1.6 Classification of rotaviruses

Rotavirus is one of the six genera within the family *Reoviridae* as classified by the International Committee of Taxonomy of Viruses (Mathews, 1979b, 1982). *Orthoreovirus, Orbivirus, Cypovirus, Coltivirus, Aquareovirus, Phytoreovirus* and *Fijivirus* are the other genera of *Reoviridae*. Classification of rotavirus into various Groups, Subgroups, and serotypes is summarised in Table 2.

1.6.1 Rotavirus Groups

Rotavirus have been classified into various Groups on the basis of fulfilment of at least two of the three criteria namely : i) antigenic distinction ii) RNA genome profile distinction in polyacrylamide gels and iii) differences in the fingerprints of RNA segments (Pedley *et al.*, 1983). The Group antigen lies on VP6 polypeptide. Five distinct groups (A to E) have been described (Bridger, 1987; Pedley *et al.*, 1983; 1986; McCrae, 1987). Group A, B, and C have been found in both human and animals, group D, and E have been found only in animals.

1.6.2 Rotavirus Subgroups

Early reports argued that it was possible to classify virus serotypes by non-neutralisation assays such as complement fixation and ELISA with selected post infection sera (Yolken *et al.*, 1978; Zissis and Lambert, 1978, 1980). However more rational analyses have revealed that these tests do not recognise the same antigens detected in serum-neutralisation tests and therefore they do not identify serotypes (Kapikian *et al.*, 1981). It has now been recognised that ELISA and immune adherence haemagglutination assay (IAHA) can be used to distinguish a subgroup antigen that is encoded by sixth gene segment (Kalica *et al.*, 1981). At

Table 2 : Classification of rotaviruses[a]

Serologic division	Protein responsible	Numbers
Groups	VP6	5
Subgroups	VP6	4
G Serotypes	VP7	14
P Serotypes	VP4	11

[a]Adapted from Kapikian and Chanock, (1990) and Hoshino and Kapikian, (1994).

present there are four subgroups in group A rotaviruses namely subgroup I, subgroup II, subgroup I+II, and non-I non-II (Greenberg *et al.*, 1983a; Hoshino *et al.*, 1987a; Svensson *et al.*, 1988).

1.6.3 Serotypes

Rotavirus serotypes have been determined by i) neutralisation assays in cell culture such as inhibition of cytopathic effect, plaque reduction and fluorescent foci inhibition (Hoshino *et al.*, 1984; Sato *et al.*, 1982; Taniguchi *et al.*, 1985), ii) solid-phase immune electron microscopy (SPIEM) (Gerna *et al.*, 1985), iii) ELISA employing serotype specific monoclonal antibodies (Taniguchi *et al.*, 1985; Coulson *et al.*, 1987; Beards, 1987; Unicomb *et al.*, 1989), iv) nucleic acid hybridisation under stringent conditions (Lin *et al.*, 1987; Flores *et al.*, 1989a) and v) sequencing (Green *et al.*, 1988; 1989). In the earlier studies, the major neutralisation antigen was shown to be VP7, an outer capsid glycoprotein, and it was therefore considered to be the determinant of rotavirus serotype (Kalica *et al.*, 1981). A reciprocal 20-fold or greater difference in serum neutralising antibody titre has been taken as the criterion to distinguish serotypes (Hoshino *et al.*, 1984; Wyatt *et al.*, 1982). Hoshino *et al.*, (1984) proposed a unified serotyping scheme based on the observation of shared neutralisation epitopes between human and animal rotaviruses in cross-neutralisation studies. Currently 14 VP7 serotypes among animal and human rotavirus have been defined (Table 3) (Browning *et al.*, 1991a,b; Shen *et al.*, 1993; Hoshino and Kapikian, 1994).

VP4 has also been demonstrated to induce the production of neutralisation antibodies (Offit and Blavat, 1986; Greenberg *et al.*, 1983a,b) and appears to be important in immune response to rotavirus infection (Matsui *et al.*, 1989). At least 11 VP4 P serotypes (based on

Table 3 : Serotypic and genotypic classification of Group A rotavirus VP7[a]

VP7 G type[b,c]	Prototype strain (name/ species)
G1	Wa / human
G2	S2 / human
G3	SA11 / simian
G4	Gottfried / porcine
G5	OSU / porcine
G6	NCDV / bovine
G7	Ch2 / chicken
G8	B37 / human
G9	WI61 / human
G10	B223 / bovine
G11	YM / porcine
G12	L26 / human
G13	L338 / equine
G14	F123 / equine

[a]Adapted from Browning *et al.*, (1991b) and Hoshino and Kapikian., (1994). [b]VP7 G serotypes determined by reciprocal cross neutralisation.

[c]In addition VP7 genotype has been determined by comparative amino acid sequence analysis and/ or nucleic acid hybridisation. Genotyping has been used to distinguish among rotavirus strains and, where evaluated, has correlated with serotyping differences by neutralisation.

neutralisation assays), and 19 VP4 genotypes (determined by comparative amino acid sequence analysis and/or nucleic acid hybridisation) present among animal and human rotavirus strains have been reported (Table 4) (Estes and Cohen, 1989; Qian and Green, 1991; Hardy *et al.*, 1992; Shen *et al.*, 1993; Hoshino and Kapikian, 1994). The neutralisation specificities of VP4 and VP7 can segregate independently due to the segmented nature of rotavirus genome (Hoshino *et al.*, 1985). A binary system of classification has been suggested and it has been proposed that VP7 serotypes be referred to as G serotypes (for glycoprotein) and VP4 serotypes be referred to as P serotypes (for protease-sensitivity) (Estes and Cohen, 1989).

Strains within a serotype which vary in their capacity to be neutralised by serotype-specific monoclonal antibodies have been termed 'monotypes' analogous to 'subtypes' defined by polyclonal hyperimmune antisera (Coulson, 1987; Coulson *et al.*, 1987; Liprandi *et al.*, 1991; Green *et al.*, 1992).

1.7 Epidemiology

Rotaviruses have been detected throughout the world wherever they have been sought (Kapikian and Chanock, 1990). Even the most isolated human populations have antibodies as evidence of past infections, and occasional epidemics occur (Foster *et al.*, 1980). Rotaviruses have been associated with acute gastro-enteritis and diarrhoea in a wide range of mammalian (Flewett and Woode, 1978; Estes *et al.*, 1983) and avian species (McNulty *et al.*, 1978; 1979).

The route of transmission is generally faecal-oral but a respiratory route has also been suggested (Ward *et al.*, 1986; Gurwith *et al.*, 1981). The rotavirus is relatively resistant to environmental factors but

Table 4 : Serotypic and genotypic classification of Group A rotavirus VP4[a]

VP4 P serotype[b]	VP4 genotype[c]	Strain
1A[d]	8	Wa
1B[d]	4	DS-1
2A	6	M37
2B	6	Gottfried
3A	9	K8
3B	14	Mc35
4	10	69M
5A	3	K9
5B	3	MMU 18006
6	1	NCDV
7	5	UKtc
8	11	B223
9	7	OSU
10	16	Eb
11	18	PA169
	2	SA11
	12	FI23
	13	MDR-13
	15	Lp 14
	17	993/83
	19	L338

[a]Adapted from Hoshino and Kapikian, (1994). [b]VP4 P serotype determined by reciprocal or one-way cross neutralisation. [c]VP4 genotype determined by comparative amino acid sequence analysis and/ or nucleic acid hybridisation. [d]Letters indicate subtypes within a serotype.

contaminated water is unlikely to play a significant role in transmission (Smith and Gerba, 1982).

In temperate countries, rotavirus gastro-enteritis shows a definite seasonal pattern, with the peak incidence during the winter months (Bryden *et al.*, 1975), whereas in tropical climates, rotavirus infections occur throughout the year (Heiber *et al.*, 1978).

Rotavirus gastro-enteritis mainly affects children from 6 months to 2 years of age. Both males and females are susceptible. Malnutrition is thought to play an important role in increasing the severity of the clinical manifestations (Kapikian and Chanock, 1990). Group B rotavirus can produce diarrhoea in adults.

Molecular epidemiology of rotavirus strains has mainly been done by comparison of the electrophoretic migration patterns of the segmented genomic dsRNAs. Analysis of the RNA electropherotypes provides some evidence for the genetic diversity and heterogeneity of the rotaviruses circulating in the population (Estes *et al.*, 1984).

1.8 Clinical features

Rotavirus infection produces a variety of disease patterns varying from subclinical infection, to mild diarrhoea, to a severe and fatal dehydrating illness. After an incubation period of 1-2 days, vomiting and then diarrhoea occurs with a mild fever. The diarrhoea lasts for 3-4 days and moderate dehydration is common (Kapikian and Chanock, 1990). Death when it occurs is usually due to severe dehydration (Rodriguez *et al.*, 1987).

1.9 Diagnosis

The clinical manifestations of rotavirus illness are not sufficiently distinctive to permit diagnosis on this basis alone. The diagnosis is

usually made by detection of virus, viral antigen, or nucleic acid in faeces and/ or the demonstration of a virus specific serological response (Kapikian and Chanock, 1990). Detection of rotavirus in the faeces by electron microscopy is the gold standard test (Flewett *et al.*, 1974b). Rotavirus antigen can be detected in the faeces by a variety of tests such as ELISA (Yolken *et al.*, 1978), counter-immuno-electrophoresis test (Grauballe *et al.*, 1981), immune adherence haemagglutination test (Kapikian *et al.*, 1981), reverse passive haemagglutination assay, solid-phase radioimmuno assay (Middleton *et al.*, 1977), latex agglutination test (Ushijima *et al.*, 1986), coagglutination test (Kang *et al.*, 1985). Similarly, serological responses can be demonstrated by a variety of tests (Estes *et al.*, 1983; Kapikian and Chanock, 1990).

Detection of rotavirus in stools can also be carried out by molecular biological techniques (Flores *et al.*, 1983; Pedley and McCrae, 1984; Lin *et al.*, 1987; Xu *et al.*, 1990).

1.10 Treatment

The primary aim of treatment of rotaviral gastro-enteritis is the replacement of fluids and electrolytes lost by vomiting and diarrhoea. This can be done either by intravenous fluid administration or by giving oral rehydration therapy. Several broad-spectrum antiviral agents have been examined as inhibitors of rotavirus replication *in vitro* (Kitaoka *et al.*, 1986). In animal experiments several antiviral drugs showed some initial promise for the specific treatment of rotavirus infection but have not been actively pursued (Smee *et al.*, 1982).

1.11 Immune response and immunity to rotaviruses

Protective immunity to rotavirus gastro-enteritis can be induced. However, for several reasons the mechanisms of rotavirus immunity are

not fully understood. Firstly, in most studies measurement of immune response has been done on sera rather than intestinal washings or coproantibodies, despite the fact that circulating antibodies are not correlated with protection in man and animals (Woode *et al.*, 1975; Snodgrass and Wells, 1976; Bishop *et al.*, 1983; Ward *et al.*, 1986). Secondly, the extent of antigenic diversity among rotaviruses in natural conditions is not well known. Thirdly, there is difficulty in ascertaining the state of immune response as well as of infection in man and animals. Therefore what we actually measure in natural conditions may be a primary, secondary or subsequent immune response and/or infection.

1.11.1 Humoral immune response

Although antibodies to most rotavirus proteins can be detected after infection (Svensson *et al.*, 1987), a protective effect has only been reproducibly demonstrated with antibodies to the outer capsid proteins, VP4 and VP7 (Hoshino *et al.*, 1988; Mackow *et al.*, 1990; Matsui *et al.*, 1989; Offit *et al.*, 1986b). VP4 and VP7 segregate independently (Hoshino *et al.*, 1985a) and the location of a number of homotypic and heterotypic neutralisation epitopes on them has been defined (Dyall-Smith *et al.*, 1986; Mackow *et al.*, 1988a,b; Taniguchi *et al.*, 1988a,b). Studies on the relative immunogenicities of VP4 and VP7 have yielded conflicting results. VP7 appears to be immunodominant in hyperimmunised animals, while the VP4 protein was found to be immunodominant in humans who had been orally inoculated with virus (Clark *et al.*, 1990; Perez-Schael *et al.*, 1990; Ward *et al.*, 1988c) or after natural infection (Ward *et al.*, 1993). In contrast, vaccination of infants with the WC3 vaccine elicited neutralising antibody responses almost solely to VP7 (Ward *et al.*, 1990c). Likewise, immune responses to epitopes on VP7 showed significant correlations to protection against

rotavirus infection or disease in adults challenged with a virulent serotype 1 human rotavirus (Green and Kapikian, 1992).

A wide variety of animal models and a substantial number of distinct rotavirus strains have been used in both passive and active models of immunity to study protection from rotavirus infection. The importance of local immune mechanisms in mediating protection has been shown by a number studies (Bishop *et al.*, 1983; Bridger and Oldham, 1987; Hoshino *et al.*, 1988; Losonky *et al.*, 1986; Offit and Clark, 1985a; Snodgrass and Wells, 1976; 1978; Snodgrass *et al.*, 1984; Torres and Ji-Huang, 1986; Woode *et al.*, 1983). The presence of neutralising antibodies in the intestinal tract has also been correlated with protection against rotavirus infection in these studies. However, mechanisms other than humoral immunity such as cell mediated immunity may also play a role in preventing and resolving infection (See cell mediated immune response). The serological specificity of immunity appears to be rather complex. There are data that demonstrate both the presence (Bishop *et al.*, 1986; Hoshino *et al.*, 1988; Matsui *et al.*, 1989; Offit *et al.*, 1986b; Torres and Ji-Huang,1986; Vesikari *et al.*, 1987; Wyatt *et al.*, 1979; 1983) and the absence of heterotypic immunity (Bohl *et al.*, 1984; Losonsky *et al.*, 1986; Murakami *et al.*, 1986; Offit and Clark, 1985b; Woode *et al.*, 1983). Homotypic immunity appears to be the rule following active infection or passive immunisation.

1.11.2 Cell mediated immune response

A role for the cell-mediated immune response (CMI) in protective immunity was postulated on the basis of observations in human infants and in calves that heterologous protection against challenge occurred in the absence of serotype-specific neutralising antibodies (Bridger and Oldham, 1987; Woode *et al.*, 1987; Clark *et al.*, 1988; Ward *et al.*,

1992). There are several pieces of evidence supporting a role for rotavirus-specific cytotoxic T lymphocytes (CTLs) in protection against disease. Rotavirus-specific CTLs generated after inoculation of mice with bovine, simian or human rotavirus cross-react with target cells infected with different rotavirus serotypes (Offit and Dudzik, 1988; Offit and Svoboda, 1989). Rotavirus-specific CTLs have been detected at the intestinal mucosal surface after oral inoculation of mice with rotavirus (Offit and Dudzik, 1989), and virus-specific CTLs passively protect mice against rotavirus-induced gastro-enteritis or chronic rotavirus shedding (Dharakhul *et al.*, 1990; Offit and Dudzik, 1990).

Cross-reactive rotavirus-specific CTLs generated in mice after oral inoculation with rotavirus recognised VP7 (Offit *et al.*, 1991; 1994; Dharakhul *et al.*, 1991; Franco *et al.*, 1993) or VP1, VP4, VP6 and VP7 (Dharakhul *et al.*, 1991). Quantitative differences in the generation of serotype-specific and cross-reactive CTLs have been found after oral inoculation of two candidate rotavirus vaccine strains RRV and WC3. Thus, RRV elicited the production of predominantly strain-specific CTLs, WC3 elicited mainly cross-reactive CTLs (Offit and Svoboda, 1989). The rotavirus-specific CTLs can be induced both at the mucosal and non-mucosal surface after oral or intraperitoneal inoculation (Offit *et al.*, 1994). An immunodominant cytotoxic T cell epitope on VP7 protein which overlaps the H2 signal peptide has recently been identified (Franco *et al.*, 1993).

1.12 Rotavirus vaccinology

1.12.1 The need for a rotavirus vaccine

The economic importance of rotavirus is well established. (see section 1.1). The development and application of an effective rotavirus vaccine should have a major public health impact by lowering the overall

diarrhoeal disease morbidity and mortality. De Zoysa and Feachem, (1985) estimated that a vaccine with 100% efficacy administered to all infants under 6 months of age in the developing world would decrease the occurrence of diarrhoeal episodes by 5% and the mortality by 16% thereby reducing the number of deaths by 160 000 per year. Under more realistic conditions, in which a vaccine has 80% efficacy and only 45% coverage of the same age groups, they estimated a reduction of diarrhoeal morbidity by about 1.8% and mortality by 6% per year. The impact of the vaccine on diarrhoeal morbidity in developed nations would be much more pronounced since the relative role of rotavirus in comparison to other agents as a cause of severe diarrhoea is greater in developed countries than in developing countries.

1.12.2 Factors to be considered when planning a rotavirus vaccination strategy

Several factors must be considered for developing and deploying a successful rotavirus vaccine: the need to induce a local, mucosal immune response; the presence of maternal antibody either in colostrum or by transplacental transfer, as well as other nonspecific inhibitors in breast milk and serum that could interfere with vaccine efficacy (Edelman *et al.*, 1989); the presence of multiple virus serotypes circulating in the population (Green *et al.*, 1990; Kapikian *et al.*, 1986; Lanata *et al.*, 1989; Snodgrass *et al.*, 1984), the compatibility between various vaccination regimens implemented in young children; and concurrent infections by rotavirus and other enteropathogens (Edelman *et al.*, 1989).

Strategies for vaccinating humans have been aimed to-date at children less than 2 years of age (Green *et al.*, 1990). In animals the susceptible age for virus disease is 3-30 days. Therefore a strategy of dam vaccination, to raise protective antibody levels that can passively

immunise suckling neonates, (Saif *et al.*, 1983; Wyatt *et al.*, 1983) has been employed.

1.12.3 Approaches for rotavirus vaccine development

A variety of approaches have been proposed and/ or pursued for rotavirus vaccine development. These can be grouped into four broad categories.

1.12.3.1 Live attenuated virus vaccines

Major research efforts have been put into the development of live attenuated vaccines for a number of reasons. It is believed that they would have the best chance of stimulating local mucosal immunity after oral administration. They would retain the native antigenic characteristics, elicit a strong immune response, without causing clinical disease. Three classes of live attenuated vaccine are currently under consideration: live attenuated or naturally avirulent strains; live attenuated heterologous strains; and attenuated, genetically reassorted (human x animal) strains.

The first live rotavirus vaccine was a calf rotavirus vaccine given orally to calves at birth (Mebus *et al.*, 1973). The Wa human rotavirus strain passaged in piglets and then in African green monkey kidney cells was the first live attenuated vaccine tested in human volunteers. However, the testing of this vaccine has been stopped due to elevations of the serum transaminase levels in the volunteers (WHO, 1984).

A naturally attenuated rotavirus nursery strain, M37, was developed as a candidate rotavirus vaccine in the USA (Flores *et al.*, 1986). However, it does not provide heterotypic protection and is presently being formulated with other live attenuated candidates (Flores *et al.*, 1990).

Live attenuated, heterologous strains of rotavirus from non-human hosts are also being evaluated for their potential in the so called Jennerian vaccines. Three such vaccine candidate are RIT 4237 (isolate of bovine NCDV origin), WC3 (bovine origin), and MMU 18006 (RRV-1 strain of rhesus monkey origin). All of the above described vaccines are effective only in the developed countries and are much less effective in developing countries. Moreover, they provide only homologous protection.

Reassortants that are attenuated may be the solution to the problem of achieving heterotypic protection. Single gene substitution rotavirus reassortants have been produced in which the VP7 of human serotypes 1, 2, 3, and 4 was incorporated into rhesus or bovine parent strains (Midthun *et al.*, 1985). Naturally attenuated rotavirus nursery strains (Bishop *et al.*, 1983; Flores *et al.*, 1986) and cold adapted human strains (Matsuno *et al.*, 1987) are also being considered as reassortant donor strains. Several RRV and WC3 reassortants are currently in phase I clinical trial testing for safety and immunogenicity (Flores *et al.*, 1989b). The VP4 can also be substituted from the same serotype if single gene substitution reassortants prove less satisfactory or from a different serotype for heterotypic protection. Multivalent vaccines either a mixture of cell culture adapted rotavirus strains of different serotypes or gene substitution reassortants are also being tested (Perez-Schael *et al.*, 1990).

1.12.3.2 Inactivated vaccines

It has recently been shown that parenteral administration of inactivated rotavirus vaccine induced active immunity and protection against a virulent rotavirus challenge in a rabbit model (Conner *et al.*, 1993).

1.12.3.3 Live vectored vaccines

Oral rotavirus vaccines are currently under development using cloned rotavirus genes in attenuated enteric bacteria such as Salmonella or *E. coli* (WHO, 1986). The advantages of using such bacteria are many: the bacteria will colonise the gut without causing disease, reduced chance of neutralisation by antirotavirus antibodies in the gut, reduced chance of reversion or reassortment. However, the disadvantage of prokaryotic expression systems is that these can not glycosylate or post-translationally modify the proteins and consequently protein folding is frequently incorrect. Also, epitopes on VP7 are known to be conformational (Dyall-Smith *et al.*, 1986).

1.12.3.4 Subunit vaccines

Subunit vaccine candidates are based on individual rotavirus antigens, either produced in culture by cells containing cloned rotavirus DNA or prepared synthetically. In vaccine formulations these antigens may either be used alone or in combination to produce immunity to a broad spectrum of rotaviruses.

1.12.3.4.1 Cloned rotavirus proteins

Both VP4 and VP7 have been cloned into prokaryotic and eukaryotic expression vectors. The use of prokaryotic expressed protein has met with limited success (Arias *et al.*, 1987; McCrae and McCorquodale, 1987).

Rotavirus proteins have also been expressed in other systems, such as vaccinia and baculovirus (Andrew *et al.*, 1987; Estes *et al.*, 1987; Mackow *et al.*, 1989). There are a number of advantages of using baculovirus expression vectors. They are not pathogenic to vertebrates and do not use transformed cells or transforming elements, and the

abundant expression of recombinant proteins (Lucknow and Summers, 1988). Unfortunately, protein glycosylation by baculovirus is not always identical to mammalian processed proteins and the difference in the carbohydrate composition may affect the folding and presentation of the recombinant molecules. Individual rotavirus proteins from various strains have also been expressed in vaccinia virus and tested *in vitro* and *in vivo* for both humoral and cellular immune response with varying degrees of success (Offit *et al.*, 1994).

1.12.3.4.2 Synthetic Vaccines

Another approach to the development of rotavirus subunit vaccine involves the identification and synthesis of protective epitopes. Peptides from the cleavage region of VP4 induced neutralising monoclonal antibodies (nmAb's) (Streckert *et al.*, 1988), whereas peptides of VP7 did not induce nmAb's (Gunn *et al.*, 1985; Streckert *et al.*, 1986), and mAb to VP7 did not bind to synthetic VP7 peptides (Streckert *et al.*, 1986; Taniguchi *et al.*, 1988a). However, heterotypic passive protection was shown to be induced by VP7 peptide (amino acids 275-295) and VP4 peptide (amino acids 232-255) either alone or in combination, when conjugated to expressed VP6 (Ijaz *et al.*, 1991), which is yet to be confirmed by others. The synthetic peptides are attractive candidates for use in vaccines, however, they must be conjugated to large carrier proteins or polymers suitable for human and veterinary applications for producing acceptable levels of antibody titres or cellular immunity.

1.12.4 Rotavirus vaccines : current position

The current vaccine candidates and their respective stage of development are shown in Table 5. Some of the possible causes of failure of these vaccines are : failure to swallow the vaccine or emesis; gastric

acid; presence of maternal transplacental, or colostrum and milk antibodies; or presence of antibodies due to previous exposure; interference by replicating enterovirus in the bowel; changes in the cellular viral receptors with age, and rapid bowel transit time (Edelman *et al.*, 1989).

Recently, Glass *et al.*, (1994) have reported that reassortant vaccines emerging from clinical trials are nearest to approval by the Food and Drug Administration for use in USA. Once approved, they could be incorporated into the routine schedule of childhood immunisation recommended by the Advisory Committee on Immunisation Practices.

Table 5 : Summary of rotavirus vaccines currently under development[a]

Vaccine type	Designation	Origin	Delivery	Development Phase
Live attenuated	_	WA	Ent/ Par	Discontinued
	M37	Nursery isolate	Ent/ Par	Completed Phase 1
	RIT 4237	NCDV	Ent/ Par	Completed Phase 1
	WC3	WC3	Ent/ Par	Completed Phase 1
	MMU18006	RRV	Ent/ Par	Completed Phase 1
Reassortants	_	RRV	Ent/ Par	Early phase 1
	_	WC3	Ent/ Par	Early phase 1
Live bacterial vectored	_	*E.coli/ Salmonella*	Ent	R/D
Live viral vectored	_	Adenovirus	Ent	R
Recombinant protein	_	Baculovirus	Par	R
Viruslike particles	_	Baculovirus	Ent/ Par	R/ D
Peptide	_	_	Par	R

[a] Taken from Redmond *et al.*, (1993).

Ent- enteric/ orally; Par- parenteral; R- research phase; D- preclinical development

Chapter 2

Reassortment in rotaviruses

2.1 Reassortment and reassortants

When two closely related segmented viruses coinfect cells either *in vivo* or *in vitro*; then some of the progeny viruses will receive genome segments from both the parents producing what are termed reassortants. The process is called reassortment. Reassortants are genetic mosaics of coinfecting strains and can be identified by their particular array of genome segments, usually through their electrophoretic mobilities during polyacrylamide gel electrophoresis. The biological properties of reassortants will depend on which segments are inherited from which parent and the functional behaviour of each particular combination of segments and their protein products.

2.2 Mechanism of genome assortment

The rotavirus genome is made up of 11 segments of dsRNA. Isolated RNA is not infectious as the virus requires a virion associated RNA-dependent-RNA polymerase for replication. Plus strand RNA is transcribed from dsRNA and is indistinguishable from viral mRNA; it acts as the template for the synthesis of negative strand as well as being translated to generate the viral proteins. The low particle:pfu ratio that can be obtained with rotaviruses indicates that each progeny virion contains one copy of all 11 genomic RNA segments. The mechanism by which assortment of genome segments in a singly infected cell and reassortment in a mixedly infected cell occurs is not understood. The fact that rotaviruses contain two complete shells of protein made up of a total of six different proteins, bud through the endoplasmic reticulum as a step in morphogenesis, and have a genome that consists of 11 unique molecules of dsRNA indicate that there are a number of distinct intermediates formed during replication cycle. One such intermediate is the replicase particle, which has the capacity to synthesise all 11

genomic dsRNA (Helmberger-Jones and Patton, 1986; Patton and Gallegos, 1988). However, it has not yet been possible to separate replicase particles into subsets, each responsible for a different genome segment. This strongly suggests that replication follows assortment i.e. assortment occurs with single stranded plus sense RNA and not the dsRNAs. It further indicates that the recognition signals for assortment/ packaging lie on viral mRNAs. The fact that reassortant viruses are readily formed in coinfected cells suggests that, during genome assortment and the assembly of the replicase particles, the viral mRNAs that are destined to serve as templates for replication are assorted independently. Successful reassortment between different strains of viruses should logically require that they share common recognition signals for assortment/ packaging.

It seems likely that two signals will be required for the assortment/ packaging process to be completed successfully. First a recognition signal which facilitates the distinguishing of viral mRNAs from host mRNAs and a second, sorting signal, by which 11 rotavirus mRNAs are individually recognised. The 5'- and 3'- ends of dsRNA genome segments are conserved (McCrae and McCorquodale, 1983). These conserved sequences and the lack of a 3'- terminal poly (A) tail may help to differentiate the viral and host mRNAs during assortment. These terminal conservations cannot alone function as sorting signals as they are identical on all 11 species of viral mRNAs. However, it is possible that the sorting signals may be located near the 5'- and 3'- ends of viral mRNAs since these are also highly conserved (Clarke and McCrae, 1983; McCrae and McCorquodale, 1983) but in a segment specific fashion. The fact that rotaviruses with rearranged segments can form reassortants (Graham *et al.*, 1987) indicates that the length of viral mRNA probably does not constitute part of the sorting signal.

2.3 Features of rotavirus reassortment

Rotavirus reassortants have been produced *in vitro* (Greenberg *et al.*, 1981; 1983a; Kalica *et al.*, 1981; 1983; Offit *et al.*, 1986; Liu *et al.*, 1988; Chen *et al.*, 1989; Snodgrass *et al.*, 1992), *in vivo* (Gombold and Ramig, 1986; 1989; Broome *et al.*, 1993), and also have been reported to occur in nature (Clarke and McCrae, 1982; Hoshino *et al.*, 1985a;1987b; Ward *et al.*, 1990a). Reassortants have been produced between two ts mutants (Ramig, 1983), between two wild-type (Snodgrass *et al.*, 1992), between ts mutants and wild-type (Greenberg *et al.*, 1981), and between wild-type and viruses with rearranged segments (Graham *et al.*, 1987).

Kinetic studies indicate that reassortment is an early event in the infectious cycle (Ramig, 1983; Gombold and Ramig, 1986). More than 25% of the progeny clones were reassortants by 12 hr p.i. in *in vivo* experiments. The frequency of reassortment increased to 80-100% by 72-96 hr p.i. (Gombold and Ramig, 1986). The finding of a significant frequency of reassortant progeny (>25%) early in the infectious cycle *in vivo* was similar to early reassortment noted for mixed infection with rotavirus ts mutants *in vitro* (Ramig, 1983; Gombold and Ramig, 1986). However, two important differences between *in vivo* and *in vitro* reassortment were noted. Significant numbers of reassortants were detected at 12 hr p.i. *in vivo* whereas the first significant reassortants were detected at 16 hr p.i. *in vitro* (Gombold and Ramig, 1986). This slight difference in the kinetics may simply reflect the adaptation of rotaviruses for replication in enterocytes compared with more restricted growth in cultured cells or the low temperature (31^{o}C) at which the *in vitro* experiments were conducted. The frequency of the reassortant progeny *in vivo* increased throughout the infectious cycle whereas maximal frequencies of reassortants were observed at the earliest time in standard *in vitro* crosses performed at a high m.o.i. (Ramig, 1983;

Gombold and Ramig, 1986). This observation suggests that either multiple cycles of replication occur *in vivo* or that certain genotypes have selective advantage *in vivo* or both (Gombold and Ramig, 1986).

Reassortment is a random process, and reassortants with 2048 different genotypes are possible in the reassortment process involving two different strains of rotaviruses. However, non-random segregation of genome segments may occur under certain circumstances (see later).

2.4 Factors affecting reassortment or reassortant frequency

2.4.1 Antigenic relatedness

Reassortant frequencies varying from 0% to 100% in rotaviruses have been documented. The formation and detection of reassortants is probably most efficient when the coinfecting strains are closely related. This is expected since the genome segments or the proteins they encode must function together. Studies with influenza viruses have indicated that more closely the gene products are related, the more likely a replacement will be tolerated (Klenk and Rott, 1988). In rotaviruses, no reassortant has been isolated between rotavirus and reovirus. Both are different genera of *Reoviridae*. Similarly, no reassortants have been reported between rotaviruses belonging to different rotavirus groups (Yolken *et al.*, 1988), and very few reassortants have been reported between different genogroups of rotaviruses (Nakagomi *et al.*, 1989; Ward *et al.*, 1990a). The progeny of coinfection between subgroup I and subgroup II rotavirus strains yielded comparatively smaller % reassortants, <1% (Urasawa *et al.*, 1986), <3.5% (Garbarg-Chenon *et al.*, 1984), or 14% (Ward and Knowlton, 1989). In contrast, coinfection with cultured cells with different pairs of subgroup II human rotaviruses resulted in high % (33-48%) of plaque picked colony being a reassortant (Ward *et al.*, 1988a). Reassortant frequency, between two serotype 3 strains, of 5%

(Broome *et al.*, 1993), and 38-100% (Gombold and Ramig, 1986) have been reported from *in vivo* crosses.

2.4.2 Host effects on rotavirus reassortment

Specific host effects on reassortant genotypes have been observed following mixed infection of cells *in vitro* (Graham *et al.*, 1987). Thus some reassortant genotypes could be isolated using either MA104 or BSC-1 cells, although the frequency of isolation of a given genotype was quite different between the two cell lines. By contrast other reassortant genotypes were isolated at a relatively high frequency on one cell line and not at all on the other (Graham *et al.*, 1987).

2.4.3 Effect of immune status of animal on reassortment

Immune responses may modulate the frequency of reassortment by reducing the effective m.o.i. by neutralisation and other immune mechanisms, thereby preventing efficient mixed infection of enterocytes. No reassortant was detected among progeny clones examined from mixedly infected, homotypic immune mice (Gombold and Ramig, 1989). By contrast about 11% were identified among progeny clones from mixedly infected, heterotypically immune mice. Reassortment was reduced more than 50-fold by homotypic immunity and approximately three fold by heterotypic immunity relative to the frequency observed in non-immune mice (Gombold and Ramig, 1986; 1989).

2.4.4 Effect of multiplicity of infection (m.o.i.) on reassortment

M.o.i can affect the frequency of reassortment both *in vitro* (Ward *et al.*, 1988a), and *in vivo* (Gombold and Ramig, 1989). In *in vitro* studies it was found to increase in parallel with m.o.i until an m.o.i of 2.5-5.0 pfu/cell of each parent was attained.

2.4.5. Reassortment and superinfection exclusion

The frequency of isolation of viruses with mixed electropherotypes from infected individuals is approximately 10% (Lourenco *et al.*, 1981; Spencer *et al.*, 1983; Nicolas *et al.*, 1984), that indicates either simultaneous or sequential infection. However, if the infection is not simultaneous, the question arises of how much temporal separation between the two viruses is consistent with the production of reassortant. It has been shown that rotaviruses fail to establish superinfection exclusion (Ramig, 1990). Recently, it has been shown that reassortment in rotaviruses can occur *in vivo*, when as much as 24 hr separates the time of parental strain inoculation (Broome *et al.*, 1993). This is in contrast to another member of the family Reoviridae, bluetongue virus (Ramig *et al.*, 1989; El Hussein *et al.*, 1989).

2.5 Non-random segregation of genome segments

Reassortment is a random process. However, if experiments are done under conditions where selective pressure could occur, then non-random segregation is both expected and observed (Graham *et al.*, 1987). Thus, in a cross between natural genetically rearranged human rotavirus and bovine rotavirus, non-random selection of single gene segments 2, 5, 6, 9, and 11 was observed. A gene association of 5+9 and a stronger selection for triple gene association among 5+9+11 was also observed. The selection was linked with the parental gene constellation and host cell type used (Graham *et al.*, 1987).

Non-random segregation has also been observed in the absence of intentional selective pressure (Gombold and Ramig, 1986; Ward *et al.*, 1988a). Gombold and Ramig, (1986), using two heterologous viruses in mice, conducted a study wherein they obtained samples from mice at

several time points post infection. Although the gene segment reassortment was quite random overall, they found a compellingly non-random distribution of SA11 gene 5, and to a lesser degree SA11 gene 3 in all the mice and at all time points examined. They also found the gene pair 5+3 to be strongly represented in their *in vivo* heterologous reassortant system. The selection for genome constellations more favourable than the parental constellations for replication *in vivo* may be the reason for this non-random segregation.

In another study, coinfection with three different pairs of subgroup II human rotaviruses followed by multiple blind passages caused a reproducible selection of genome segments from each set of parents in progeny viruses (Ward *et al.*, 1988a). One of the parents dominated in the final selection in each pair of viruses used. Although more segments were selected from the virus of a pair that grew to higher titre, certain segments were selected independently of the relative growth properties or m.o.i. of the coinfecting viruses; whereas selection of other segments was dependent on both (Ward *et al.*, 1988a). Although the segment selection was reproducible for each pair of viruses, no obvious pattern was discernible regarding the particular segments selected. It was also observed that i) the selection of specific reassortants following coinfection was due to differences in the infectivities of progeny viruses and not in their assembly and, ii) the selection of new virus strains did not occur because the selected reassortants grew better than the parents but because they grew better than other reassortants (Ward *et al.*, 1988a).

2.6 Reassortment as a tool to identify rotavirus gene product and gene function

Reassortment has proved to be the primary tool for the identification of gene function, and an alternative system to *in vitro*

translation for identifying gene product. Thus, segregation analysis has been used to map a number of phenotypes in rotaviruses. For example, reassortment has been used to identify VP4 as the rotavirus haemagglutinin determinant and determinant of protease-enhanced plaque formation (Kalica *et al.*, 1983), VP6 as the subgroup antigen (Kapikian *et al.*, 1981) rotavirus proteins as neutralisation antigens (Greenberg *et al.*, 1983a; Offit and Blavat, 1986), identifying determinants of viral virulence (Offit *et al.*, 1986; Broome *et al.*, 1993; Hoshino and Kapikian, 1994), rescuing non-cultivable viruses (Greenberg *et al.*, 1981), and mapping temperature-sensitive mutations (Gombold *et al.*, 1985).

2.7 Evolution of rotaviruses through reassortment

Reassortant formation in cells coinfected with viruses having segmented genomes has been viewed as a potentially rapid mechanism of viral evolution (Chanock *et al.*, 1983). Pandemics of influenza A virus have occurred every 10-20 years during this century through the emergence of human strains with new haemagglutination properties by a mechanism called antigenic shift. The antigenic shift event is believed to occur as a result of reassortment between human and animal or avian strains (Desselberger *et al.*, 1978).

In rotaviruses, the serotype 3 strains of human rotavirus are serotypically related to rotaviruses of several animal species and at least three strains of porcine rotavirus are classified with serotype 4 human strains (Hoshino *et al.*, 1984). This sharing of antigenic determinants by rotaviruses isolated from different species may have resulted from reassortment. Furthermore, this process may have led to and could continue to lead to the production of new human rotavirus serotypes to which man has little if any immunity. The evidence for evolution of rotavirus through reassortment comes from the finding of naturally

occurring intertypic reassortants like M37 and SB-1A (Hoshino *et al.*, 1985a; 1987b) and isolation of natural reassortants of human rotaviruses belonging to different genogroups (Ward *et al.*, 1990a) and a human rotavirus belonging to G10 serotype (Beards *et al.*, 1992).

Reassortment not only has the capability of shuffling previously recognised genes but also affecting the expression of those genes in subtle ways by masking or unveiling of epitopes. For example, in the case of neutralisation antigens, the simple direct expression of the parental antigenicity of a reassortant would have the potential to create new combinations of previously recognised antigens, but would be unlikely to create a virus recognised as a new serotype. However, it has become clear that recipient genetic background can affect the expression of antigenicity, through the interactions of the donor and recipient proteins (Chen *et al.*, 1989; 1992; Kool *et al.*, 1992).

2.8 Limitations of reassortment

Reassortment is a random process. The probability of getting the desired reassortants without any selection pressure is very low and therefore, identifying the desired reassortant is very time consuming. Also, reassortment experiments will not map phenotypes with precision greater than assigning them to a segment(s). The recipient genetic background can affect the expression of the phenotypes of donor segments (Chen *et al.*, 1989; 1992; Kool *et al.*, 1992). Therefore, the interpretation and application of results of reassortment mapping studies with one pair of parents and its comparison with a different pair of parents must be approached with caution.

Chapter 3

Molecular basis of disease

3.1 Molecular basis of viral disease

Disease resulting from virus infection is a complex event depending on the close interaction of viral and host factors. In the 1880s, Robert Koch produced his postulates for correlating disease with micro-organisms in his 'Germ Theory Of Disease'. These criteria still hold good for associating a micro-organism with a disease. However, the central drive at present is to understand the disease at the molecular level. Technological developments over the last two decades have enhanced our understanding of the biochemical basis of heredity and have led to an increasing understanding of the organisation and regulation of eukaryotic genes, with much of this understanding being based on the analysis of viral systems. By contrast the complex nature of the interactions between viral agents and eukaryotic hosts that occur during the infectious process have hampered efforts to decipher the molecular mechanism involved in viral diseases. Thus, while many studies have been performed on the descriptive aspects of pathogenesis of viral diseases, little is known about the precise molecular events responsible. Recently, increased knowledge of the function of individual components of viruses and their interactions in the ultimate production of infection have begun to allow research directed towards defining molecular mechanisms of viral pathogenesis and pathogenicity/ virulence.

3.2 What is virulence?

Virulence or pathogenicity is defined as the capacity of a virus to produce disease in a particular host. It is often used incorrectly to refer to infectiousness (transmissibility) of a virus. Highly virulent viruses are not always readily transmissible from individual to individual (e.g. rabies), and readily transmissible virus are not necessarily very virulent (e.g. rhinovirus). A measure of the virulence is given by dose of the virus

required to cause disease or death. This figure differs for different hosts because a wide range of factors, genetic and non-genetic, immunological and non-immunological, influence resistance of a host to a given virus. In fact, the virulence of a virus and susceptibility/resistance of the host cannot be considered in isolation as it is their interaction that is relevant. Serious disease or death can be caused by avirulent viruses if administered via an atypical route, or in a very large dosage, e.g. avirulent ectromelia virus given by the footpad route to susceptible Bagg mice. On the other hand, C57 B1 mice are resistant to even virulent ectromelia virus when given through footpad route (Schell, 1960).

There is a wide range of pathogenicity/ virulence. At one extreme the infection is harmless, asymptomatic, with very low pathogenicity, e.g. cytomegalovirus in man. At the other extreme infection can lead to a rapidly or uniformly lethal disease, e.g. rabies is uniformly lethal in most vertebrates once the disease becomes apparent. Between these extremes are viruses that have a recognised clinical disease associated with them, e.g. poliomyelitis, but in which disease pattern ranges from asymptomatic infection to full blown clinical disease.

3.3 Why study virulence?

3.3.1 For developing better vaccines

The ideal objective for prevention of viral disease is the eradication of the virus. Such an approach has been accomplished with the world-wide eradication of the virus responsible for smallpox. This was achieved by a highly successful campaign of surveillance and containment by vaccination in order to prevent its spread, rather than by universal routine immunisation. Because eradication is desirable does not mean it is always feasible.

At present, vaccines are the best tools for control and prophylaxis against viruses. The safety and efficacy records of the existing licensed virus vaccines are outstanding. Attenuating live viruses form the basis of many of the currently used vaccines for humans. However, the basis for attenuation of these viruses is poorly understood. Thus, occasional risk of reversion to virulence after poliovirus vaccine either in recipients or in susceptible contacts (WHO Consultative Group, 1982) or other side-effects such as rubella vaccine-associated arthritis in women (Chantler *et al.*, 1982) or development of meningitis in children following live mumps virus vaccine (Forsey *et al.*, 1990) have been reported.

Attenuating live virus has traditionally been an empirical process, without the ability to control where mutations occur in the genome. Understanding of the mechanisms responsible for attenuation of viruses is important for prevention of viral disease. Current molecular techniques allow one to engineer specific mutation into the genome of most DNA viruses and some RNA viruses at any given site. Additional information will be helpful for designing new attenuated live vaccines. First, it will be important to identify the areas in the genome which are crucial for viral virulence. Second, selected sites within these areas, which are not critical for viral replication, should be located. Third, it would be important to verify that the mutations at these sites do not interfere with the development of the host's immune response to the virus. If such sites can be identified, then mutations could be engineered into the genome at these locations, resulting in attenuated viruses. Thus, a better understanding of the molecular basis of virulence may ultimately result in new candidate live virus vaccines.

The same viral gene may have antigenic and virulence determinants (e.g. haemagglutinin in influenza and reo virus) which do not overlap. The understanding of virulence will provide necessary

information to manipulate the gene in such a way so as to reduce its virulence without affecting its antigenicity.

3.3.2 A Penicillin!! for viral disease

There is a wide range of viral infections where immune prophylaxis either by vaccine or gamma-globulins is impracticable or unavailable. While it is theoretically possible to make vaccine against any virus disease, there are often serious difficulties in practice due to a variety of reasons. Some viruses will grow to a very low titre or may be poorly antigenic. Human rhinoviruses are found in more than 100 different serotypes and therefore, it is most unlikely that vaccination would be a successful procedure for prevention because of the specificity of the immune reaction. An alternative method of control is by antivirals. Viruses are obligate intracellular parasites which take genetic control of the host cell's metabolism to achieve their replication. A thorough understanding of the molecular and genetic determinants of virus replication and virulence may help to develop efficient antivirals which selectively inhibit or block viral biosynthesis but have little or no adverse effect on host cell biosynthesis or function.

Thus, a better understanding of the molecular basis of virulence is important for the development of new viral vaccines and antivirals for prevention, control and eradication of viral diseases.

3.4 Rotavirus virulence

3.4.1 Why study rotavirus virulence?

The economic importance of rotavirus is well established (see chapter 1). Rapid progress has been made in the characterisation of rotavirus genome and viral polypeptides, their antigenic properties, and immune responses to them. However, the important parallel area of

rotavirus virulence remains under-explored. Virtually nothing is known about the precise molecular mechanisms determining the relative capacity of rotaviruses to produce disease in an infected host.

Understanding rotavirus virulence may help to control rotavirus disease by providing a direction for developing better vaccines. For example, if the protein involved in virulence is also found to be antigenic and immunogenic, then that can serve as a candidate for future vaccine development. The development of a rotavirus vaccine is a priority area of the WHO. However, the candidate rotavirus vaccines do not confer heterotypic immunity. This may be due to the multiplicity of serotypes. Fourteen G serotypes and 11 P serotypes (19 P types based on sequencing; Hoshino and Kapikian, 1994) have been reported and additional serotypes continue to be reported. Since VP4 and VP7 can segregate independently (Hoshino *et al.*, 1985a; Offit and Blavat, 1986), there are 154 potential G/P serotype combinations at the moment. Also, the interaction between VP4 and VP7 may mask or unmask some neutralisation epitopes (Chen *et al.*, 1992; Kool *et al.*, 1992), thereby increasing the number of potential serotypes predicted by reassortment alone.

An alternative (or a second defence strategy) for the control of rotavirus disease is by drug intervention. If a common pathway of disease production by different strains of rotavirus is found, antivirals may be developed that can be effective against all of them. The common mechanism of assortment in a singly infected cell or reassortment in a mixedly infected cell is one such pathway against which drugs and/ or vaccines may be developed. However, at present the mechanism of genome segment assortment is not known. The interaction of rotavirus proteins with host cell receptor is another area against which drugs may

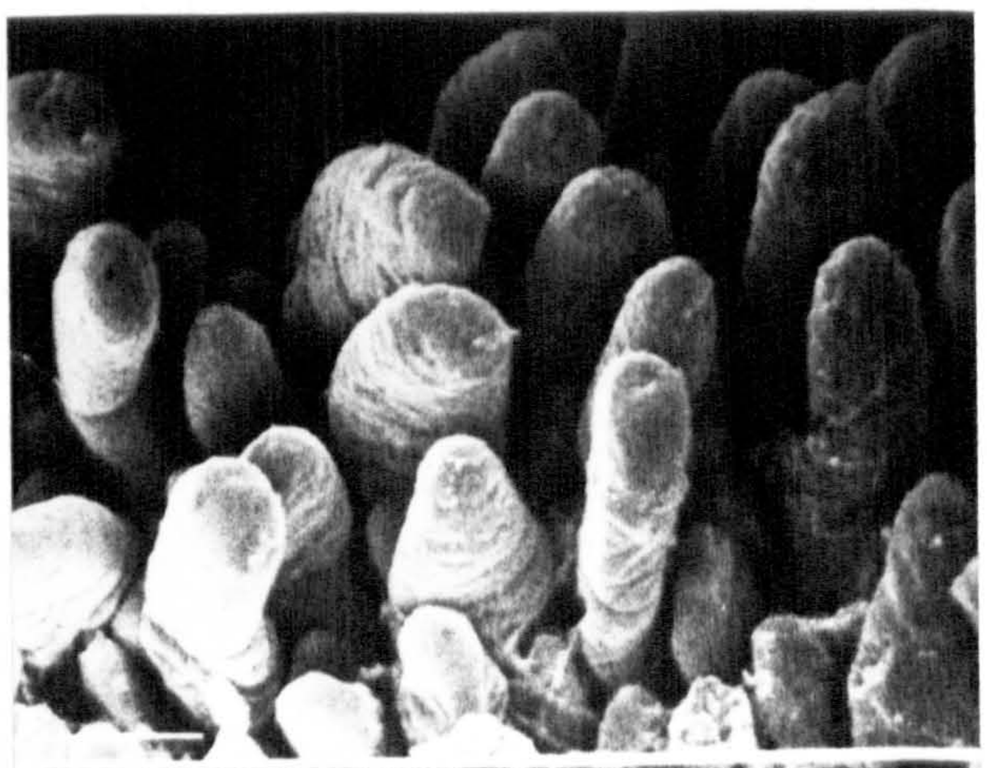
be developed. However, the exact nature of the rotavirus receptor is not known.

3.4.2 Pathogenesis and pathophysiology of rotaviruses

The studies on pathogenesis and pathophysiology of rotavirus disease in humans is limited, but many detailed studies of natural and experimental rotaviral infections in other animals have been reported. The general pattern of infection involves virus penetration and infection of the differentiated enterocytes on the tips of the microvilli of small intestines (McNulty, 1978; Mebus *et al.*, 1971; Mebus and Newman, 1977; Theil *et al.*, 1978). Rotaviruses multiply in the cytoplasm of these enterocytes and damage the absorptive cells, resulting in damage to both the digestive and absorptive functions. There is premature shedding of cells and shortening of villi leading to villus atrophy (Figure 3) (Starkey *et al.*, 1986). The lysis of infected cells releases virus into the intestine, resulting in the large quantities of virus being detected in the faeces of infected subjects.

The major pathophysiological mechanism for diarrhoea in rotavirus patients appears to be decreased absorption of salt and water related to the selective infection of absorptive intestinal villus cells, resulting in net fluid loss (Middleton, 1978). The replacement of the mature epithelial cells with immature cuboidal cells, that have retained their secretory activity as well as have reduced levels of disaccharidases, also increases the secretion. A secondary contribution comes from carbohydrate malabsorption which results in osmotic diarrhoea due to the presence of undigested lactose in the gut (Graham *et al.*, 1982; Sack *et al.*, 1982). Some reports have described replication of rotavirus in hepatocytes of mice under certain experimental conditions and unusual clinical conditions (Riepenhoff-Talty *et al.*, 1987; Uhnoo *et al.*, 1990).

a



b

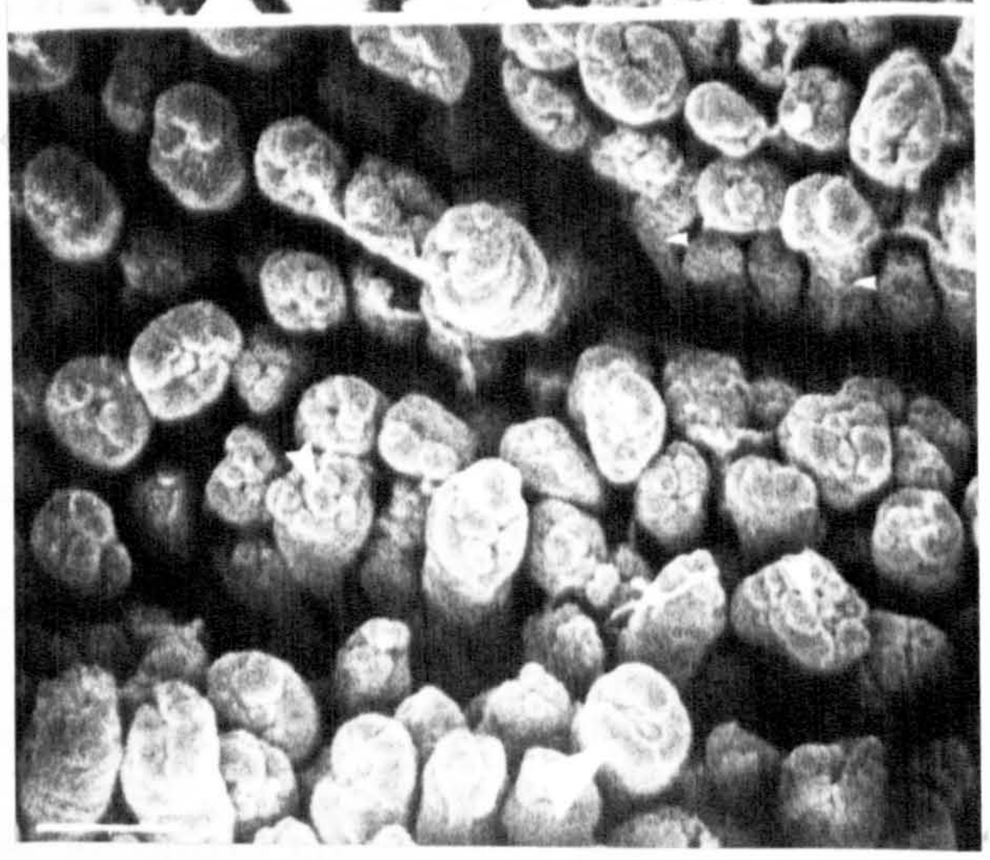


Figure 3 : Scanning electron micrograph of (a) control middle small intestine showing finger or cone-shaped normal villi with regular smooth appearance and (b) middle small intestine 48 hr after infection with EDIM virus showing the appearance of infected villus tips (medium and large arrows) and shrunken appearance of middle to base regions (small arrows)

From Starkey *et al.*, (1986).

3.4.3 Genes involved in rotavirus virulence

Within the past few years, rotavirus strains have been recovered from new-born nurseries in which the virus has persisted and in which most of the infants failed to develop significant symptoms (Perez-Schael *et al.*, 1984). Also infection of gnotobiotic calves with some bovine rotaviruses does not cause disease (Bridger and Pocock, 1986). It is possible that rotavirus strains which produce silent infection differ from the virulent strains as suggested by Perez-Schael *et al.*, (1984), based on electropherotyping. A marked conservation of sequence in gene 4 was observed by RNA:RNA hybridisation for virus strains recovered from infants with asymptomatic infection, while a different set of gene 4 sequences was conserved amongst virulent human isolates (Flores *et al.*, 1986). Sequence analysis confirmed and extended the observations made by RNA:RNA hybridisation suggesting that VP4 has an important role in virulence (Gorziglia *et al.*, 1988b). However, the M37- like VP4 gene reported to occur only in strains of the virus recovered from asymptomatically infected neonates strains (Flores *et al.*, 1986), has recently been recovered from neonates infected symptomatically (Gerna *et al.*, 1990; Steele *et al.*, 1993). The putative rotavirus strains studied by Gorziglia *et al.*, (1988b) were not directly tested in an animal model or in a volunteer trial to determine whether these phenotypes were a genetic property of the viruses in question or due to host factors.

Animal experiments for studying the genes involved in rotavirus virulence have produced conflicting results. The dose which causes diarrhoea in 50% of the inoculated animals is defined as 50% diarrhoea dose, (DD_{50}). The difference in 50% diarrhoea dose, (DD_{50}) between rotavirus strains is described as a difference in virulence (Offit *et al.*, 1986). In the suckling mouse model, the simian strain SA11 was found to

induce diarrhoea in 50% of the inoculated pups at 50-fold lower dose than did bovine strain NCDV. Analysis of the DD_{50} of a large number of SA11/ NCDV reassortants revealed that the virulence segregated with gene 4 of SA11 (Offit *et al.*, 1986).

3.4.4 Putative mechanisms of rotavirus virulence

The trypsin cleavage site of surface glycoproteins is of considerable significance in the pathogenesis among the myxoviruses and paramyxoviruses, because one or two amino acid substitutions in this region of the haemagglutinin of influenza A virus or the fusion glycoprotein of Newcastle disease virus can bring about a marked change in virulence (Kawaoka and Webster, 1988; Toyoda *et al.*, 1987). The trypsin activation of paramyxoviruses and orthomyxoviruses generates a highly conserved apolar amino terminus, and infectivity of these activated viruses can be blocked by oligopeptides that mimic this sequence (Richardson *et al.*, 1980; Richardson and Choppin, 1983).

In rotavirus, VP4 is cleaved into VP8* (28 kD) and VP5* (60 kD) polypeptides in the presence of trypsin and this cleavage results in enhanced viral infectivity (Clark *et al.*, 1981; Espejo *et al.*, 1981; Estes *et al.*, 1981). Direct amino acid sequence analysis of VP4, VP5*, VP8* of the SA11 4fM strain identified the sites of trypsin cleavage as arginine 241 and arginine 247, with the latter position being a preferred site (Lopez *et al.*, 1985). Subsequent nucleotide sequence analysis across the region of gene 4 revealed the conservation of two arginines at the cleavage site. However, the new amino terminus generated in the VP4 of rotavirus is not hydrophobic; instead it contains many polar amino acids some of which may be charged at neutral pH. Therefore, the mechanism of activation of rotavirus infectivity is different from that postulated for other viruses. It is thought that cleavage of VP4 activates an early step in

the replication which may be triggered by one or both of the terminal regions generated by the cleavage or possibly by conformational changes that occur in the cleaved VP4 molecule.

The cleavage of VP4 by trypsin has been shown to enhance internalisation of rotavirus (but not binding) into the cells which is an essential step in the replication process. Evidence has been put forward for both endocytosis and direct cell penetration of viral particles as being the normal route of viral infection (Clark *et al.*, 1981; Petrie *et al.*, 1984; Kaljot *et al.*, 1988). It has been postulated that the VP4 cell membrane interactions may occur at a hydrophobic protein region distant from the cleavage site. This region (aa 384-401) is homologous to the internal fusion sites of Semliki Forest virus and Sindbis virus (Mackow *et al.*, 1988a). Conservation of sequence in this putative fusion region of VP4 of different rotavirus strains supports the suggestion that it may play an important role in the replication cycle and hence virulence.

VP4 in the virion can also be cleaved by chymotrypsin, generating a polypeptide with a molecular weight similar to that of VP5* but such cleavage does not enhance viral infectivity (Estes *et al.*, 1981) and serves to illustrate the specificity of cleavage sites of VP4 for activation of infectivity. The region flanking the trypsin cleavage sites is relatively highly conserved in all strains and it may serve to hold the cleavage sites in the proper conformation for cleavage. The cleavage sites and intervening peptide are considered to have a role in virulence.

However, other investigators have questioned the role of trypsin cleavage site in the rotavirus virulence on the basis of sequence analysis or studies with neutralisation escape mutants. Gottfried strain of rotavirus is virulent in pigs. However, its VP4 is more closely related to the VP4 of the asymptomatic neonatal human rotaviruses than to the VP4 of symptomatic human or animal rotavirus strains based on amino acid

homology, identical potential trypsin cleavage sites, and similarity in the connecting peptide (Gorziglia *et al.*, 1990b).

The amino acid sequence of VP4 of two virulent NCDV bovine strains and an attenuated NCDV strain (RIT4237) differs in only five amino acids which are scattered throughout the protein but do not involve the trypsin cleavage site. It indicates that either a gene(s) other than VP4 gene sustained mutations which are responsible for attenuation of the RIT strain or VP4 amino acid substitutions outside the cleavage region could affect the role of VP4 in determining the virulence phenotype (Nishikawa *et al.*, 1988).

Antigenic mutants of rhesus rotavirus selected with VP4 specific neutralising monoclonal antibodies directed against variable region do not demonstrate altered virulence in mice (Shaw *et al.*, 1986). Also, among the rhesus rotavirus (RRV) variants selected by escape from broadly acting heterotypic neutralising anti-VP4 monoclonal antibodies or with homotypic anti-VP4 monoclonal antibodies, no mutants were identified in or near the trypsin cleavage site (Mackow *et al.*, 1988a).

VP4 and VP7 interact closely and such interaction may influence many more biological properties of these viruses than currently thought and recognised (Estes and Cohen, 1989). VP4 forms spikes on a smooth outer capsid formed by VP7 (Prasad *et al.*, 1988). The region downstream from the long α helical stretch at the carboxy terminal of VP4 may contain sites of VP4 that closely interact with VP7 (Lopez *et al.*, 1991). Whether or not these interactions have any role in virus infectivity and virulence has not yet been explored.

3.4.5 Determinants of host range restriction of rotavirus

Subgroup I human rotaviruses have been found to have short electropherotypes in all but a few cases (Nakagomi *et al.*, 1987; Brown *et*

al., 1988). On the other hand, almost all known strains of animal rotavirus belong to subgroup I but have long electropherotypes (Kalica *et al.*, 1978; Rodger and Holmes, 1979; Thouless *et al.*, 1982; Albert *et al.*, 1987), and subgroup I human isolates are not serotypically related to these animal strains (Hoshino *et al.*, 1984). These observations indicate that animal rotaviruses do not readily infect humans. It is known, however, that vaccine strains of bovine (Clark *et al.*, 1988) and simian (Losonsky *et al.*, 1986; Anderson *et al.*, 1986) rotaviruses will infect humans but the amount of virus shed appeared to be orders of magnitude less than can occur during infection with human strains (Ward *et al.*, 1984). Although reassortant formation between animal and human rotaviruses occurs readily in cultured cells after coinfection, evidence of reassortants between human and animal strains within natural human isolates has only recently been reported (Sukumaran *et al.*, 1992; Das *et al.*, 1993; Dunn *et al.*, 1993).

The host range restriction which is associated with the spread of the virus amongst the population, has been suggested not to be entirely related with virulence. The gene 5 which encodes the non-structural protein VP5 (NS53/NSP1) was indicated to be involved in host range restriction (Broome *et al.*, 1993). Support for this experimental observation has come recently from the identification and characterisation of asymptomatic human rotavirus strains isolated from neonates in India, which had sequence homology of genes encoding VP4 and VP7 with bovine B223 strain (Sukumaran *et al.*, 1992; Das *et al.*, 1993; Dunn *et al.*, 1993) but gene 5 sequence was representative of human rotavirus. The gene 5 selection of human rotavirus type over bovine rotavirus type presumably helped the Indian strains to survive and circulate in the population, albeit asymptomatically. The host range restriction was found to be associated with gene 4 in a virus overlay protein blot assay (Bass *et*

al., 1991). About 10^5-fold higher inocula of heterologous viruses are required to produce the disease in mouse (Greenberg *et al.*, 1986) indicating host range restriction.

3.4.6 Rotavirus receptor

The specific cell and tissue tropism of rotavirus infection as well as the host range and host age restrictions could be partially mediated by specificity of interactions between rotavirus attachment proteins and host receptors. Reduced binding of rotavirus to adult mouse enterocytes as compared to those of suckling mice (Riepenhoff-Talty *et al.*, 1982) has been reported. Two large molecular weight glycoproteins in murine intestinal brush border that specifically bind to infectious double-shelled rotavirus in a Western blot virus affinity overlay assay have recently been identified (Bass *et al.*, 1991). Expression of these glycoproteins correlates with rotavirus cell and tissue tropism as well as the host range and host age restriction seen in rotavirus infection. However, the development of a similar assay for cultured cells was not successful (Bass *et al.*, 1992a). The binding of rotaviruses to target cells is sensitive to neuraminidase treatment (Yolken *et al.*, 1987; Bass *et al.*, 1991) indicating involvement of sialic acid. However, the exact nature of rotavirus receptor remains unknown.

3.4.7 Limitations on virulence studies in rotavirus

There are two limitations on virulence studies.

1. Lack of suitable animal model.

2. Inability to manipulate the rotavirus gene(s) *in vitro* and then rescue this information into an infectious particle.

3.5 Determinants of poliovirus neurovirulence/ attenuation

3.5.1 Introduction

Poliovirus, a member of *Picornaviridae* family, is classified into three serotypes. The virion contains a single-stranded positive sense RNA molecule of 7.5 kilo bases coding for a large polyprotein from which viral capsid and non-structural proteins are derived. The icosahedral viral capsid is made up of 60 copies of each of the four structural polypeptides: VP1, VP2, VP3 and VP4. The complete sequence of the viral genome is known and it is possible to construct viral recombinants and mutants by manipulating cloned infectious cDNA (Racaniello and Baltimore, 1981).

3.5.2 Molecular basis of attenuation of poliovirus neurovirulence

Poliovirus poliomyelitis has been greatly reduced due to the two excellent vaccines. One of these is Sabin's live attenuated preparation which contains all three viral serotypes. In the past the three Sabin vaccine strains have been extensively studied to obtain information on the mechanisms of attenuation.

Analysis of viral proteins has been used to reveal differences between wild-types and attenuated viruses. While a difference in the amino acid sequence between a wild-type and attenuated viruses may be associated with virulence, this difference is not necessarily responsible for virulence (Diamond *et al.*, 1985). These studies focus more on the surface proteins that react with antibodies or proteins that can be obtained in large quantities from virions. Therefore, the role of non-structural proteins as well as the non-coding regions of the genome may be overlooked.

Differences in the RNA genomes of attenuated polioviruses and their neurovirulent parents were first detected by the method of RNAase

T1 oligonucleotide fingerprinting (Nomoto *et al.*, 1979). A comparison of the genomes of the P1/Sabin vaccine strain and its neurovirulent parent, P1/Mahoney, indicated that the two viruses differ at approximately 35 nucleotide positions. Complete nucleotide sequencing of their genomes showed that these two viral RNAs differ by 55 nucleotide substitutions out of the total genome length of 7441 (Nomoto *et al.*, 1982), with the differences being scattered throughout the viral genome. This study showed that a relatively small number of mutations accompany the attenuation process, although they did not allow the precise localisation of the mutations responsible for the attenuated phenotype. A number of different approaches have been used to identify these mutations. These include the analysis of the sequences from the viral revertants, the construction of recombinant viruses containing regions of wild-type and attenuated genome, and more recently mutants produced by site-directed mutagenesis.

Insights into the molecular basis of virulence has been gained by comparing nucleotide sequence between neurovirulent revertants and the attenuated parent virus. Nucleotide sequence analysis of the strain P3/119, isolated from a vaccine associated fatal case of poliomyelitis, and comparison of it with P3/Leon (neurovirulent progenitor) and P3/Sabin (vaccine strain) indicated it to be a true vaccine revertant. At 8 out of 10 base positions that differed between P3/Sabin and P3/Leon, the sequence of P3/119 was identical to the vaccine strain. The only base in the non-coding region that had back-mutated to P/Leon sequence was 472 : which was C in P3/Leon, a U in P3/Sabin, and a C in P3/119 (Cann *et al.*, 1984). Back-mutation of nucleotide 472 to C was also observed in RNA from five other isolates of vaccine-associated cases of poliomyelitis (Evans *et al.*, 1985). Nucleotide sequence comparison of P2/ Sabin and its revertants indicates that the reversion occurs by A-G mutation at

nucleotide 481 in the 5' non-coding region and Ile-Val mutation in VP1-143 (Macadam *et al.*, 1991; Equestre *et al* ., 1991). Sequence analysis of neurovirulent derivatives of P1/Sabin suggests that as few as two mutations may be sufficient for reversion to neurovirulence (Christodoulou *et al.*, 1990).

The general approach used to identify mutations responsible for attenuation phenotype has been to construct viral recombinants between neurovirulent and attenuated strains and determine the neurovirulence of the recombinants in monkeys. By coinfecting cells with two genetically distinct viruses, Agol *et al.*, (1985) obtained four recombinant polioviruses that were the mixtures of 5' half and 3' half in various combinations from the parents. Intracerebral inoculation of these recombinants into monkeys demonstrated that the 5' half of the genome was primarily responsible for viral virulence, the 3' half did contribute to virulence, but to a much lesser extent. The demonstration that poliovirus type 1 cDNA is infectious in cell culture (Racaniello and Baltimore, 1981) provided a new method for preparing recombinant poliovirus. Subsequently, recombinants were constructed by exchanging DNA restriction fragments between infectious clones and recovering virus by transfection of cultured cells with recombinant plasmids.

Analysis of the neurovirulence of recombinants between P1/Sabin and P1/ Mahoney by intrathalamic inoculation of cynomolgus monkeys showed that the attenuating determinants are scattered throughout the genome. However, a strong attenuating mutation was located in the 5' non-coding region at nucleotide 480. Analysis of the recombinants between P3/Sabin and P3/Leon using intraspinal inoculation of monkeys indicated that two mutations accounted for the attenuated phenotype of P3/Sabin: a base change from C to U at nucleotide 472 in the 5' non-coding region, and a change from serine to phenylalanine at

nucleotide 2034 in the capsid protein VP3 (Westrop *et al.*, 1989). Although recent evidence suggests that another mutation at nucleotide 2493 in the capsid protein VP1 may also be involved (Tatem *et al.*, 1992).

The type 2 vaccine strain (P2/Sabin) was derived from a naturally attenuated wild strain P2/712 (Sabin and Boulger, 1973) which meant that a different approach than described for type 1 and 3 had to be taken to analyse attenuating determinants. Analysis of neurovirulence in primates by recombinants between P2/Sabin and P2/117 (a neurovirulent derivative isolated from a vaccine associated case of poliomyelitis) indicated that mutation at nucleotide 481 is responsible for neurovirulence (Macadam *et al.*, 1991).

An alternative way of analysing poliovirus neurovirulence has been the conversion of vaccine strains into neurovirulent strains and conversely attenuating the neurovirulent strains by site-directed mutagenesis. Using site-directed mutagenesis of P2/Sabin strain, Macadam *et al.*, (1993) have recently shown that mutations at just two positions, nucleotide 481 in the 5' non-coding region and amino acid 143 in the VP1 capsid protein, resulted in a highly neurovirulent virus in monkeys. Other nucleotide changes may have weaker phenotypic effects. These results are consistent with those reported in the mouse model by Ren *et al.* (1991).

It, therefore, appears that all three poliovirus vaccine strains have in common at least one mutation in the 5' non-coding region around nucleotide 480, that can attenuate the neurovirulence of polioviruses inoculated into the central nervous system (CNS) of experimental animals. This attenuation may be due to reduced viral translation and failure to replicate efficiently or disruption of RNA secondary structure in this region (Skinner *et al.*, 1989; Macadam *et al.*, 1991; 1992).

3.5.3 Molecular determinants of host range restriction of poliovirus

Humans are the only known natural hosts of poliovirus. Experimentally, poliomyelitis can be transmitted to chimpanzee, monkeys and mice by inoculation of poliovirus into CNS. Wild strains of all three serotypes are spontaneously neurovirulent in primates. In contrast, only a limited number of poliovirus strains are able to induce poliomyelitis in mice by intracerebral inoculation. A number of viral strains, such as P1/Mahoney strain are clearly host restricted and cause paralysis in primates but not in mice (La Monica *et al.*, 1986). However, transgenic mice expressing human poliovirus receptor are susceptible to infection with all three serotypes of poliovirus (Ren *et al.*, 1990; Koike *et al.*, 1991). These result show that the primary block to infection in normal mice by these strains is at the level of cell entry.

The molecular basis of poliovirus neurovirulence in mice has shed light on the genetic determinants involved in host range restriction. The construction of viral recombinants between the mouse-adapted P2/Lansing and mouse-avirulent P1/Mahoney has revealed that the capsid coding region of the Lansing strain is responsible for its mouse-neurovirulent phenotype (La Monica *et al.*, 1986). Further studies have shown the important role played by the B-C loop (amino acid 94-102) of the capsid protein VP1 in host range restriction (La Monica *et al.*, 1987; Martin *et al.*, 1988; Murrey *et al.*, 1988; Couderc *et al.*, 1991; Martin *et al.*, 1991). Various single amino acid substitutions in the VP1 B-C loop of P2/Lansing reduce the pathogenicity of the virus in mice (La Monica *et al.*, 1987). A significant finding concerning poliovirus host range is that variants of P1/Mahoney carrying VP1 B-C loop of P2/Lansing are neurovirulent in mice (Martin *et al.*, 1988; Murrey *et al.*,

1988). As for P2/Lansing, amino acid changes in VP1 B-C loop of this mouse-adapted P1/P2 chimeric virus impairs its neurovirulence in mice (Couderc *et al.*, 1991; Martin *et al.*, 1991). More recently, neurovirulence determinants of P2 were identified by analysing mutants of P2/Lansing which had been attenuated by introducing mutations in the B-C loop and then passaged in mice. Mouse-selected mutants were subsequently found to be more virulent. The increased neurovirulence of these strains was mapped to two different suppressor mutations in the N terminus of VP1. Whereas the B-C loop of VP1 is highly exposed on the surface of the capsid, the suppressor mutations are in the interior of the virion. Moreover, the introduction of suppressor mutations into the genome of the mouse-avirulent P1/Mahoney resulted in neurovirulent viruses (Moss and Racaniello, 1991). These results demonstrate that the VP1 B-C loop of P2/Lansing is sufficient but not absolutely necessary to render P1/Mahoney neurovirulent for mice, indicating that mechanism of poliovirus adaptation to mouse is more complex. Recently, Couderc *et al.*, (1993) isolated two mouse-virulent poliovirus type 1 Mahoney mutants in the mouse CNS after a single passage of P1/Mahoney inoculated by the intracerebral route. The nucleotide sequence of the mutant genomes were determined. Mutations were introduced into the parental P1/Mahoney genome by single-site mutagenesis. Mutated P1/Mahoney viruses were then tested for their neurovirulence in mice. Both independent mutations in VP1 (Thr 22-Ile) and VP2 (Ser-Thr) conferred mouse virulent phenotype to the mouse-avirulent P1/Mahoney strain. Thus, Couderc *et al.*, (1993) have identified a new mouse adaptation determinant on VP1 and they have also shown that at least one other capsid protein, VP2, could also express a mouse adaptation determinant.

3.6 Virulence determinants of Influenza A virus

3.6.1 Introduction

Influenza A, a member of *Orthomyxoviridae* family, is an enveloped virus. The genome consists of eight segments of single-stranded RNA of negative polarity. The mRNAs are transcribed from the virion RNA by a virion-associated-RNA-dependent-RNA-polymerase. The eight gene-segments code for eight structural (PB1, PB2, PA, HA, NA, NP, M1 and M2) and two non-structural proteins (NS1 and NS2) (Lamb, 1983). At present there are 14 HA (haemagglutinin) and 9 NA (neuraminidase) subtypes of influenza A virus.

Influenza A viruses have been isolated from humans, horses, pigs, mink, seals and whales as well as from a variety of different avian species. Although there is variation in the severity of illness in mammalian species, infection is usually localised to the respiratory tract. Majority of the avian influenza viruses also cause local infection in the respiratory tract or in the gut, which frequently remains asymptomatic. In contrast other avian strains are highly pathogenic causing fowl plague, a systemic infection which is often fatal. The avian system is particularly useful for virulence studies because a large number of naturally occurring strains can be analysed in their natural host (Schulman, 1983).

Unlike poliovirus, infectious influenza viruses having all the genes derived from cDNAs can not be obtained. However, the particular genome organisation of influenza viruses permits genetic reassortment when a single cell is infected by two different parents. This approach has been used to segregate the virulence phenotype with gene segment(s).

3.6.2 Influenza virus pathogenicity is multigenic

Employing the highly virulent fowl plague virus (FPV) in *in vitro* crosses with non-pathogenic viruses of mammalian or avian origin, it was

demonstrated that the pathogenicity is of polygenic nature (Rott *et al.*, 1979). These findings were further confirmed by genetic analysis of a large number of reassortants obtained after mixed infection with ts mutants of FPV and different human or animal influenza viruses (Scholtissek *et al.*, 1977).

The constellation of genes coding for viral RNA polymerase complex (PB1, PB2, PA, NP) appears to have an important role in virulence. When the complete set of these genes was derived from one or the other avian parent virus, the reassortant was, in general, pathogenic. In contrast all non-pathogenic reassortants had a mixed polymerase complex. This was the case regardless of whether these genes ultimately came from pathogenic or non-pathogenic strains (Rott *et al.*, 1979; Giesendorf *et al.*, 1986). Reassortment, even between highly pathogenic strains may lead to pathogenic as well non-pathogenic reassortants (Rott *et al.*, 1979). By contrast, virulent reassortants can be derived following mixed infection with two avirulent parents. Also, certain reassortants derived from crosses between a non-neuropathogenic FPV and a non-neuropathogenic human influenza virus have been shown to be highly virulent for mice (Scholtissek *et al.*, 1979). It has been demonstrated that NA, M, and NS genes of WSN virus could contribute to neurovirulence, however, the NA gene is essential (Sugiura and Ueda, 1980).

The human H1N1 prototype strain, A/FM/1/47 (FM), was adapted to the mouse by serial lung passage to produce a virulent variant, FM-MA that had increased in virulence by $10^{4.3}$-fold as measured by LD_{50}. Segments 4, 5, 7, and 8 were identified as being associated with virulence in reassortant studies (Brown, 1990). Sequence analysis revealed single amino acid replacements in gene 4 and gene 7 to be responsible for the increased virulence (Smeenk and Brown, 1994).

3.6.3 Functional basis of virulence : role of individual gene segments

The results obtained with viral reassortants has revealed that the expression of virus pathogenicity is dependent upon the functional integrity of each gene and on a gene constellation optimal for infection of a given host. Changes that result in alterations in the function of any gene or in genome composition can cause alterations in pathogenicity. As a consequence virtually every gene product of influenza virus has been reported to contribute to pathogenicity, but evidence is steadily growing to assign a key role to haemagglutinin.

Three different functions of haemagglutinin have been shown to play a role in influenza virus pathogenicity. First, antigenic shift and drift in the virus allows it to infect individuals who are already immune to previous strains. Second, studies on receptor-binding sites also suggest that changes in this region of haemagglutinin of the virus can lead to changes in the pathogenicity. Third and most important is that the proteolytic cleavage of the haemagglutinin by trypsin-like proteases at arginine residues is indispensable for influenza virus to initiate infection. Since the activating proteases are cellular enzymes, the infected cell type determines whether the haemagglutinin is cleaved (Klenk *et al.*, 1975; Klenk and Rott, 1980). The haemagglutinins of mammalian influenza viruses and non-pathogenic avian influenza viruses, which cause a local infection, are susceptible to proteolytic cleavage only in a restricted number of cell types. On the other hand, haemagglutinins of pathogenic avian influenza viruses among the H5 and H7 subtypes causing a systemic infection are cleaved by proteases present in a broad range of different host cells (Bosch *et al.*, 1979). Thus, there are differences in the host range resulting from differences in haemagglutinin cleavability which can be correlated with the pathogenic properties of the virus. The difference

in cleavability is due to differences in the structure of cleavage site of the haemagglutinins. The HA1 and HA2 fragments of haemagglutinin of the apathogenic avian and of all mammalian influenza viruses are linked by a single arginine. This is in contrast to the pathogenic avian strains, which have a sequence of several basic amino acids at the cleavage site. This fact was derived from the comparisons of naturally occurring strains (Bosch *et al.*, 1981; Garten *et al.*, 1981; Klenk and Rott, 1988) and was corroborated when acquisition of high cleavability paralleled by an increase in the number of basic residues at the cleavage site was observed in studies on haemagglutinin mutants generated by site-directed mutagenesis (Kawaoka and Webster, 1989) or on virus mutant adapted to new host cells (Li *et al.*, 1990). Determination of amino acid sequence at the HA cleavage site is essential for assessing the potential virulence of avian influenza virus isolates. The important role, in the cellular proteases-induced cleavage, of the first (Vey *et al.*, 1992), second, and fourth (Kawaoka and Webster, 1988) basic residues from the carboxyl end of the HA1, and the irrelevance of the third (Kawaoka and Webster, 1988) basic residue, led (Vey *et al.*, 1992) to recognise a conserved sequence motif Arg-xxx-Arg/Lys-Arg, found at the cleavage site of H7 avian virus HAs. This is further supported (Walker and Kawaoka, 1993) for H5 avian influenza viruses. In order for a HA to be cleaved completely by the endogenous proteases in cell culture, at least six amino acids have to be present at the cleavage site if a carbohydrate side chain is nearby. Otherwise, only four amino acids are needed. Using reverse genetics, it has recently been demonstrated directly that the cleavability of haemagglutinin determines influenza virus virulence (Horimoto and Kawaoka, 1994). They have further proposed that viruses with the Arg-Arg/Lys-Arg-Lys-Thr-Arg motif (Wood *et al.*, 1993) should be considered in the same category as virulent viruses with xxx-xxx-

Arg/Lys-xxx-Arg/Lys-Arg motif. The European Community definition of highly pathogenic avian influenza viruses for control purposes (Council of European Communities, 1992) specifies an intravenous pathogenic index (IVPI) in six week old chickens of 1.2 or higher out of a score of 3.00 (a score of 3.00 in this test indicates that all birds died within 24 hours of infection and 0.00 indicates that no bird died or showed any clinical signs over the observation period of ten days), or for H5 and H7 viruses if *in vivo* testing shows a lower IVPI, then the presence of multiple-basic amino acids at the haemagglutinin cleavage site is sufficient. However, the highly pathogenic and avirulent isolates from a recent outbreak in turkeys in Norfolk, England in 1991/92 had the same cleavage site sequence with multiple-basic amino acids, but unlike the outbreaks of avian influenza in Pennsylvania in 1983, the effect of the multiple-basic amino acids was not masked by an adjacent oligosaccharide at asparagine 11 (Wood *et al.*, 1994) in avirulent strains.

The second important determinant appears to be a carbohydrate side chain that is present in the vicinity of the cleavage site and interferes with the protease accessibility. Loss of this carbohydrate resulted in enhanced haemagglutinin cleavability and viral pathogenicity (Deshpande *et al.*, 1987; Kawaoka *et al.*, 1984), and the effect of steric hindrance was abolished when the number of basic amino acids at the cleavage site increased (Kawaoka and Webster, 1989; Ohuchi *et al.*, 1989). It has also been observed that insertion of a relatively long foreign peptide composed of nonbasic amino acid residues renders a single arginine at the cleavage site susceptible to ubiquitous cellular proteases (Katchikian *et al.*, 1989). There is evidence that mutations in sequences different from the cleavage site can also affect the activation of the molecule. Such a mutation has been found to be responsible for an altered protease sensitivity of the H3

haemagglutinin, which in turn resulted in a change in host cell range (Rott *et al.*, 1984).

Examination of the amino acids of the amino-terminal sequence of HA2 of several influenza A strains of human and avian origin has revealed a highly conserved region of 10 residues (Waterfield *et al.*, 1979). This sequence is homologous to the amino-terminus region of the fusion glycoprotein F of Sendai virus (a paramyxovirus), which mediates fusion of the Sendai virus envelope with the plasma membrane of the host, a function which is considered to be essential for virus infectivity (Gething *et al.*, 1978). It has, therefore, been postulated that the infectivity of influenza virus is dependent on the fusion function of haemagglutinin analogous to that of Sendai virus fusion protein. Newcastle disease virus, another paramyxovirus, comprises of various strains which, like the avian influenza viruses, differ widely in virulence for chicken. Differences in the pathogenicity can be correlated to the cleavability of the F protein (Nagai *et al.*, 1976) and recent sequence analysis has revealed that apathogenic strains have a single arginine residue and the pathogenic strains have paired basic residues at the cleavage sites (Toyada *et al.*, 1987).

While it is clear from the above studies that susceptibilities of the haemagglutinin of avian influenza viruses to cleavage is determined by the structure of the haemagglutinin, in other systems differences in the viral neuraminidase may determine whether or not cleavage of haemagglutinin occurs. Influenza A/WSN/33 (H1N1) virus is unique in its capacity to undergo multi-cycle replication and to form plaques in MDBK cells. Analysis of reassortants demonstrated that this property is dependent on the neuraminidase of the virus (Schulman and Palese, 1977). Infectivity was found to be dependent on a cleaved haemagglutinin and that the infectivity of viruses lacking WSN neuraminidase can be

activated by *in vitro* treatment with trypsin. In related studies it was shown that the virulence for 1-day-old chickens (Bean and Webster, 1978) and neurovirulence in mice (Sugiura and Ueda, 1980) can also be correlated with the possession of the WSN neuraminidase gene in certain reassortants. The nucleic acid sequence of the neuraminidase of WSN virus has been determined (Hiti and Nayak, 1982). It showed that a potential glycosylation site at position 130 conserved in other influenza viruses neuraminidase is absent in this strain. Using reverse genetics methods, recently it has been shown that the absence of this glycosylation site at position 130 of the neuraminidase plays a key role in the neurovirulence of WSN virus in mice (Li *et al.*, 1993a).

3.7 Molecular basis of reovirus virulence

Mammalian reoviruses, as members of the *Reoviridae* family, have icosahedral symmetry. The virion comprises of two protein shells wrapped around the segmented dsRNA genome. Each of the 10 genome segments represents a single gene which is transcribed and translated into a unique mRNA molecule and a primary polypeptide, respectively (McCrae and Joklik, 1978) with the exception of S1 which encodes two proteins σ1 and p14.

The mammalian reoviruses are separable into three serotypes (Rosen, 1960). Although structurally quite similar, these serotypes interact with mammalian hosts with very distinct patterns of disease (Fields, 1981).

The general approach to study reovirus virulence has been to inoculate prototype strains, especially the laboratory strains Type 1 (Lang), Type 2 (Jones), Type 3 (Dearing) and to compare their patterns of infections. If differences between the serotypes were detected then reassortants were generated and a genetic analysis performed to

determine if the observed differences were properties of one or more of the viral genes (Fields, 1982).

Intracerebral inoculation of newborn mice with type 3 reovirus leads to the development of an acute encephalitis that is uniformly fatal and associated with specific viral replication in neurones (Margolis *et al.*, 1971; Raine and Fields, 1973). In contrast, inoculation of type 1 reovirus causes a non-lethal acute ependymitis without involvement of neurones (Kilham and Margolis, 1969). Localisation of different types of reovirus to different cells was responsible for difference in pathogenicity, which in turn was found to be determined in both cases by the σ1 protein encoded by the S1 gene (Weiner *et al.*, 1977; 1980) from reassortant analysis.

Antigenic escape mutants selected using a monoclonal antibody directed at the major neutralisation site on σ1 of type 3 reovirus provided further evidence for the critical role of the σ1 protein in neurovirulence. Some of these variants were at least 4 $\log_{10}$ less neurovirulent than the type 3 Dearing strain from which they were derived. Other variants were found to be essentially avirulent ($LD_{50} > 10^7$ PFU) and impaired in their capacity to grow in the brains of mice (Spriggs and Fields, 1982). Sequence analysis of the S1 genes of several of these variants indicated that they have single amino acid substitutions at amino acid positions 340 or 419 of σ1 protein within the predicted outward facing globular head of the protein. The significance of the single amino acid substitutions in σ1 in producing attenuated neurovirulence and altered tropism has been confirmed in studies using a reassortant virus containing the S1 gene derived from one of the σ1 antigenic variants (Kaye *et al.*, 1986).

The Dearing strain of type 3 is highly lethal when inoculated intracerebrally into suckling mice, a typical LD_{50} being ~1 $\log_{10}$ (Weiner *et al.*, 1977). However, when this strain is given orally, even in doses of 10^7 PFU of purified virus, it is avirulent (Rubin and Fields,

1980). This quantity of type 3 virus fails to grow in intestinal tissue after oral inoculation directly into the stomach of newborn mice and is inactivated when mixed with intestinal homogenates. In contrast, reovirus type 1 (Lang) strain inoculated by the same route multiplies in the intestine, and then spreads to the brain where it produces a benign ependymitis similar to that which develops after intracerebral inoculation. Another difference between the two viruses is that the infectivity of type 1 virus is unchanged or slightly enhanced by treatment with pancreatic protease chymotrypsin while that of type 3 is greatly reduced. A genetic analysis using reassortant viruses between type 1 and type 3 revealed that the ability to grow in the intestinal tissue and the response to pancreatic proteases are properties of M2 gene and hence μ1/μ1C proteins (Rubin and Fields, 1980).

In addition to determining growth in intestinal tissue, the M2 gene also plays a part in controlling the relative degree of neurovirulence of a natural isolate of type 3 reovirus (Hrdy *et al.*, 1982). A large number of type 3 isolates were screened to ascertain their relative neurovirulence after intracerebral inoculation. One strain retained the neurotropism of type 3 (i.e. infection of neurone and not ependyma) but was markedly less virulent (LD_{50} of $\sim 10^{4.5}$ compared with type 3 Dearing LD_{50} of $\sim 10^{1}$). Genetic analysis revealed that the attenuation in neurovirulence in this isolate was also related to its M2 gene. The results of these studies indicate that the nature of M2 gene is important in determining virulence after inoculation by the natural oral route and by the experimental intracerebral route. Therefore, the S1 gene determines the cell and tissue tropism and the M2 gene determines the relative capacity to grow in the CNS as well as at mucosal surfaces (Hrdy *et al.*, 1982).

The results in the aforementioned studies with poliovirus recombinants and revertants, and reassortants of influenza and reo viruses

indicate that virulence is a multigenic trait, while certain genes may play a primary role in determining virulence, other genes contribute and may enhance or attenuate the virulence phenotype. All the three viruses, polio, influenza, and reo discussed above are RNA viruses. Poliovirus is a positive sense ssRNA virus and the availability of vaccine revertants and the ability to obtain infectious virus from cDNA clones has greatly contributed to the understanding of its virulence. Unlike poliovirus, influenza and reo viruses are segmented viruses. Influenza virus is an enveloped negative sense ssRNA virus, that is an important human pathogen and where the techniques of genetically manipulating the virion have recently been developed. On the other hand, reovirus is a non-enveloped dsRNA virus that does not cause any disease in humans and the techniques to rescue a gene into an infectious virus are not available. Studies with reassortants of both influenza and reo viruses have indicated that the virulence is multigenic. Different reovirus genes contribute to different aspects of its virulence, whereas in influenza virus though each gene has been known to be involved in virulence, however, the role of HA and to a lesser extent NA is known.

Rotaviruses are segmented dsRNA viruses like reovirus but are important pathogens of humans, animals, and birds like influenza virus. It would be interesting to find out whether its virulence resembles influenza virus or reovirus or both or neither.

3.8 Aims

1. To establish a suitable animal model for monitoring rotavirus virulence.

2. To construct and characterise rotavirus reassortants.

3. To begin mapping of rotavirus virulence to its gene(s).

Chapter 4

Materials and Methods

4.1 Materials

4.1.1 A list of the biochemicals, chemicals, and radiochemicals utilised in this study is given below :

Amersham International plc. Amersham, Buckinghamshire :

Amplify, ^{35}S dATP, ^{32}P dGTP

BDH chemicals Ltd., Poole, Dorset :

Boric acid, β- mercaptoethanol, Bromophenol blue, Calcium chloride, Crystal violet, Dimethyl sulphoxide (DMSO), Glycerol, Liquid paraffin, Lithium chloride, Nonidet P-40 (NP40), Potassium chloride, Sodium chloride, Sodium dihydrogen phosphate, Sodium dodecyl sulphate (SDS), Sodium hydroxide, Tris-base, Triton-X

Bio 101 Inc., Joshua Way, Vista CA :

Gene Clean kit

Bio-Rad Laboratories Ltd., BioRad house, Hemel, Hempstead, Hertfordshire :

Ammonium persulphate, N,N'-methylene bisacrylamide, TEMED

Boehringer Mannheim UK Ltd., Lewes, East Sussex :

Proteinase K

Flow Laboratories Ltd. Scotland :

GMEM

FSE, Loughborough, Leicestershire :

Acetic acid, Acrylamide, Ethanol, Formaldehyde, Formamide, Glycine, Magnesium chloride, Methanol, Phenol, Silver nitrate, Urea, Xylene cyanol

Life Technologies Ltd. P.O. Box 35, Renfrewshire, Scotland :

1kb DNA ladder marker, Restriction enzymes, Tissue culture plates

Northumbria Biologicals Ltd., Cramligton, Northumberland :

Foetal calf serum

NBL Gene Sciences Ltd., Cramligton, Northumberland :

Avian myeloblastosis reverse transcriptase (AMV RT)

Polaroid UK Ltd., St Albans, Hertfordshire :

Type 665 (positive/negative) and 667 (positive) films

Prolabo, Manchester :

Diethyl ether

Promega, Chilworth Research Centre, Southhampton :

Taq DNA polymerase

Sigma Chemical Co. Ltd., Poole, Dorset :

Agarose medium EEO (type II), Ampicillin, Deoxynucleoside triphosphates, Dideoxynucleoside triphosphates, Dithiothreitol (DTT), Ethidium bromide, Gelatin, Kanamycin, Nystatin, Salmon sperm DNA

Whatman International Ltd., Maidstone, Kent :

Filter paper number 1

4.1.2 Media and solutions

The following media and solutions were prepared by the media preparation staff in the Department of Biological Sciences, University of Warwick :

2x Agar, 2x GMEM, 200 mM Glutamine, Neutral red, Phosphate buffer saline ([PBS], 8g NaCl, 0.2g KCl, 1.15g Na_2HPO_4, 0.2g KH_2PO_4 per litre of sterile distilled water), Penicillin, Streptomycin (100g and 100,000,000 units respectively per 2 litre sterile distilled water), Trypsin solution (0.25% in versene), Versene

4.2 Cells

The continuous cell lines BSC-1 (derived from African green monkey kidney) and MA104 (derived from foetal rhesus monkey kidney) were grown as monolayers in Glasgow modified minimal essential medium with non-essential amino acids (GMEM), supplemented with 5% foetal calf serum (FCS), glutamine (4mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). The split ratio for BSC-1 and MA104 cells was 1:4, and 1:5, respectively.

4.3 Viruses

The Compton UK tissue culture adapted (UKtc) bovine strain of rotavirus was originally obtained from M. Thouless. The OSU strain of rotavirus was originally obtained from Professor E. H. Bohl. The B223 strain of bovine rotavirus was originally obtained from Dr D. R. Snodgrass. Seed stock of UKtc, OSU, B223, and a Birmingham strain of

murine rotavirus (EBR) were provided by Professor M. A. McCrae. An EDIM isolate at passage 16 in MA104 cells was kindly provided by Professor R. L. Ward.

4.4 Antisera and monoclonal antibodies

Small quantity of the monoclonal antibodies # 5632 specific for UKtc VP7 (Beards *et al.*, 1992), and # 4907 specific for OSU VP7 produced in mouse, and monospecific antiserum # 5925 specific against UKtc VP4 produced in rabbits by inoculating a reassortant containing UKtc VP4 gene on ST3 rotavirus background, were kindly provided by Dr D. R. Snodgrass, Mordun Research Institute, Edinburgh, UK. The bovine hyperimmune antiserum against UKtc was a kind gift of Dr J. C. Bridger, Royal Veterinary College, University of London, UK.

4.5 Virus stocks

Plaque purified stocks of UKtc, OSU, and B223 viruses were prepared in BSC-1 cells grown in 800-cm^2 roller bottles on a rotating roller apparatus (Modular cell production, Model III). Confluent monolayers of BSC-1 cells were infected with a multiplicity of infection (m.o.i.) of 0.1 plaque forming unit (pfu) per cell (McCrae and Faulkner-Valle, 1981). Infection was allowed to proceed until at least 70 % of the cells showed a cytopathic effect (cpe) (3-6 days). The cultures were then freeze-thawed three times, sonicated for 20 seconds (sec) in a sonic waterbath (Townson and Mercer Ltd. Croyden, England), and centrifuged at 2000 rpm for 10 minutes (min) to remove cell debris. The resulting supernatant was concentrated 100 times by centrifugation (SW 28 rotor, Beckman) at 22K at 4^oC for 2 hours (hr). The pellet was resuspended in virus resuspension buffer (50 mM Tris-HCl pH 8.0, 10 mM NaCl, 1.5 mM β-mercaptoethanol, 3mM $CaCl_2$) (McCrae, 1985).

The titre of virus stocks of UKtc, B223, and OSU was determined by plaque assay (see section 4.6) in BSC-1 cells (McCrae and Faulkner-Valle, 1981; Offit *et al.*, 1983). The virus stocks were aliquoted (200 µl) and stored at -70°C until use.

Virus stocks of the EBR and EDIM strains of murine rotaviruses were prepared by inoculating 7 day old suckling CD-1 pups. The pups were sacrificed 48 hr post inoculation (p.i.) and the entire gut was collected in ice-cold PBS. The intestines were processed as described below in section 4.18. The gut from one mouse was resuspended in a final volume of 3 ml PBS. This intestinal homogenate was stored at -70°C and served as inoculum for further studies with murine rotaviruses.

4.6 Plaque assay

The plaque assay was done according to previously published methods (McCrae and Faulkner-Valle, 1981; Offit *et al.*, 1983) with minor modifications. Confluent BSC-1 cell monolayers in 12-well tissue culture plates were infected (in duplicate) with serial 10-fold dilutions of the trypsin-pretreated virus. After virus adsorption for 1 hr at 37°C, the plates were overlaid with a medium containing final concentrations of 1% agar, 2% FCS, 1 µg/ml trypsin. The plates were incubated in a humid atmosphere under 5% CO_2 tension for six days. Then, the cells were fixed in formol saline (30% formaldehyde in PBS [v/v]) and stained with crystal violet (0.1% [w/v] crystal violet in 20% ethanol [v/v]), and the titre determined (Reed and Muench, 1938).

The plaque assays for plaque to plaque purification or for picking progeny clones following coinfection with parent viruses in reassortant construction experiments, were performed as above except that they were done in 6-well tissue culture plates and at the end of the 6 day incubation

they were stained with neutral red (5% [v/v] in PBS) without prior fixation of the cells.

4.7 Plaque reduction neutralisation assay (PRN)

It was performed as described previously (Offit *et al.*, 1983) with minor modifications. Serial 2-fold dilution of heat inactivated serum was mixed with 100 pfu of homologous virus and incubated in a waterbath at 37°C for 1 hr. The serum-virus mixture was inoculated onto BSC-1 cell monolayers and the test carried out as described under plaque assay. The neutralising titre of the serum was expressed as reciprocal of the highest dilution giving 50% reduction in the plaque counts.

4.8 Extraction of rotavirus RNA from cells

Rotavirus RNA from the infected cells was extracted as described previously (Ballard *et al.*, 1992; Beards *et al.*, 1992). Briefly, confluent monolayer of BSC-1 cells in one well of a six-well tissue culture plate was infected at a m.o.i. of 1 to 5. When the cells showed 50% cpe, they were harvested by scraping them into the medium and concentrated by centrifugation. The cell pellet was resuspended in lysis buffer (100 mM Tris-HCl [pH 8.0], 50 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40), and incubated at 4°C for 10 min. Nuclei and unlysed cells were removed by centrifugation for 10 min at 2000 rpm at 4°C. The cytoplasmic extract present in the supernatant was then incubated at 37°C with 200 µg/ml of proteinase K in the presence of 0.2% SDS for 3 hr before being extracted twice with phenol saturated with 50 mM Tris-HCl buffer (pH 8.0). The residual phenol was removed by ether extraction (4x) and the nucleic acid was precipitated as described below.

4.9 Precipitation of nucleic acids

Nucleic acids were precipitated by LiCl (final concentration 67 mM) and 3 volumes of 100% ethanol. Following incubation at -70°C for 30 min, nucleic acid was pelleted by centrifugation at 10000 rpm for 10 min. The pellet was washed with 80% ethanol, vacuum dried, and finally resuspended in sterile distilled water (50 µl for one well of six-well plate).

The variations of this basic method of nucleic acid extraction and precipitation are described in detail in chapter 6.

4.10 Extraction of rotavirus RNA from intestinal homogenates

The intestinal homogenates were prepared as described in 4.18. The intestinal homogenates were extracted with phenol and then ether and the nucleic acid was precipitated as described in section 4.9 for infected cells.

4.11 Oligonucleotide primers

The primers used for amplification of VP7 genes are those described by Xu *et al.*, (1990) : 5' CCC GGG ATC CAT GGC CGG CTT TAA AAG CGA GAA TTT 3' (5' end of gene) and 5' CGA TCG CGA ATT CTG CAG GTC ACA TCA TAC AAC TCT A 3' (3' end of gene). The primers used for the amplification of gene 11 were : 5' TCC GGA TCC AGA TCT GGC CAT GGC TTT TAA AGC GCT 3' (5' end of gene) and 5' GGG CCC GGG CAC GTG GCC AGC TGA AGG TCA CAA AAC GGG A 3' (3' end of gene). The virus specific sequences are underlined. Oligonucleotides were synthesised on an Applied Biosystems 381A DNA synthesiser.

4.12 Reverse transcription and PCR amplification of rotavirus genes

It was performed as described by Xu *et al.*, (1990), with slight modifications. Typically, the RT-PCR amplification was carried out in a 25 µl volume that contained 12.5 µl of sample mix (3-9 µl of RNA, 100 ng of each primer, 10% DMSO) and 12.5 µl of 2x reaction mix. The final concentrations in the reaction were 30 mM Tris-HCl buffer (pH 8.3), 55 mM KCL, 3.5 mM $MgCl_2$, 0.25 mM of each deoxynucleotide triphosphate, 0.3 mM DTT, 0.5 mg/ml gelatin, 6 U avian myeloblastosis virus reverse transcriptase, 1 U Taq DNA polymerase.

The sample mix was heated in a thermocycler (Perkin Elmer Cetus) to 94°C for 2 min to denature the dsRNA and then cooled to 42°C. An equal volume (12.5 µl) of the 2x reaction mix was added and the reactions were overlaid with mineral oil, and incubated at 42°C for 30 min followed by 46°C for 10 min to generate cDNA copies of rotavirus dsRNA. This reverse transcription step was immediately followed by PCR amplification of cDNA using 20 cycles of a regime in which each cycle involved denaturation at 94°C for 2 min, annealing at 55°C for 1 min, and synthesis at 70°C for 2 min.

Amplified bands were separated in 1% agarose gels and visualised by ethidium bromide staining. On many occasions the RT-PCR amplified DNA was reamplified to generate more cDNA. The first PCR products were purified from agarose gels using silica based DNA binding Gene Clean™ kit according to manufacturer's recommendations, to produce a clean seed for a second PCR and also for direct sequencing of the PCR product.

4.13 Restriction enzyme digestion

The restriction enzyme digestions were carried out according to the manufacturer's instructions. Buffers used were those supplied with the enzymes (as a 10x concentrate). Typically, the DNA was digested in a 20 µl reaction volume containing appropriate buffer at 1x concentration. Enzyme (4-10 U) was added to no more than 10% of the final volume. The sample was incubated at 37°C for at least 3 hr. Following digestion the DNA was analysed by agarose gel electrophoresis.

4.14 Agarose gel electrophoresis

Large and small (mini) horizontal agarose gels were used for analysis of DNA after RT-PCR, or PCR amplification, and restriction digestion. Typically, the concentration of agarose used was 1% (w/v) in 1x TBE or TAE buffer (Sambrook *et al.*, 1989), but concentrations between 0.6 - 1.5% were also employed. The commercial apparatus used was Biorad and Uniscience for minigels and Sci-plas or Koch-Light Ltd (New Brunswick Scientific Co) for large gels.

DNA samples for electrophoresis were prepared by adding 1/5 the volume of 5x TBE agarose gel loading buffer (50% glycerol, 5x TBE, 0.02% bromophenol blue and 0.02% xylene cyanol). The electrophoresis was carried out for 1 hr at 70 mA. DNA bands were stained with ethidium bromide (0.5 µg/ml) and then visualised on an ultraviolet (uv) illuminator and the gel photographed using a polaroid camera and # 665 or # 667 type Polaroid films.

4.15 Polyacrylamide gel electrophoresis (PAGE) of rotavirus dsRNA

The viral genomic dsRNA was fractionated on vertical slab gels (1.5 x 200 mm) using the Laemmli discontinuous buffer system

(Laemmli, 1970). The stacking gel consisted of the acrylamide monomer (5% [w/v]), N,N'-methylenebisacrylamide (0.13% [w/v]), 0.125 M Tris base (pH 6.8), N,N,N',N'-tetramethylenediamine (0.125% [v/v]), ammonium persulphate (0.07% [w/v]), and SDS (0.2% [w/v]). The resolving gel consisted of the acrylamide monomer (6% [w/v]), N,N'-methylenebisacrylamide (0.16% [w/v]), 0.375 M Tris base (pH 8.8), N,N,N',N'-tetramethylenediamine (0.05% [v/v]), ammonium persulphate (0.07% [w/v]), and SDS (0.2% [w/v]). Approximately 50 µl of each sample in electrophoresis sample buffer (25mM Tris HCl [pH 6.8], 10% glycerol, 2% SDS, 5% β- mercaptoethanol, bromophenol blue 0.02%) was loaded and electrophoresis was performed at 4°C at 35 mA for 8 hr.

4.16 Silver staining (SS)

Following electrophoresis, the PAGE gels were stained with silver nitrate (Svensson *et al.*, 1986) with minor modifications. Briefly, the gels were fixed with 40% (v/v) methanol with 10% (v/v) acetic acid in distilled water for 45 min, followed by 10% (v/v) methanol with 0.5% (v/v) acetic acid in distilled water for 45 min, and finally soaked in 11 mM silver nitrate for 45 min. The gels were then washed with distilled water and developed with developer (0.75 M sodium hydroxide, 0.32% [v/v] formaldehyde), until the bands were clearly visible and the background was bright yellow. The reaction was stopped with 5% acetic acid and the gels photographed on a fluorescent light box.

4.17 Mice

Eight week old adult Balb/c mice were obtained from the Animal House, Department of Biological Sciences, University of Warwick, Coventry. Seven day old suckling pups of various inbred mouse strains (Balb/c [H-2^d], C3H/HE [H-2^k], C57/BL-6 [H-2^b], or the outbred CD-1

strain were obtained from Charles River Breeding Laboratories. The animals were pre-bled randomly and screened for the presence of rotavirus specific antibodies by PRN. The adult mice or dams along with their litters were housed in a negative pressure isolation units in a Microisolator (Isotec Systems, Olac, Bicester) and given sterile food and water *ad libitum*.

4.18 Inoculation of the animals

Each animal was orally inoculated with 100 µl of inoculum that contained 10% blue food colouring dye (J.Sainsbury plc., London) which served as a marker for the site of the inoculum and passage of the inoculum through the intestine. The eight week old adult animals were inoculated by proximal oesophageal intubation using a 2" 18 gauge metal gavage (IMS, Cheshire), whereas in the 7 day old suckling pups the inoculum was directly deposited in the stomach through mouth using a silicone rubber (1mm diameter) attached to a 100 µl Hamilton syringe. The inocula were not trypsin activated prior to inoculation. At various times post inoculation the animals were observed for external signs of disease and a pair of mice was sacrificed at each time point. At the time of post mortem, the internal signs of disease (consistency of colon contents and distended colon, hepatomegaly) were noted and the combined small and large intestines were removed in 1 ml of ice-cold PBS and frozen.

The disease in the experimental animals was scored as described previously (Gombold and Ramig, 1986) with minor modifications. On the score, - indicates no disease, + indicates semisolid faeces in the colon, +2 indicates slight diarrhoea with or without abdominal palpation, and +3 indicates overt diarrhoea.

The infectivity of the intestinal homogenates (see section 4.18) was determined by titration in plaque assay. Thirty day post inoculation sera were tested for the development of antibodies in PRN. A schematic of the study design for animal model development is shown in Figure 4.

4.19 Preparation of the intestinal homogenates

The intestines collected in ice-cold PBS were frozen-thawed three-times, homogenised by giving 50 strokes in Dounce Homogenisers (Jencons Ltd.). The homogenates were transferred to a universal and sonicated for 20 sec in a sonic waterbath (Townson and Mercer Ltd, Croyden), and then centrifuged at 600 g for 10 min at 4^{o}C. The supernatants were further microfuged for 5 min in 1.5 ml eppendorfs and supernatant made to a final volume of 3 ml in PBS that contained final concentrations of Nystatin (250 U/ml), Kanamycin (200 μg/ml), Ampicillin (1000 μg/ml). This intestinal homogenate was aliquoted (200 μl) and stored at -70^{o}C until use.

4.20 Generation of reassortants

Reassortant viruses were derived by coinfection of BSC-1 cells with different pairs of rotavirus strains, each at a m.o.i. of 5 pfu per cell. After 48 hr of incubation at 37^{o}C, the infected cultures were harvested by freezing and thawing. The cell debris was removed by centrifugation and the supernatant was plated on BSC-1 cells in 6-well cell culture plates under agar overlay. Plaques were randomly picked in 1 ml of GMEM for genotyping of the putative reassortants. Variations of this basic procedure are described in detail under chapter 7.

FIGURE 4 : A SCHEMATIC OF THE BASIC PROCEDURE FOR THE DEVELOPMENT OF AN ANIMAL MODEL FOR MONITORING ROTAVIRUS VIRULENCE

100 UL
ROTAVIRUS
ORALLY

ROTAVIRUS ANTIBODY FREE
8 WEEK OLD ADULTS OR
7 DAY OLD PUPS

PRN ASSAY AT
30 DAYS ON SERA

AT VARIOUS TIMES POINTS
POST INOCULATION

HOMOGENISE GUT
SONICATE, MICROFUGE

EXTERNAL & INTERNAL
SIGNS OF DISEASE

INFECTIVITY TITRATION ON
SUPERNATANT BY PLAQUE ASSAY

4.21 Genotypic analysis of reassortant viruses

Individual progeny plaques were initially propagated once in 6-well plate and then in a 24-well plate before genotyping. However, in the later experiments the progeny clones were directly propagated in 24-well plates.

Five hundred microlitre of inoculum from each plaque was used to infect one well of a 24-well cell culture plate. At the end of 5 days or when the cells showed about 60% cpe, the supernatant was collected for further passaging and the nucleic acid extracted by the optimised method (described in detail in chapter 6). Putative reassortants were genotyped by PAGE-SS and RT-PCR.

4.22 Lipofection mediated transfection of rotaviruses

It was done according to previously published report (Bass *et al.*, 1992a) with some modifications. Briefly, 1 ml of serum-free and antibiotics-free GMEM was vortexed with 15 µl Lipofectin^{TM}. Then, 100 µl of rotavirus (10^7 pfu) or 1:60 dilution of intestinal homogenate was vortexed along with the above mixture. Subsequently, 20 µg salmon sperm DNA was vortexed in the above mixture. This mixture was incubated at room temperature for 15 min. One well of a six-well BSC-1 monolayer (80% confluent) was washed with serum-free and antibiotics-free GMEM. The mixture was added to the well of a six-well tissue culture plate and incubated at 37^{o}C for 4 hr under 5% CO_2 tension. At the end of incubation the mixture was replaced with 3 ml GMEM containing 5% FCS. After 18 hr the cells were harvested by scraping and freeze-thawing for titration, extraction of rotavirus RNA, and for picking progeny clones in plaque assay when the lipofection was performed with a pair of rotaviruses.

4.23 Sequencing

The equivalent of 10 μl of PCR product was used for each sequencing reaction. The PCR product DNA was purified from untreated triphosphates, primers, and unexpected products by using Gene Clean kit^{TM} following fractionation of the PCR product on 0.6% agarose gels in TAE buffer. The Taq polymerase step cycle DNA sequencing was carried out by dideoxynucleotide chain termination method (Sanger *et al.*, 1977), and was performed by Lesley Ward, Department of Biological Sciences, University of Warwick.

Sequence comparisons were carried out using the Genetics Computer Group (GCG) set of programmes (Devereux *et al.*, 1984).

Chapter 5

Development of a murine model of rotavirus virulence

5.1 Introduction

Compared to some other areas of rotavirus biology, rotavirus virulence is under-explored. One of the main limitations on virulence studies has been the lack of a suitable animal model. While it can be described what happens during a virus disease, there is very little knowledge about the molecular events which distinguishes otherwise identical virulent and avirulent strains. Comparison of their nucleic acids by sequence analysis will show differences, but the essential problem still remains to correlate them with whatever controls virulence and then to understand in molecular terms how virulent virus strain interacts with its host to produce a violent end-result while an avirulent strain does not. Studies of viral genetics have given interesting results that increase the understanding of viral replication in cells and other *in vitro* phenomena, but little that helps in the understanding of virulence in the infected host.

In this chapter, the development of a mice model for conducting future experiments with genetically manipulated viruses to study rotavirus virulence will be described. Three different rotavirus strains viz. UKtc (G6P7), B223 (G10P8), and OSU (G5P9) were selected. The reasons for choosing UKtc were that it is a well characterised strain at the molecular level, grows to higher titres in cell cultures, and is an important pathogen of domestic cattle. The B223 strain was selected because it has been used in some of the classical experiments of rotavirus biology giving unexpected results (Chen *et al.*, 1989; 92). The OSU strain was chosen because it an important virus affecting pigs.

5.2 Why a mouse model?

None of the animal models currently being used to study rotavirus infection are really suitable. Gnotobiotic calves, lambs, piglets have all been used in the study of rotavirus pathogenesis and infection (Hall *et al.*,

1976; Mebus *et al.*, 1976; Snodgrass *et al.*, 1977; Crouch and Woode, 1978 ; Theil *et al.*, 1978). However, the expense, the need for specialised facilities, the limited availability and the outbred nature of these animals are serious limitations to their widespread use. The use of rabbits as a model has advantages in terms of increased susceptibility, modest cost, availability of homologous cultivable virus strains, and also transplacental transfer of antibodies (Conner *et al.*, 1988). However, the production of moderate disease is infrequent and severe clinical disease is not seen (Conner *et al.*, 1988). The lack of frank diarrhoea in rabbits is due to the effective water absorptive capacity of the large intestine. Clinical signs are also difficult to interpret in rabbits because rabbits normally excrete a soft moist faeces (Hagen, 1974) that may be indistinguishable from a soft stool caused by rotavirus infection. Mice, because of their small size, ease of handling, low cost and inbred nature offer a number of advantages for use in studies on rotavirus infection. Large number of mice can be handled and thus the significance of the results obtained can be tested statistically. The inbred nature of the mice also excludes the variations in antigen presentation due to MHC restriction. However, only a few murine rotavirus strains have been adapted to growth in cell culture. Most heterologous rotavirus strains replicate inefficiently in the intestines of mice and require an input dose 5 or 6 $\log_{10}$ higher than that of mice rotavirus in order to produce diarrhoea consistently (Greenberg *et al.*, 1986).

5.3 Establishment of seronegative status of adult mice and pups

Serum immune response to rotavirus is a good indicator of previous infection (Conner *et al.*, 1991). It was essential to establish the seronegative status of the animals used in the study in order to rule out the effect of prior antibodies on the outcome of infection and disease.

From each batch of mice, randomly selected adult mice and/ or pups were sacrificed. The serum of individual adult animals or pooled sera from pups was heat inactivated and tested for the presence of rotavirus antibodies by PRN. A neutralisation titre of the test samples was <32, whereas that of the hyperimmune antiserum to UKtc was >2048. The neutralisation antibody titre to rotavirus of >50 has been considered to be positive (Offit *et al.*, 1983; 84; Gouvea *et al.*, 1986). Therefore, all the animals used in the present study were considered to be negative for the presence of rotavirus specific antibodies.

5.4 Rotavirus virulence is age dependent

5.4.1 Introduction

Seronegative 8 week old adult Balb/c mice or seronegative litters of 7 day old suckling pups were orally inoculated with 10^9 pfu/ animal of the UKtc strain, 10^7 pfu/ animal of the B223 strain, or uninfected BSC-1 cells administered in an equivalent volume. At various times post inoculation the animals were examined for clinical signs of disease. At each time point, a pair of animals was sacrificed and observed for internal signs of disease. The entire gut of sacrificed animals was aseptically collected in cold PBS for titration of virus infectivity.

5.4.2 Results

The 7 day old suckling Balb/c pups inoculated with 10^9 pfu of UKtc or 10^7 pfu of B223 developed mild diarrhoea, whereas the adult mice remained healthy and failed to develop diarrhoea with either virus (Figure 5). The mock infected animals also remained healthy (data not shown) throughout the course of the experiment. The onset of the diarrhoea with UKtc strain occurred at 24 hr p.i. and persisted for 2 days before waning. The severity of the diarrhoea ranged between +1 to +3

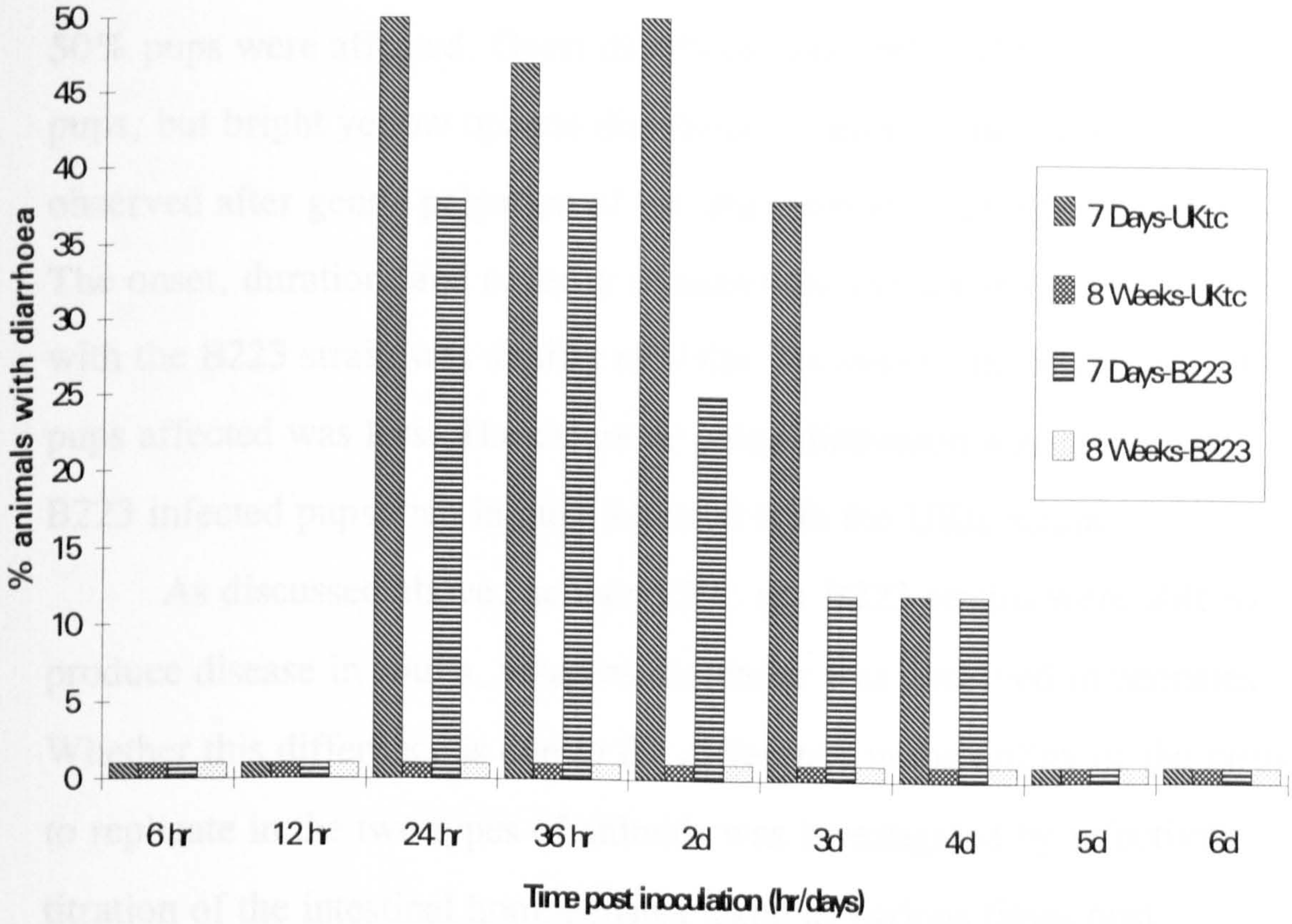


Figure 5 : Rotavirus virulence is age dependent

Seronegative 7 day old suckling Balb/c pups or 8 week old adult Balb/c mice were orally inoculated with 10^9 pfu of UKtc or 10^7 pfu of B223 on day 0. At various times post inoculation the animals were examined for clinical signs of disease. At each time point, a pair of animals was sacrificed and observed for internal signs of disease. Plotted are the mean from 2 experiments and number of pups in each experiment (n) = 5-8. The standard deviation ranged from 0 to 6.4.

(see materials and methods for the definition of the score) and only about 50% pups were affected. Overt diarrhoea was observed in a very few pups, but bright yellow opaque diarrhoea or semi-formed faeces were observed after gentle palpation of the abdomen in other affected pups. The onset, duration, and severity of diarrhoea in suckling pups infected with the B223 strain was similar to UKtc. However, the percentage of the pups affected was less. The extent of colon distension was greater in B223 infected pups than in pups infected with the UKtc strain.

As discussed above, neither UKtc nor B223 strains were able to produce disease in adults, whereas diarrhoea was observed in neonates. Whether this difference is due to the difference in the ability of the virus to replicate in the two types of animals was investigated by infectivity titration of the intestinal homogenates taken at various times post inoculation. The virus titres in adult as well as neonates decreased rapidly during the first 12 hr, though the decline in the adult mice was more abrupt than observed in neonates with both strains (Figure 6).

In the absence of evidence of either disease or virus replication in adult animals, an alternative approach was required to provide evidence that they had actually been infected by the virus. This was done by looking for immunological markers of infection i.e. seroconversion. The pre inoculation sera from both adult and neonates had a titre of <32 whereas that of the hyperimmune bovine serum was >2048. The serum titres from adults and pups 21 days p.i. both with UKtc and B223 were consistently higher than pre inoculation titres (Table 6).

5.4.3 Discussion

Diarrhoea can be induced by UKtc and B223 strains of rotavirus in 7 day old suckling Balb/c pups but not in adult mice. The age dependent induction of diarrhoea in mice has been reported previously for

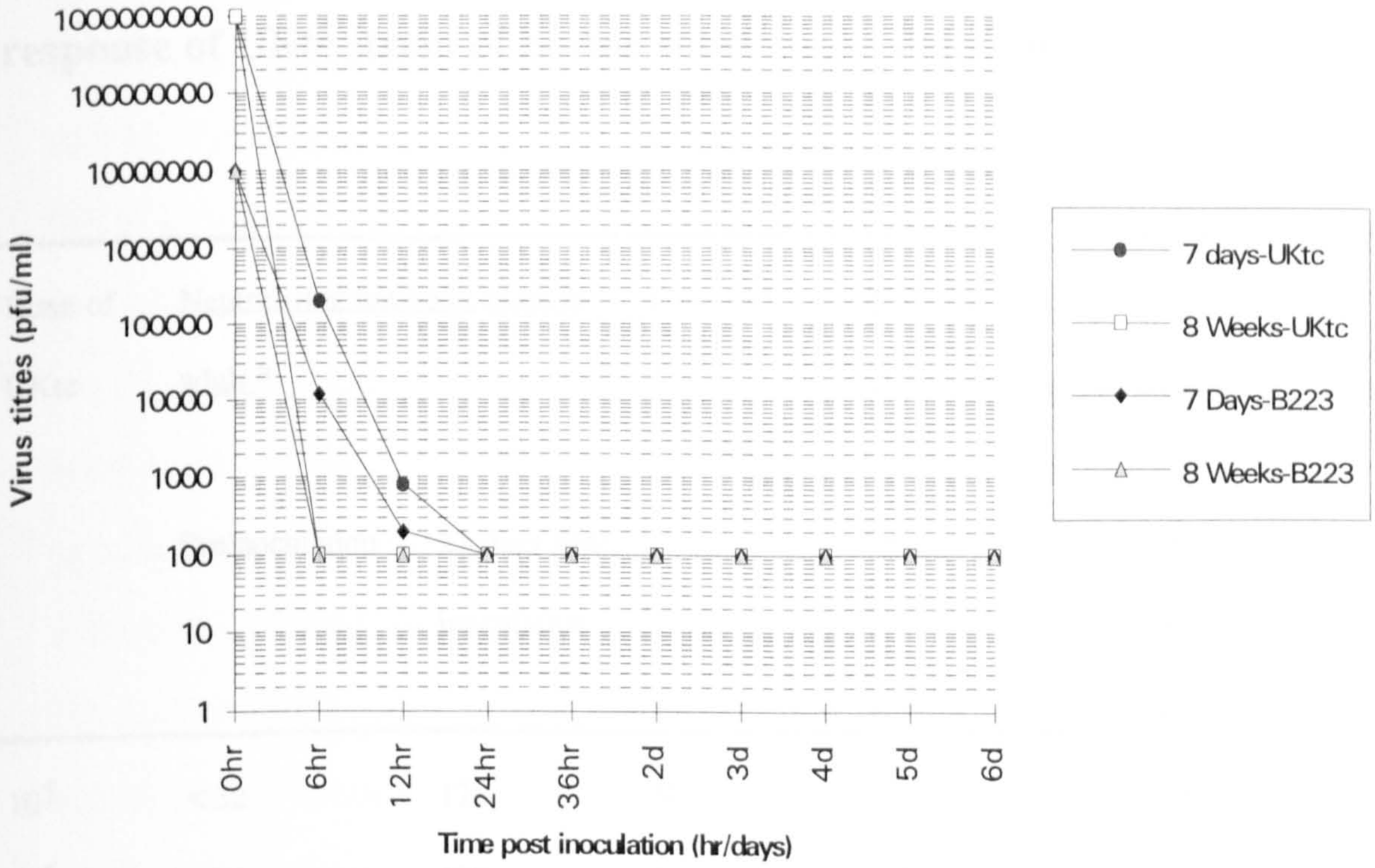


Figure 6 : Replication of UKtc or B223 strain of rotavirus in the intestines of adult or 7 day old suckling pups.

Seronegative 7 day old suckling Balb/c pups or 8 week old adult Balb/c mice were orally inoculated with 10^9 pfu of UKtc or 10^7 pfu of B223 on day 0. At each time point, a pair of animals was sacrificed and entire gut was aseptically collected in cold PBS. Titres of the infectious virus in the intestinal homogenates were determined by plaque assay at various times post inoculation. Plotted are the mean from 2 experiments and in each experiment n = 2. The limit of virus detection in plaque assay was 1×10^2.

Table 6 : Relationship between viral dose and pathological, immunological response of UKtc strain of bovine rotavirus in Balb/c mice

Dose of UKtc	Neutralising antibody titres in adult [a]		Disease [b]	Neutralising antibody titres in 7 day old suckling pups[a]		Disease [b]
	Preinoculation	21 days post inoculation		Preinoculation	21 days post inoculation	
10^5	<32	128	Nil	<32	128	12
10^6	<32	128	Nil	<32	128	23
10^7	<32	512	Nil	<32	256	30
10^8	<32	1024	Nil	<32	256	50
10^9	<32	1024	Nil	<32	256	50

[a] Randomly selected animals were sacrificed and serum was collected. Heat inactivated serum was tested for the presence of neutralising antibodies by PRN. Titres are expressed as the reciprocal of the dilution giving 50% reduction in the mean plaque count.

[b] % animals showing diarrhoea on day 2.

homologous (Wolf *et al.*, 1981; Eydelloth *et al.*, 1984) and for heterologous rotavirus strains (Offit *et al.*, 1984; Ramig, 1988; Jones, 1993). The duration of diarrhoea and the percentage pups affected by the UKtc strain of rotavirus reported by Jones, (1993) was greater even though a lower dose ($5x10^7$ pfu) was used in that study.

It is unlikely that the primary mucosal immune response initiated after UKtc or B223 inoculation of adult animals is sufficiently large or produced at a rate that would be sufficient to control and prevent virus infection and replication. This was proposed for the EDIM virus model (Eydelloth *et al.*, 1984). Also the difference in the age related susceptibility cannot be due to secondary immune response since both adult and pups used in the present study were seronegative. Therefore, non-susceptibility to UKtc or B223 induced disease in adults is likely to be a function of the gut epithelial cell. Intestinal maturation (Wolf *et al.*, 1981), expression of rotavirus-specific receptors on enterocytes (Riepenhoff-Talty *et al.*, 1982; Bass *et al.*, 1991), decreased efficiency in rotavirus antigen expression (Eydelloth *et al.*, 1984), extra cellular proteolytic activation (Vonderfecht *et al.*, 1988), or inactivation (Bass *et al.*, 1992b) have been suggested as different factors for age dependent induction of disease in rotaviruses.

In mice, the murine rotaviruses undergo an extended replication (Wolf *et al.*, 1981; Riepenhoff-Talty *et al.*, 1982; Little Shaddock., 1982; Eydelloth *et al.*, 1984) as compared to the short replication cycle observed with heterologous rotaviruses (Offit *et al.*, 1984; Gouvea *et al.*, 1986) and therefore replication of heterologous rotaviruses is generally poor when compared with homologous strains (Greenberg *et al.*, 1986). A sharp rise between 8 and 12 hr in virus infectivity in intestinal tissues has been taken as indicative of virus replication (Offit *et al.*, 1984). However, no increase in virus titres was seen in adult or Balb/c pups

infected with UKtc or B223 strains of rotavirus. It is unclear from the infectivity titration curves whether the diarrhoea which occurred in pups was due to the input virus or undetected replication. Offit *et al.*, (1984) were not able to recover any infectious rotavirus from the gut of mice inoculated with $5x10^6$ pfu of NCDV when tested 4-72 hr p.i. The possibility of toxic viral action in the animals inoculated with higher doses has also been documented (Gouvea *et al.*, 1986). Ramig, (1988) has reported that although some complete replication cycles do occur in the epithelial cells infected with heterologous rotavirus in neonatal mice, the majority of these infections are abortive i.e. many cells are infected but produce only very low levels of virus. This is indicated by shorter period of replication (when compared to the EDIM mouse model), the greater inoculum size required for heterologous rotaviruses to produce an equivalent titre to EDIM (Offit *et al.*, 1984; Ramig, 1988) and the inability to detect morphologically heterologous rotavirus structures in murine ileal enterocytes (Ramig, 1988).

The abrupt decrease in the infectivity present in the adult mouse intestines as compared to pups may be due to internalisation of majority of the virus by epithelial cells (Ramig, 1988) without subsequent production of infectious virus.

The seroconversion observed in both adult and pups also indicates that internalisation of virus without subsequent production of infectious virus, occurred in adults. The seroconversion also indicates that adult mice were infected with rotavirus as has been reported by Ward *et al.*, (1990b). Recently, similar findings have been observed (Bridger, 1994), who found age-dependent resistance to disease but not to infection in gnotobiotic calves inoculated with bovine rotaviruses. The observations on seroconversions in adult mice also questions the interpretation that the failure to detect diarrhoeal disease in infected adult animals is due to the

loss of viral receptors on enterocytes since exposure of the virus had resulted in infection, albeit asymptomatic (Sheridan *et al.*, 1983).

5.4.4 Conclusions

Adult and 7 day old suckling Balb/c pups can be infected with heterologous rotavirus, but diarrhoea can only be induced in neonates.

5.5 Rotavirus virulence is dose dependent

5.5.1 Introduction

To examine the dose dependency on disease potential and to establish a DD_{50} seronegative litters of 7 day old suckling pups of various mice strains were orally inoculated with various doses of UKtc, or B223, or OSU strains of rotaviruses.

5.5.2 Results

Seronegative litters of 7 day old suckling Balb/c pups were orally inoculated with B223 at doses of 10^3, 10^4, 10^5, 10^6, or 10^7 pfu/ pup. Diarrhoea could not be induced in pups inoculated with a dose of less than 10^5 pfu/ pup. At 10^5 pfu negligible disease could be induced. The duration of diarrhoea induced at a dose of 10^6 and 10^7 pfu/ pup was first observed at 24 hr p.i. and lasted until 4 days p.i. The severity of diarrhoea ranged from +1 to +3 and not more than 35% pups were affected (Figure 7). The infectivity titration curves are shown in Figure 8. Infectious virus could not be detected in the intestinal homogenates in any of the dose after 12 hr p.i.

Dose response of UKtc in Balb/c pups is shown in Figure 9. Very little disease could be induced at doses 10^5 and 10^6pfu in Balb/c pups. The extent of diarrhoea at doses 10^8 and 10^9 pfu was very similar with 50% pups showing diarrhoea 1-3 days p.i.; however, its severity was

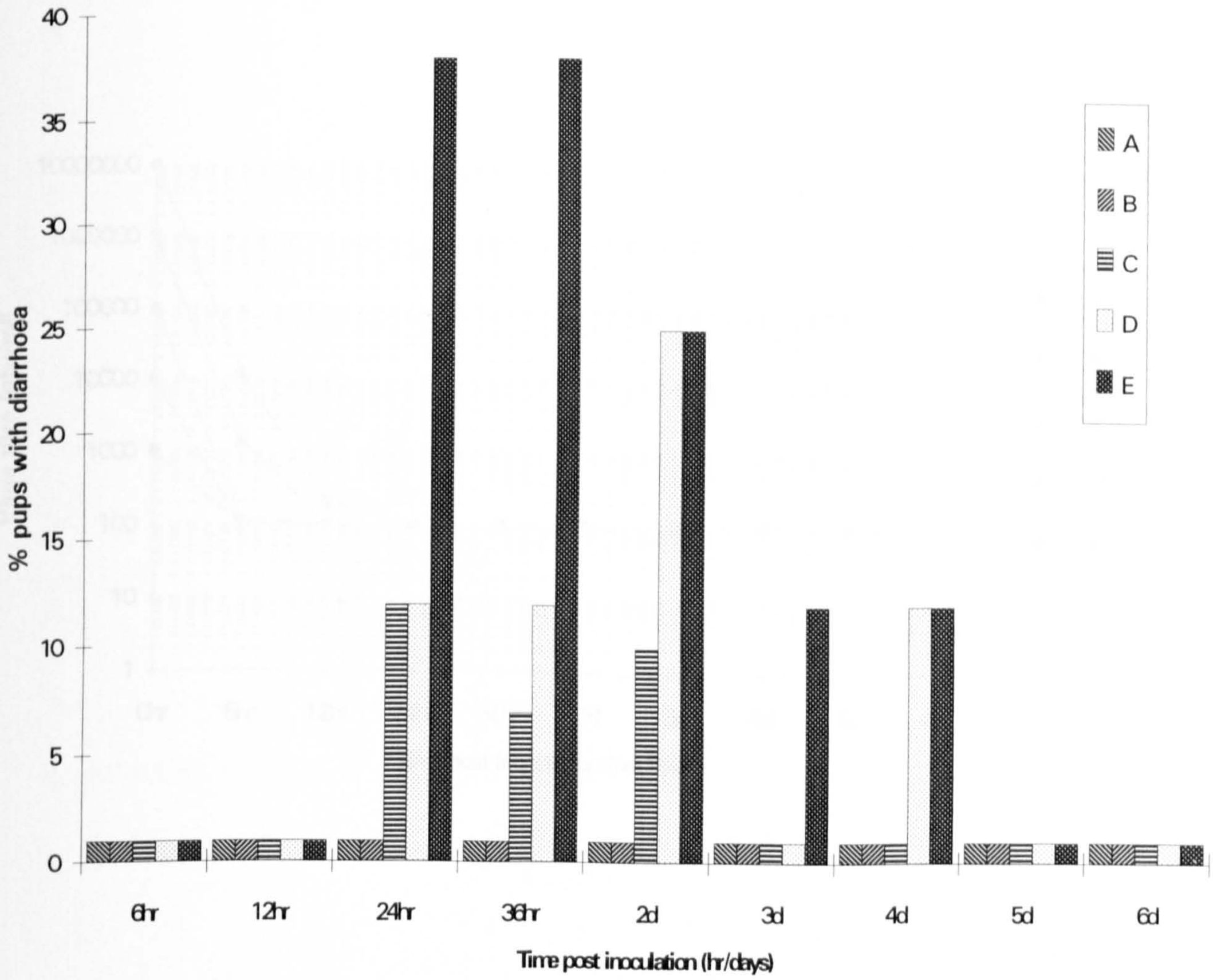


Figure 7 : Diarrhoea produced in 7 day old suckling Balb/c pups inoculated with various doses of B223 strain of rotavirus.

Seronegative 7 day old suckling Balb/c pups were orally inoculated with 10^3 pfu = A; 10^4 pfu = B; 10^5 pfu = C; 10^6 pfu = D; 10^7 pfu = E; of B223 on day 0. At various times post inoculation the animals were examined for clinical signs of disease. At each time point, a pair of animals was sacrificed and observed for internal signs of disease. Plotted are the mean from 2 experiments and in each experiment n = 5-8. The standard deviation ranged from 0 to 9.9.

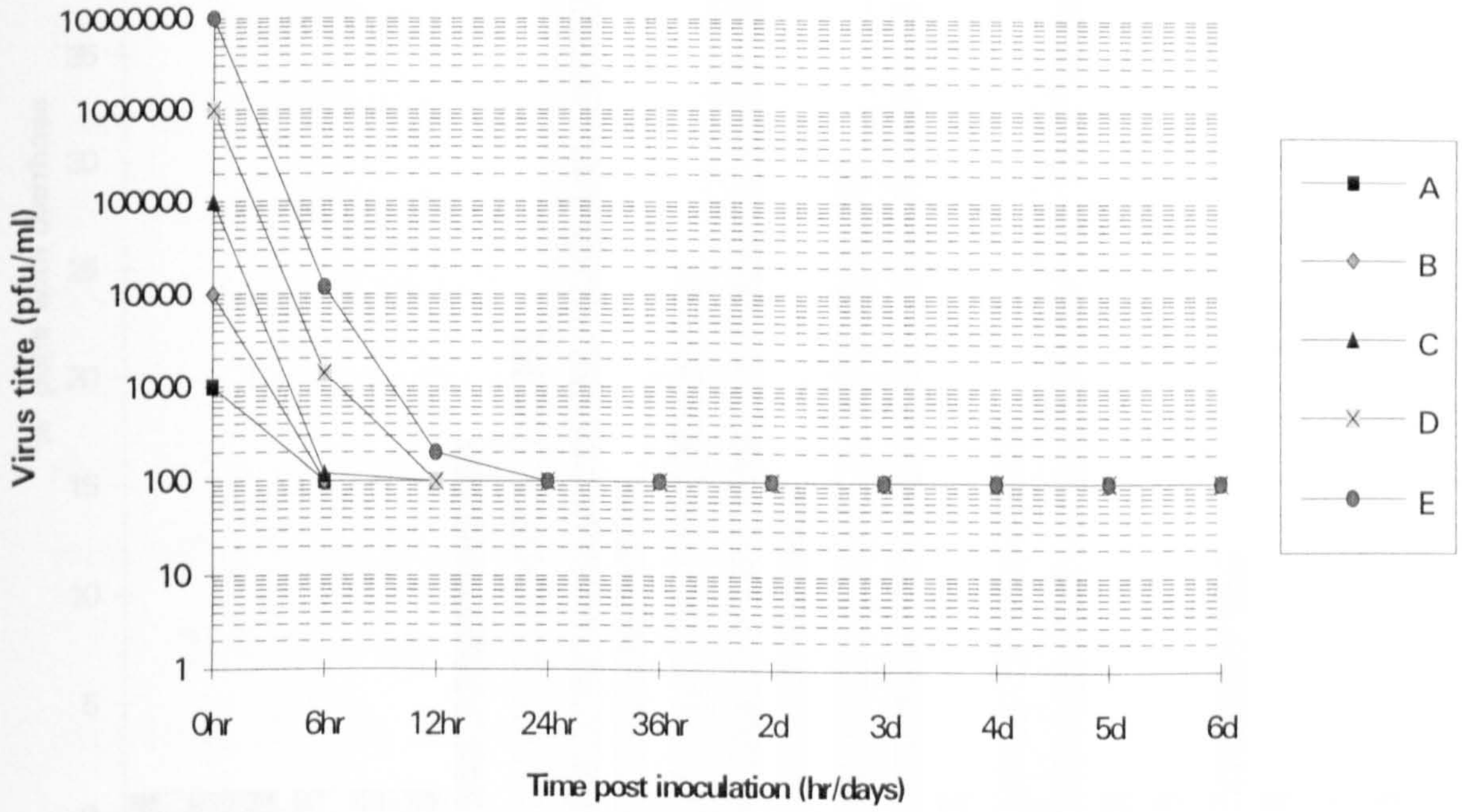


Figure 8 : Replication of B223 strain of rotavirus in the intestines of 7 day old suckling Balb/c mice pups inoculated with various doses.

Seronegative 7 day old suckling Balb/c pups were orally inoculated with 10^3 pfu = A; 10^4 pfu = B; 10^5 pfu = C; 10^6 pfu = D; 10^7 pfu = E; of B223 on day 0. At each time point, a pair of animals was sacrificed and entire gut was aseptically collected in cold PBS. Titres of the infectious virus in the intestinal homogenates were determined by plaque assay at various times post inoculation. Plotted are the mean from 2 experiments and in each experiment n = 2. The limit of virus detection in plaque assay was 1 x 10^2.

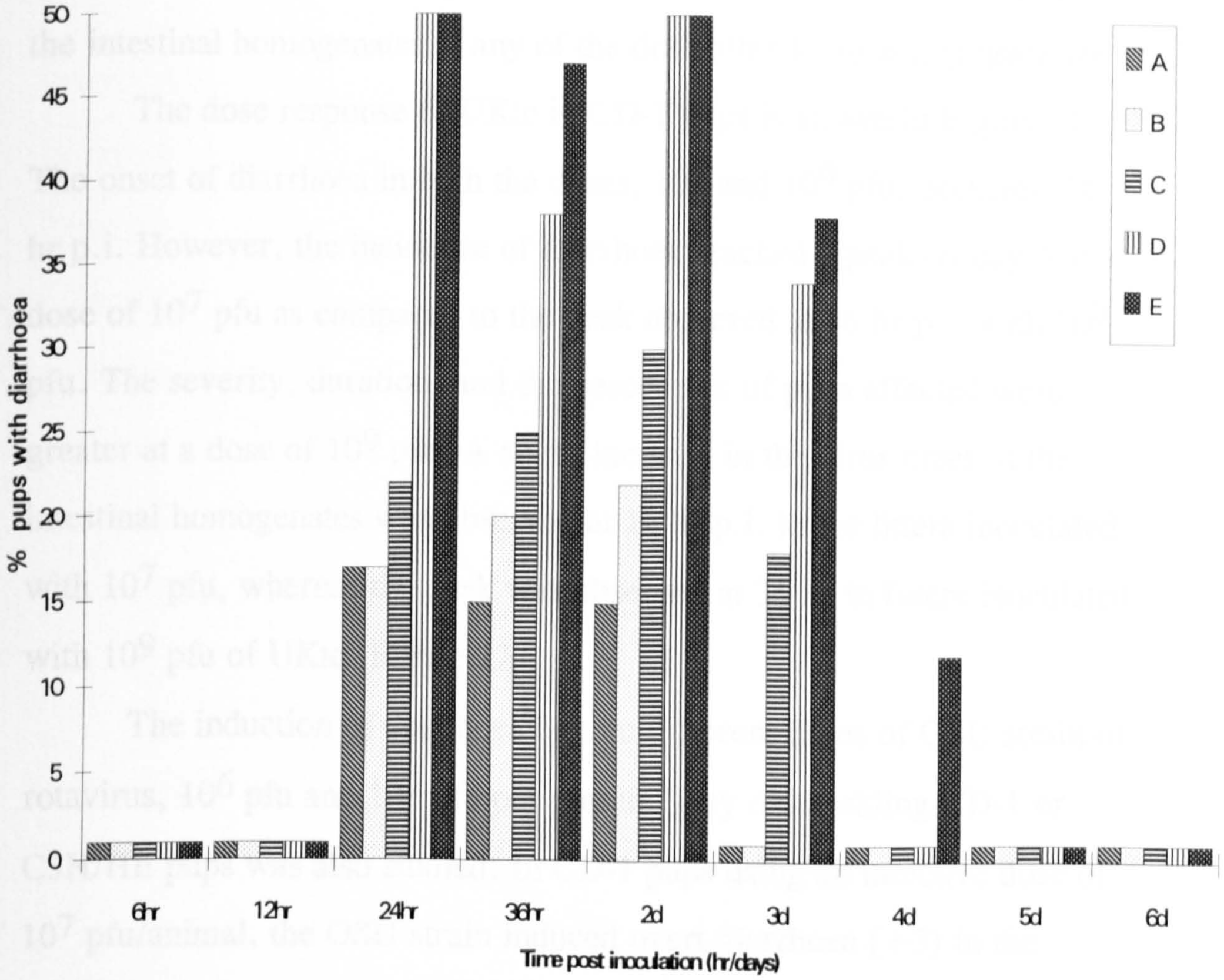


Figure 9 : Diarrhoea produced in 7 day old suckling Balb/c pups inoculated with various doses of UKtc strain of rotavirus.

Seronegative 7 day old suckling Balb/c pups were orally inoculated with 10^5 pfu = A; 10^6 pfu = B; 10^7 pfu = C; 10^8 pfu = D; 10^9 pfu = E; of UKtc on day 0. At various times post inoculation the animals were examined for clinical signs of disease. At each time point, a pair of animals was sacrificed and observed for internal signs of disease. Plotted are the mean from 2 experiments and in each experiment n = 5-8. The standard deviation ranged from 0 to 6.4.

greater with a dose of 10^9 pfu. Infectious virus could not be detected in the intestinal homogenates at any of the dose after 12 hr p.i. (Figure 10).

The dose response of UKtc in CD-1 pups is shown in Figure 11. The onset of diarrhoea in both the doses, 10^7 and 10^9 pfu, occurred 24 hr p.i. However, the incidence of diarrhoea reached a peak on day 3 at a dose of 10^7 pfu as compared to the peak observed at 36 hr p.i. with 10^9 pfu. The severity, duration, and the percentage of pups affected were greater at a dose of 10^9 pfu. A slight increase in the virus titres in the intestinal homogenates was observed at 12 hr p.i. in the litters inoculated with 10^7 pfu, whereas the peak was observed at 36 hr in litters inoculated with 10^9 pfu of UKtc (Figure 12).

The induction of diarrhoea by two different doses of OSU strain of rotavirus, 10^6 pfu and 10^7 pfu per pup in 7 day old suckling CD-1 or C3H/HE pups was also studied. In CD-1 pups using an infective dose of 10^7 pfu/animal, the OSU strain induced overt diarrhoea (+3) in the majority of pups which started at 24 hr p.i. and reached a peak at 36 hr p.i. when 100% of the pups were found to be affected with disease symptoms still evident in 70% of infected animals at day 3 p.i. (Figure 13). At the lower dose used (10^6 pfu/animal of OSU strain), the peak was observed at day 2 p.i., when about 60% of the pups were affected. The diarrhoea observed was bright yellow, opaque but in much larger volume than that observed with either UKtc or B223 strains. Infectivity titrations revealed a slight increase in virus titres at 12 hr p.i. when 10^6 pfu/animal of OSU was used for infection, with this increase being delayed until day 2 p.i. at the higher dose examined (Figure 14). A large amount of liquid faeces together with distension of colon was observed in pups day 1 through day 4 post inoculation.

The OSU strain produced a biphasic diarrhoea in C3H/HE pups with both the doses of 10^6 and 10^7 pfu (Figure 15). The peak of

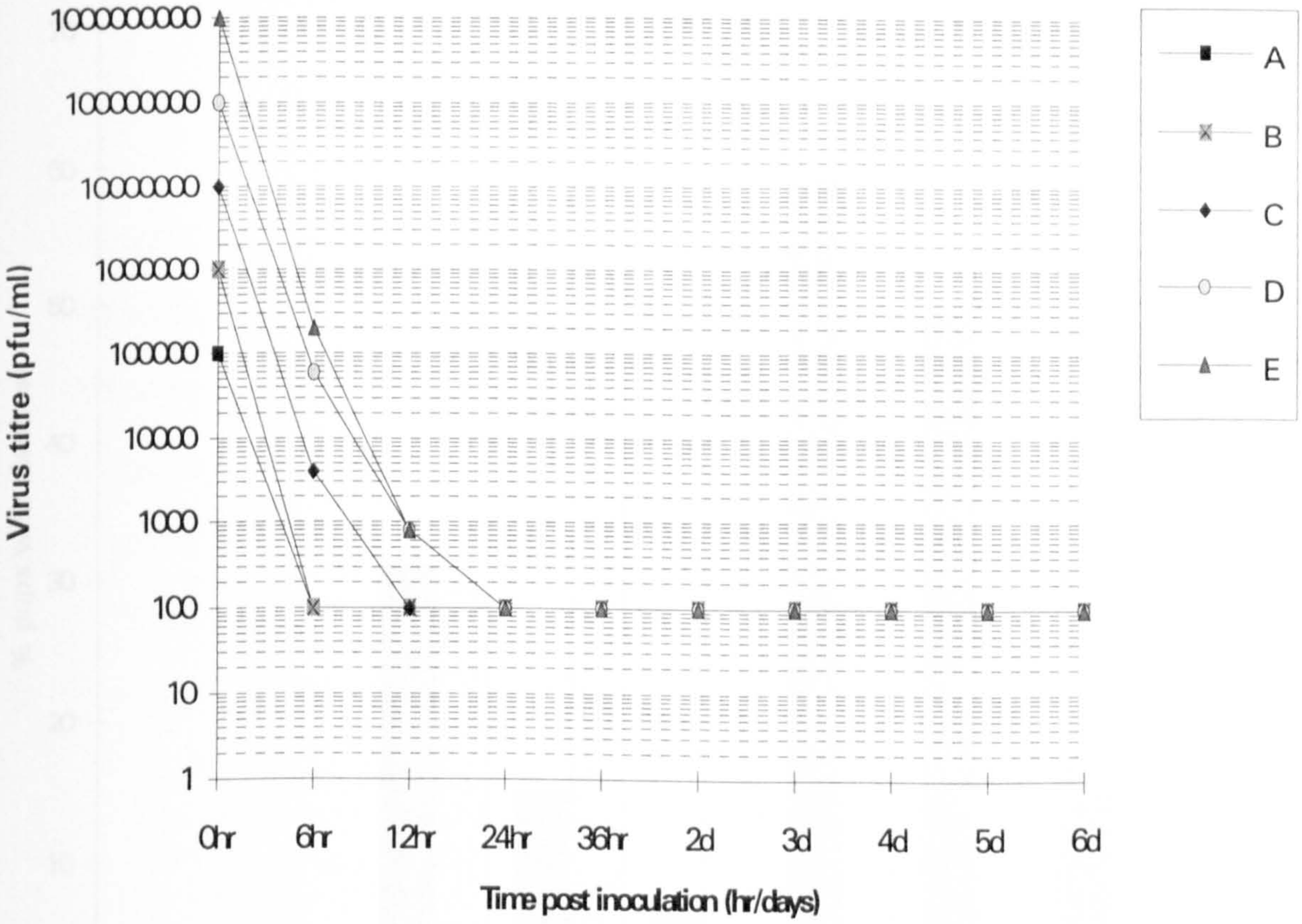


Figure 10 : Replication of UKtc strain of rotavirus in the intestines of 7 day old suckling Balb/c mice pups inoculated with various doses.

Seronegative 7 day old suckling Balb/c pups were orally inoculated with 10^5 pfu = A; 10^6 pfu = B; 10^7 pfu = C; 10^8 pfu = D; 10^9 pfu = E; of UKtc on day 0. At each time point, a pair of animals was sacrificed and entire gut was aseptically collected in cold PBS. Titres of the infectious virus in the intestinal homogenates were determined by plaque assay at various times post inoculation. Plotted are the mean from 2 experiments and in each experiment n = 2. The limit of virus detection in plaque assay was 1 x 10^2.

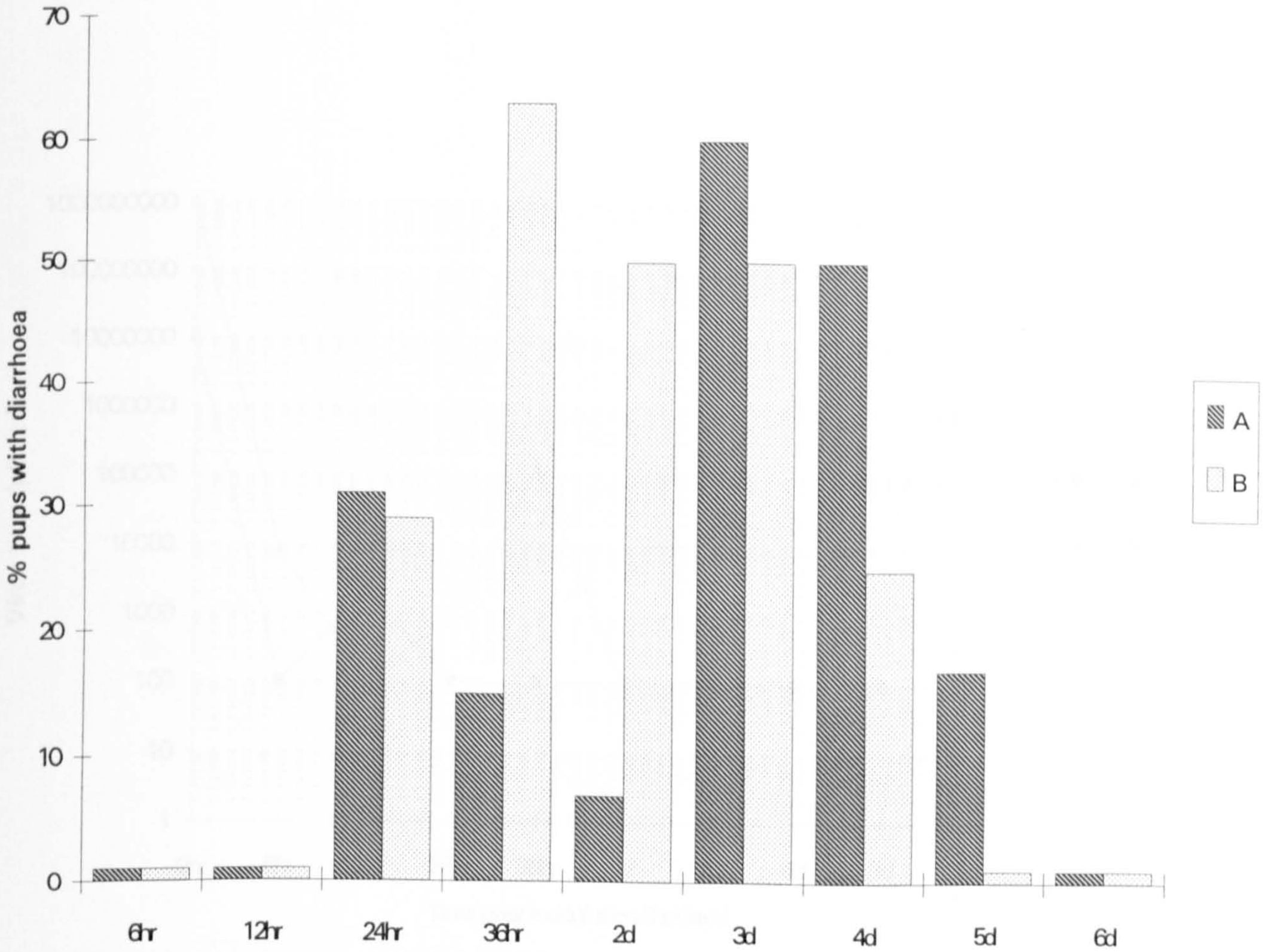


Figure 11 : Diarrhoea produced in 7 day old suckling CD-1 pups inoculated with various doses of UKtc strain of rotavirus.

Seronegative 7 day old suckling CD-1 pups were orally inoculated with 10^7 pfu = A; 10^9 pfu = B; of UKtc on day 0. At various times post inoculation the animals were examined for clinical signs of disease. At each time point, a pair of animals was sacrificed and observed for internal signs of disease. Plotted are the mean from 2 experiments and in each experiment n = 5-8. The standard deviation ranged from 0 to 5.0.

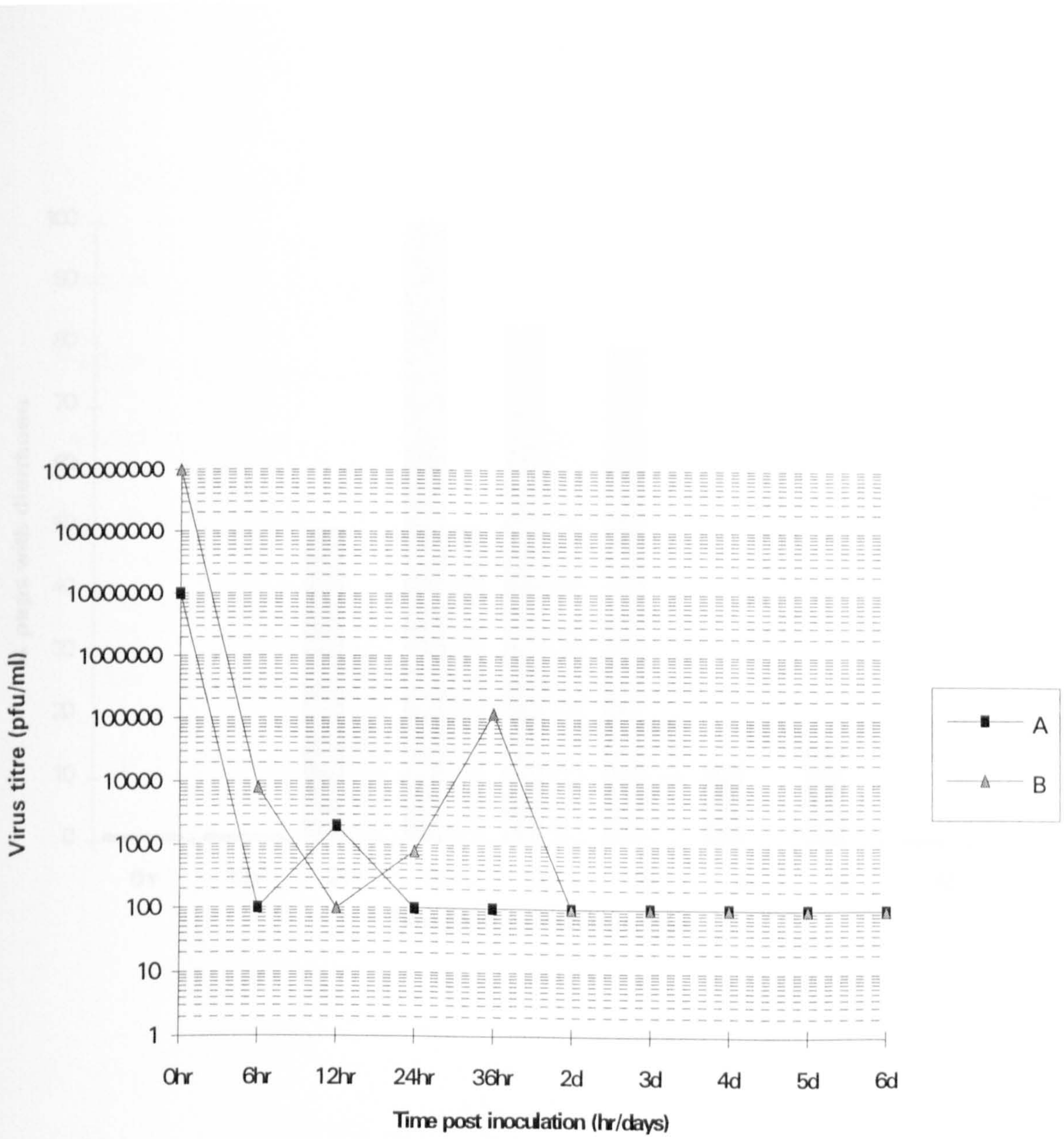


Figure 12 : Replication of UKtc strain of rotavirus in the intestines of 7 day old suckling CD-1 mice pups inoculated with various doses.

Seronegative 7 day old suckling CD-1 pups were orally inoculated with 10^7 pfu = A; 10^9 pfu = B; of UKtc on day 0. At each time point, a pair of animals was sacrificed and entire gut was aseptically collected in cold PBS. Titres of the infectious virus in the intestinal homogenates were determined by plaque assay at various times post inoculation. Plotted are the mean from 2 experiments and in each experiment n = 2. The limit of virus detection in plaque assay was 1×10^2.

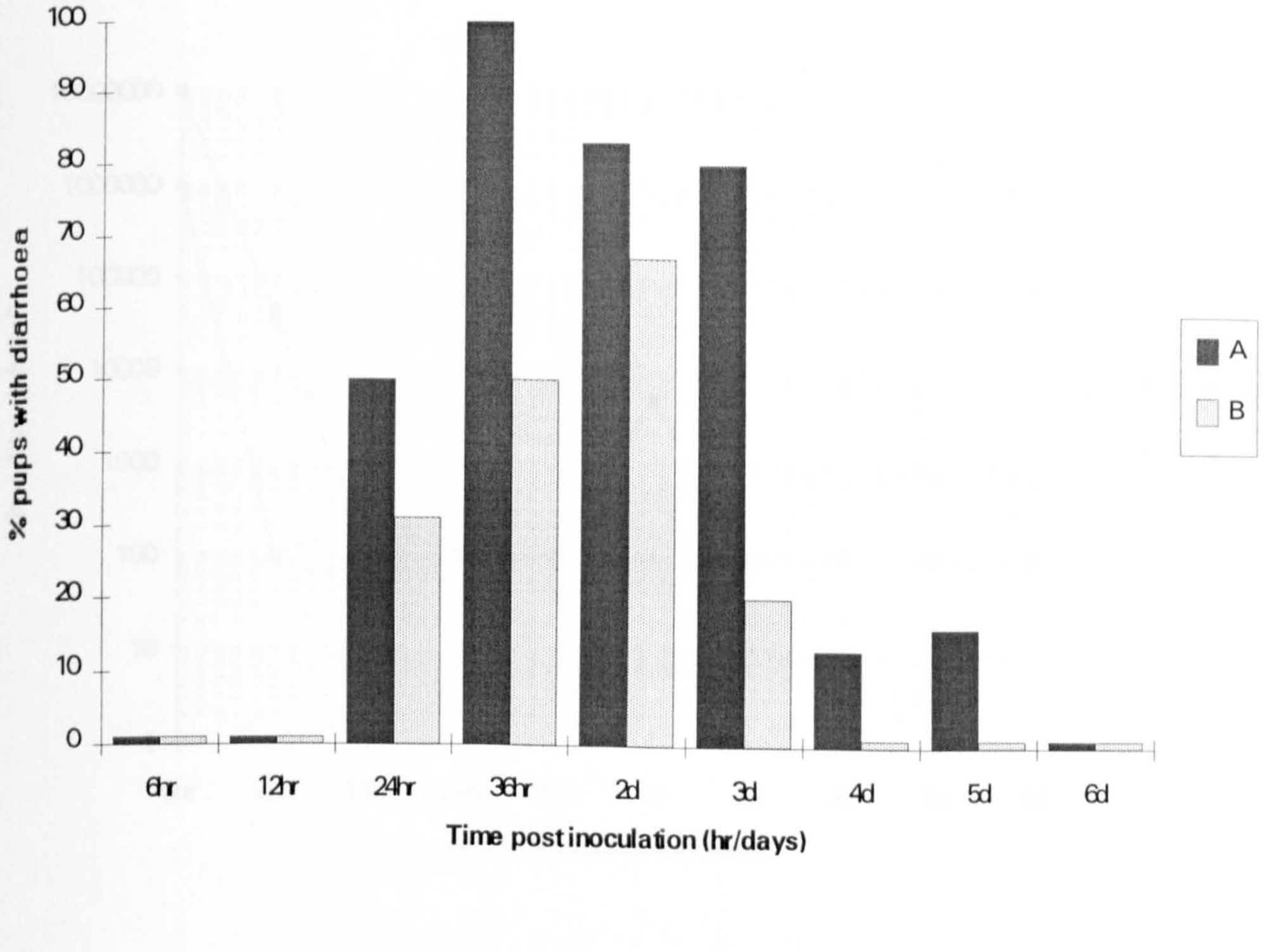


Figure 13 : Diarrhoea produced in 7 day old suckling CD-1 pups inoculated with various doses of OSU strain of rotavirus.

Seronegative 7 day old suckling CD-1 pups were orally inoculated with 10^7 pfu = A; 10^6 pfu = B; of OSU on day 0. At various times post inoculation the animals were examined for clinical signs of disease. At each time point, a pair of animals was sacrificed and observed for internal signs of disease. Plotted are the mean from 2 experiments and in each experiment n = 5-8. The standard deviation ranged from 0 to 5.7.

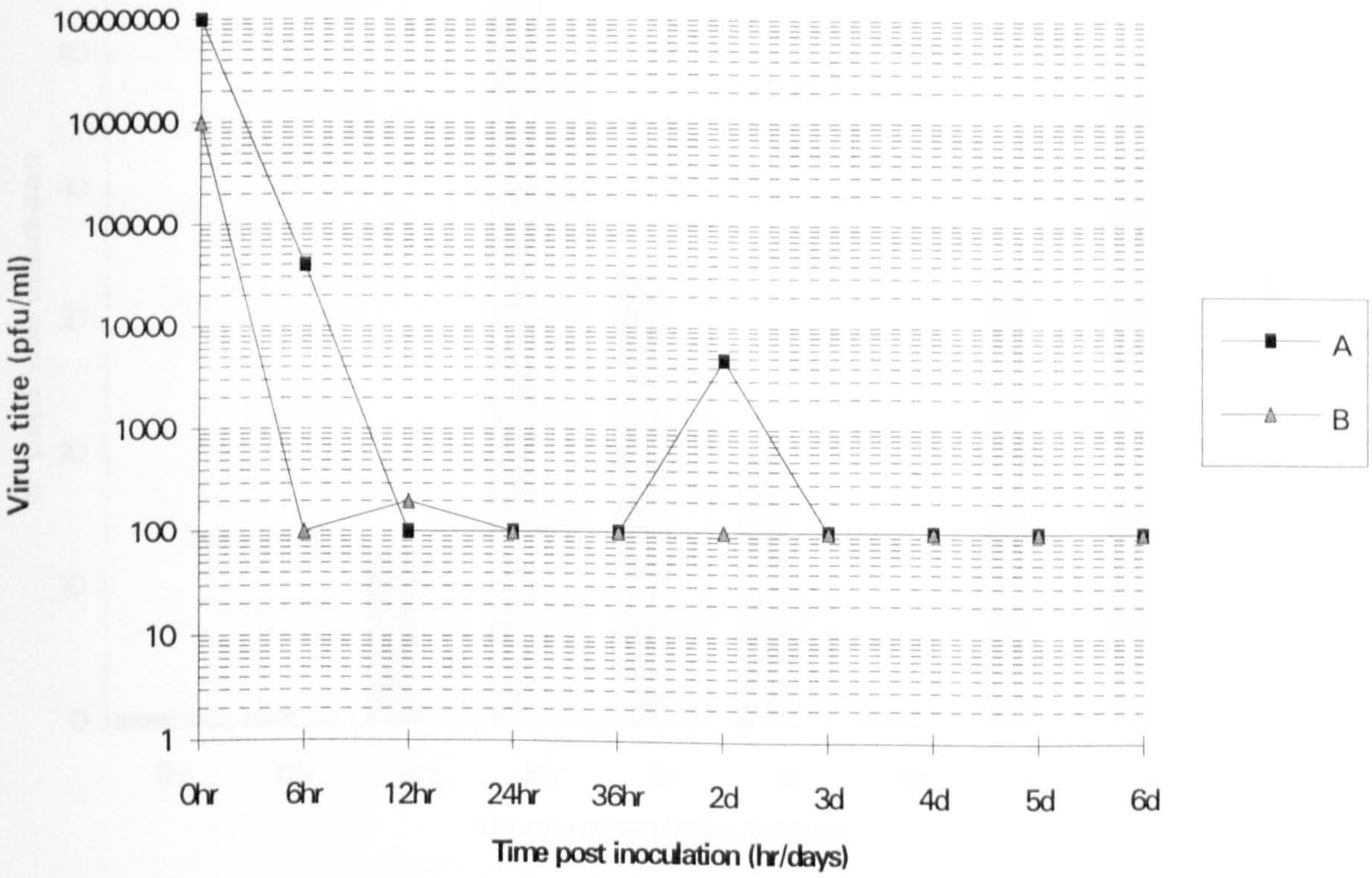


Figure 14 : Replication of OSU strain of rotavirus in the intestines of 7 day old suckling CD-1 mice pups inoculated with various doses.

Seronegative 7 day old suckling CD-1 pups were orally inoculated with 10^7 pfu = A; 10^6 pfu = B; of OSU on day 0. At each time point, a pair of animals was sacrificed and entire gut was aseptically collected in cold PBS. Titres of the infectious virus in the intestinal homogenates were determined by plaque assay at various times post inoculation. Plotted are the mean from 2 experiments and in each experiment n = 2. The limit of virus detection in plaque assay was 1×10^2.

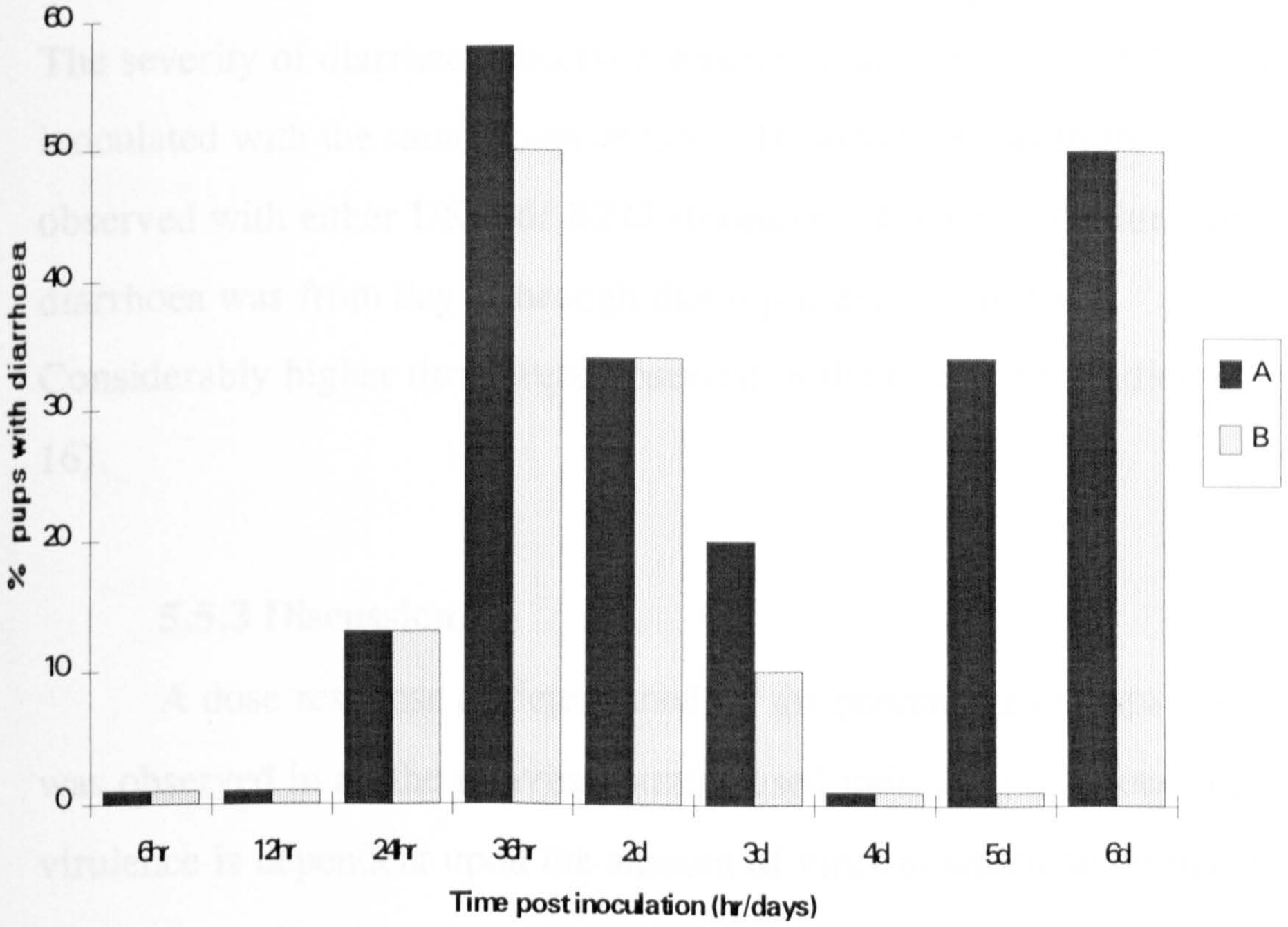


Figure 15 : Diarrhoea produced in 7 day old suckling C3H/HE pups inoculated with various doses of OSU strain of rotavirus.

Seronegative 7 day old suckling C3H/HE pups were orally inoculated with 10^7 pfu = A; 10^6 pfu = B; of OSU on day 0. At various times post inoculation the animals were examined for clinical signs of disease. At each time point, a pair of animals was sacrificed and observed for internal signs of disease. Plotted are the mean from 2 experiments and in each experiment n = 5-8. The standard deviation ranged from 0 to 6.4.

diarrhoea occurred at 36 hr p.i. when about 50% pups were affected. The severity of diarrhoea observed was less than that seen in CD-1 pups inoculated with the same doses of OSU. However, it was more than that observed with either UKtc or B223 strains of rotavirus. The duration of diarrhoea was from day 1 through day 6 p.i. except on day 4. Considerably higher titres were observed in the infectivity studies (Figure 16).

5.5.3 Discussion

A dose response as determined by the percentage of pups affected was observed in all the rotavirus strains used indicating that rotavirus virulence is dependent upon the amount of virus to which an animal is exposed. Similar dose dependent diarrhoea in mice has been reported for the MET strain (Gouvea *et al.*, 1986), EB, EW, and RRV strains (Greenberg *et al.*, 1986), and SA11 (Offit *et al.*, 1984; Greenberg *et al.*, 1986; Ramig, 1988). Diarrhoea of considerable severity was observed in 7 day old CD-1 pups inoculated with 10^5 pfu of the B223 strain, along with viral replication at 36 hr p.i. by Ramig, (1988) which is in contrast to our observations. The DD_{50} of B223 strain in Balb/c pups could not be established. Therefore, another experiment of using different strain of mice was conducted (see later sections).

Similarly the disease pattern in Balb/c pups inoculated with 10^7 pfu was different from that reported by Jones, (1993). Replication of UKtc in Balb/c pups was not observed with any of the doses used. However, it was observed in CD-1 pups as indicated by slight increase in virus titre (Offit *et al.*, 1984), though the titres were not above the input dose as observed with $1x10^2$ pfu of SA11 in CD-1 pups (Ramig, 1988). The DD_{50} for UKtc was $>10^8$, but the diarrhoea induced was very mild.

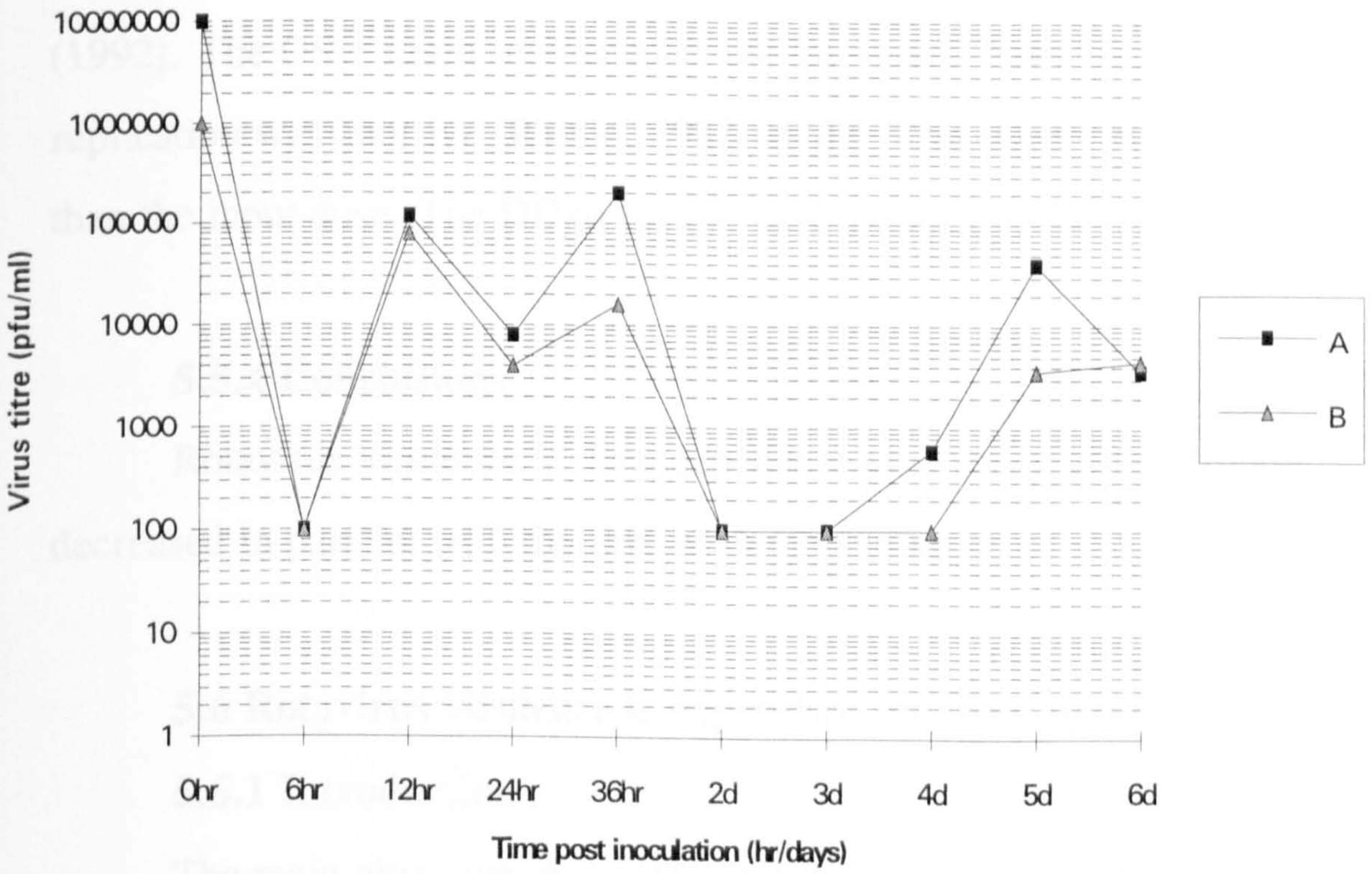


Figure 16 : Replication of OSU strain of rotavirus in the intestines of 7 day old suckling C3H/HE mice pups inoculated with various doses.

Seronegative 7 day old suckling C3H/HE pups were orally inoculated with 10^7 pfu = A; 10^6 pfu = B; of OSU on day 0. At each time point, a pair of animals was sacrificed and entire gut was aseptically collected in cold PBS. Titres of the infectious virus in the intestinal homogenates were determined by plaque assay at various times post inoculation. Plotted are the mean from 2 experiments and in each experiment n = 2. The limit of virus detection in plaque assay was 1 x 10^2.

A severe diarrhoea could be induced using the porcine OSU strain in the present study as compared to the observations of Ward *et al.*, (1992). The OSU strain replicated in the intestines, however, the replication was abortive (Ramig, 1988) as the titres observed were less than the input dose. The DD_{50} for the OSU strain was 10^7 pfu.

5.5.4 Conclusions

Rotavirus virulence is dose dependent as severity of the disease decreased in parallel with the titre of the inoculum.

5.6 Rotavirus virulence is dependent on virus strain

5.6.1 Introduction

The main objective of this study was to obtain rotavirus strains of varying virulence that would be used as parents in genetic manipulation experiments to probe the molecular basis of virus virulence. Three different rotavirus strains viz. UKtc (G6P7), B223 (G10P8), and OSU (G5P9) were selected.

A dose of 10^7 pfu of these strains was orally inoculated into seronegative 7 day old suckling pups of different mice strains.

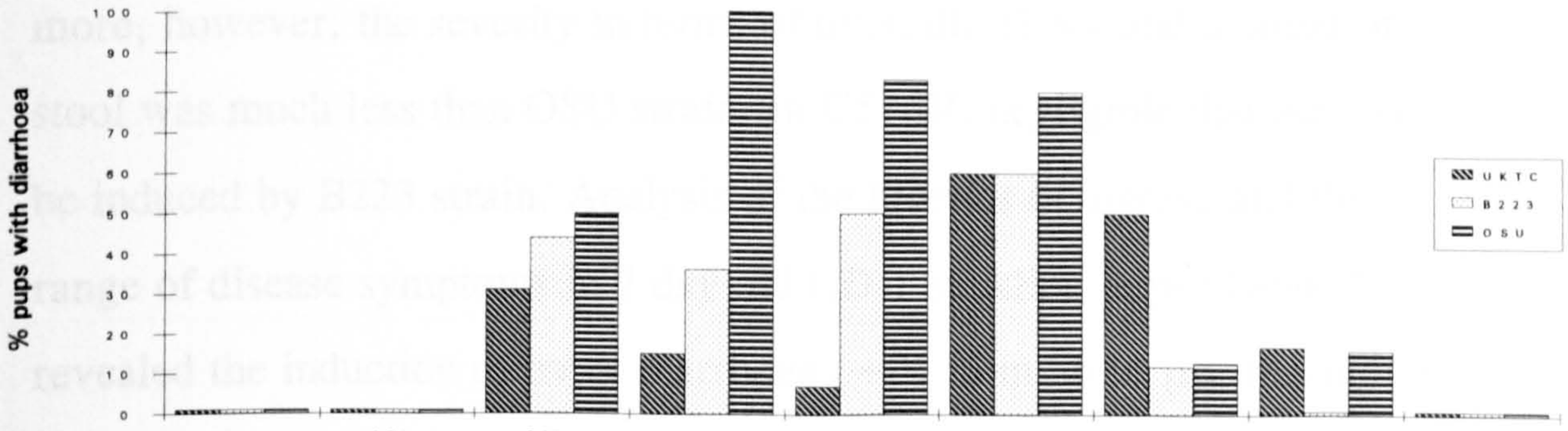
5.6.2 Results and discussion

In general, the porcine OSU strain was found to be the most virulent of the strains used followed by bovine B223 and UKtc strains (Figures 17A-D). The onset of diarrhoea in all the strains occurred at 24 hr p.i. and could be seen during the next 2-3 days except in C3H/HE mice strain in which the percentage of pups showing diarrhoea seemed to cycle over time (Figure 17C). It is not clear whether this represented multiple cycles of replication or physiologic responses to infection. The

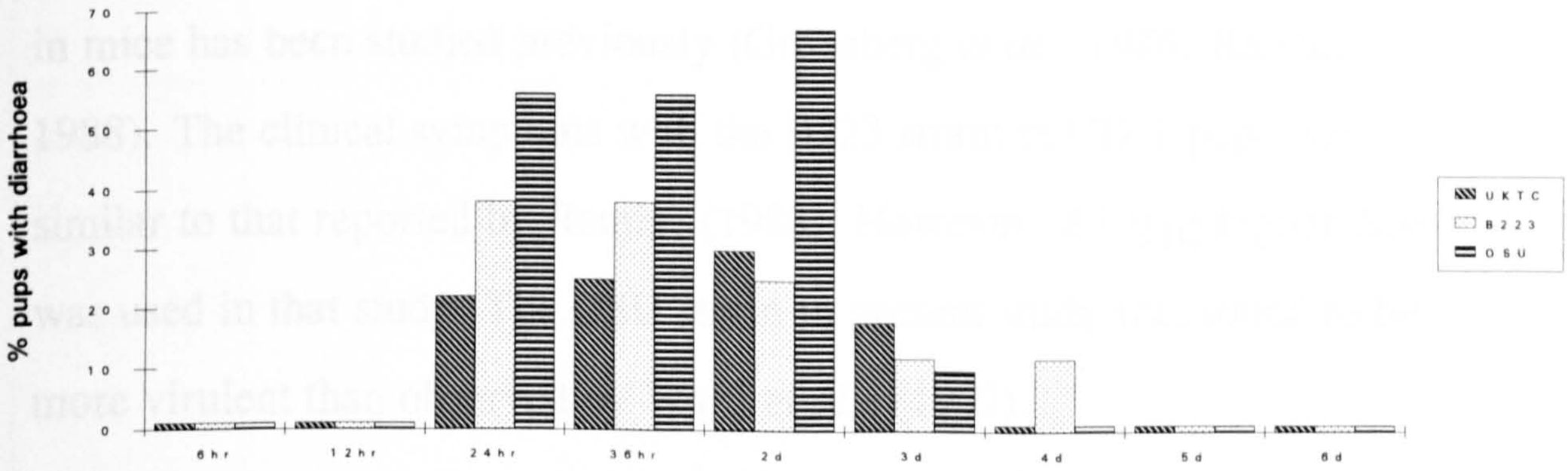
Figure 17 : Diarrhoea produced by various strains of rotavirus in 7 day old suckling pups.

Seronegative 7 day old suckling (A) CD-1; (B) Balb/c; (C) C3H/HE; (D) C57/BL, pups were orally inoculated with 10^7 pfu of various strains of rotavirus on day 0. At various times post inoculation the animals were examined for clinical signs of disease. At each time point, a pair of animals was sacrificed and observed for internal signs of disease. Plotted are the mean from 2 experiments and in each experiment n = 5-8. The standard deviation ranged from 0 to 9.9.

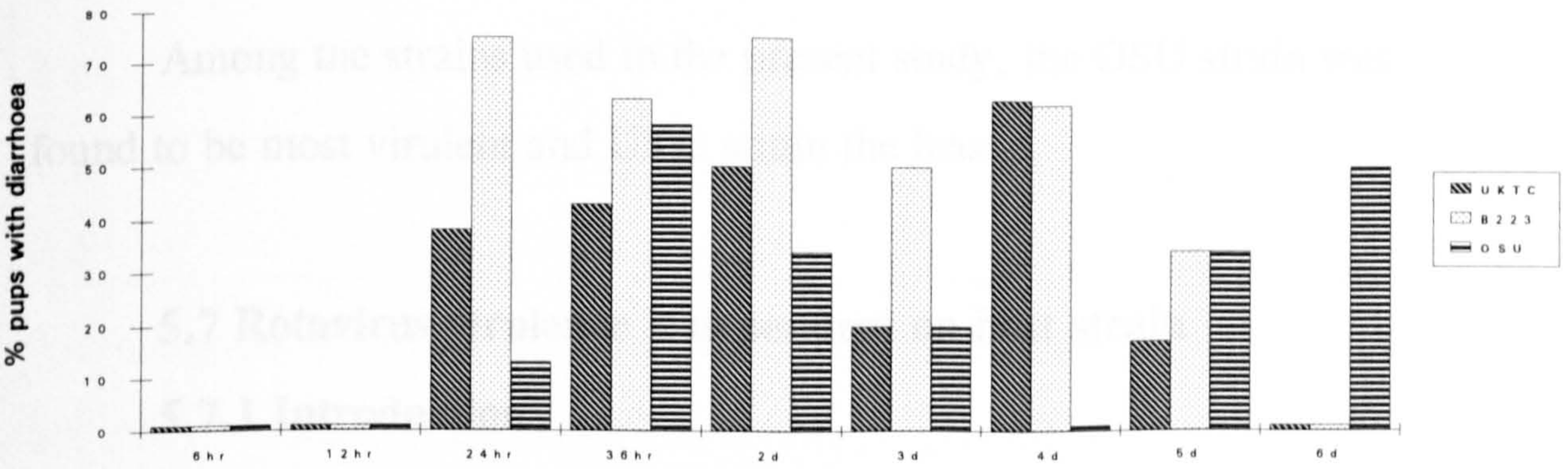
A


% pups with diarrhoea
100
90
80
70
60
50
40
30
20
10
0
6 hr
12 hr
24 hr
36 hr
2 d
3 d
4 d
5 d
6 d
UKTC
B223
OSU

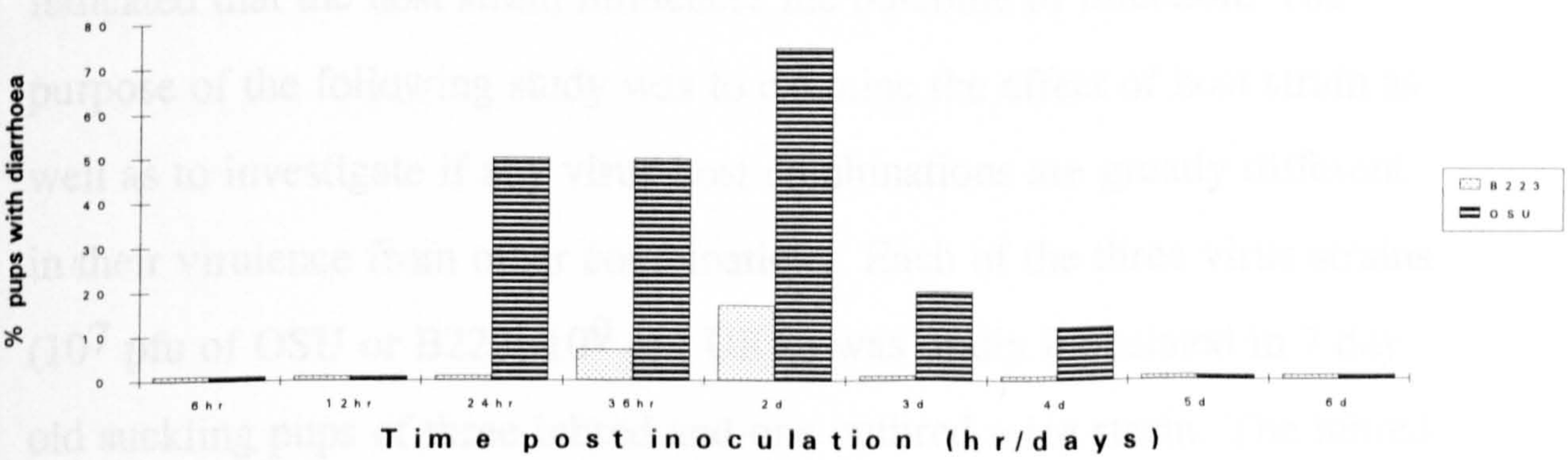
B


% pups with diarrhoea
70
60
50
40
30
20
10
0
6 hr
12 hr
24 hr
36 hr
2 d
3 d
4 d
5 d
6 d
UKTC
B223
OSU

C


% pups with diarrhoea
80
70
60
50
40
30
20
10
0
6 hr
12 hr
24 hr
36 hr
2 d
3 d
4 d
5 d
6 d
UKTC
B223
OSU

D


% pups with diarrhoea
80
70
60
50
40
30
20
10
0
6 hr
12 hr
24 hr
36 hr
2 d
3 d
4 d
5 d
6 d
B223
OSU
Time post inoculation (hr/days)

percentage of pups affected by B223 in C3H/HE pups appeared to be more, however, the severity in terms of overt diarrhoea and amount of stool was much less than OSU strain. In C57/BL negligible disease could be induced by B223 strain. Analysis of the kinetics of disease and the range of disease symptoms in 7 day old CD-1 suckling pups (Table 7) revealed the induction of overt diarrhoea (+3) from 36 hr p.i. through 3 days p.i. in pups inoculated with the OSU strain of rotavirus. The effect of using various heterologous rotavirus strains for induction of diarrhoea in mice has been studied previously (Greenberg *et al.*, 1986; Ramig, 1988). The clinical symptoms with the B223 strain in CD-1 pups was similar to that reported by Ramig, (1988). However, 2 $\log_{10}$ higher dose was used in that study. The OSU strain in present study was found to be more virulent than observed by Ward *et al.*, (1992).

5.6.3 Conclusions

Among the strains used in the present study, the OSU strain was found to be most virulent and UKtc strain the least.

5.7 Rotavirus virulence is dependent on host strain

5.7.1 Introduction

The results of the experiments conducted in the preceding sections indicated that the host strain influences the outcome of infection. The purpose of the following study was to examine the effect of host strain as well as to investigate if any virus-host combinations are greatly different in their virulence from other combinations. Each of the three virus strains (10^7 pfu of OSU or B223; 10^9 pfu UKtc) was orally inoculated in 7 day old suckling pups of three inbred and one outbred mice strain. The inbred mice strains used were C3H/HE (H-2^k), Balb/c (H-2^d), and C57/BL (H-2^b); whereas CD-1 was the outbred strain.

Table 7 : The kinetics of disease in 7 day old suckling CD-1 mice pups inoculated with various rotavirus strains[a]

Time post inoculation	Range of disease symptoms in pups inoculated with[b]		
	UKtc[c]	B223[c]	OSU[c]
6 hr	-/-	-/-	-/-
12 hr	-/-	-/-	-/-
24 hr	++/-	+/+	++/++
36 hr	+/-	++/+	+++/++
2 d	+/-	+++/+	+++/++
3 d	++/+	++/+	+++/++
4 d	++/+	+/-	+/-
5 d	+/-	-/-	+/-
6 d	-/-	-/-	-/-

[a]Seronegative 7 day old suckling CD-1 pups were orally inoculated with 10^7 pfu of various rotavirus strains on day 0. The incidence of diarrhoea in the animals was then monitored over the next 6 day period.

[b]Disease was scored as defined in Materials and Methods.

[c]2 litters were studied separately, each consisted of 5-8 pups.

5.7.2 Results

The B223 strain of virus induced negligible disease in C57/BL pups and somewhat more in Balb/c (Figure 18). However, the determination of DD_{50} in these two strains was not possible as less than 50% of the pups showed diarrhoea. Diarrhoea induction in C3H/HE strain appeared to be cyclic. Whether this is due to multiple rounds of replication or just the physiologic response is not clear from the titration of infectivity in intestinal homogenates which revealed a delayed decline in virus titres as compared to other strains of mouse (Figure 19). The B223 strain in CD-1 pups showed a second peak in virus titres at around 4 day p.i., however, in this case it was not accompanied by diarrhoea.

The C3H/HE strain appeared to be most susceptible to UKtc strain followed by CD-1 strain of mouse (Figure 20). The replication pattern of the UKtc in various strains of mouse appeared to be similar to that observed with the B223 strain (Figure 21).

The CD-1 strain of mice was most susceptible to the OSU strain with 100% of the pups affected at 36 hr p.i. (Figure 22). The duration of the overt diarrhoea was also greatest in this strain. The diarrhoea in C3H/HE strain though less severe than observed in CD-1 strain was biphasic. The infectivity titration curves also showed a second peak of virus replication (Figure 23).

5.7.3 Discussion

The present studies showed that rotavirus virulence is affected by the host strain used. The outcome of the infection can be viewed as the product of virus virulence and host resistance. A highly virulent strain of virus is less lethal for a highly resistant strain of animal than for a susceptible one; conversely, a relatively avirulent strain of virus may be

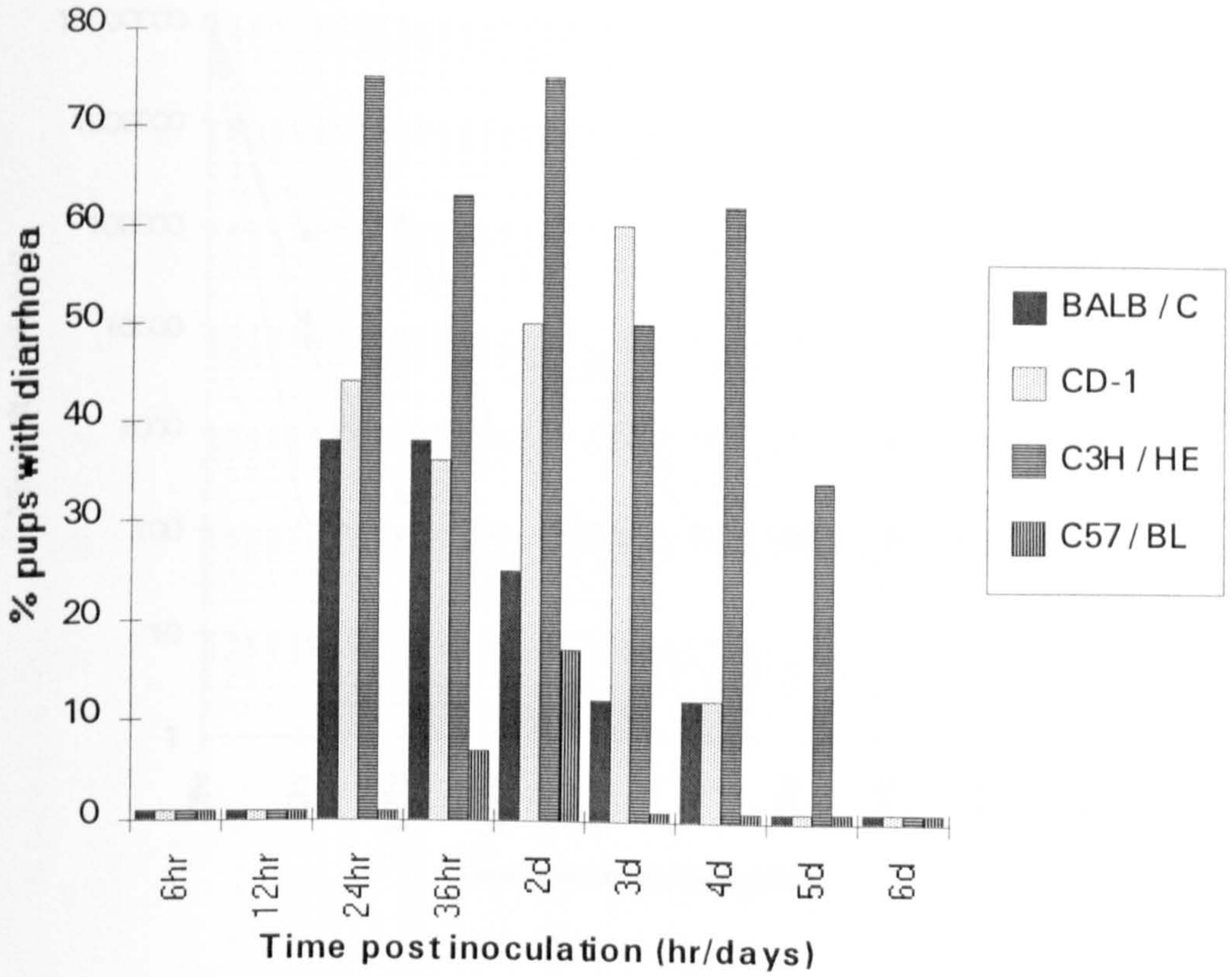


Figure 18 : Diarrhoea produced by B223 strain of rotavirus in 7 day old suckling pups of various mice strains.

Seronegative 7 day old suckling pups of various mice strains were orally inoculated with 10^7 pfu of B223 strain of rotavirus on day 0. At various times post inoculation the animals were examined for clinical signs of disease. At each time point, a pair of animals was sacrificed and observed for internal signs of disease. Plotted are the mean from 2 experiments and in each experiment n = 5-8. The standard deviation ranged from 0 to 6.4.

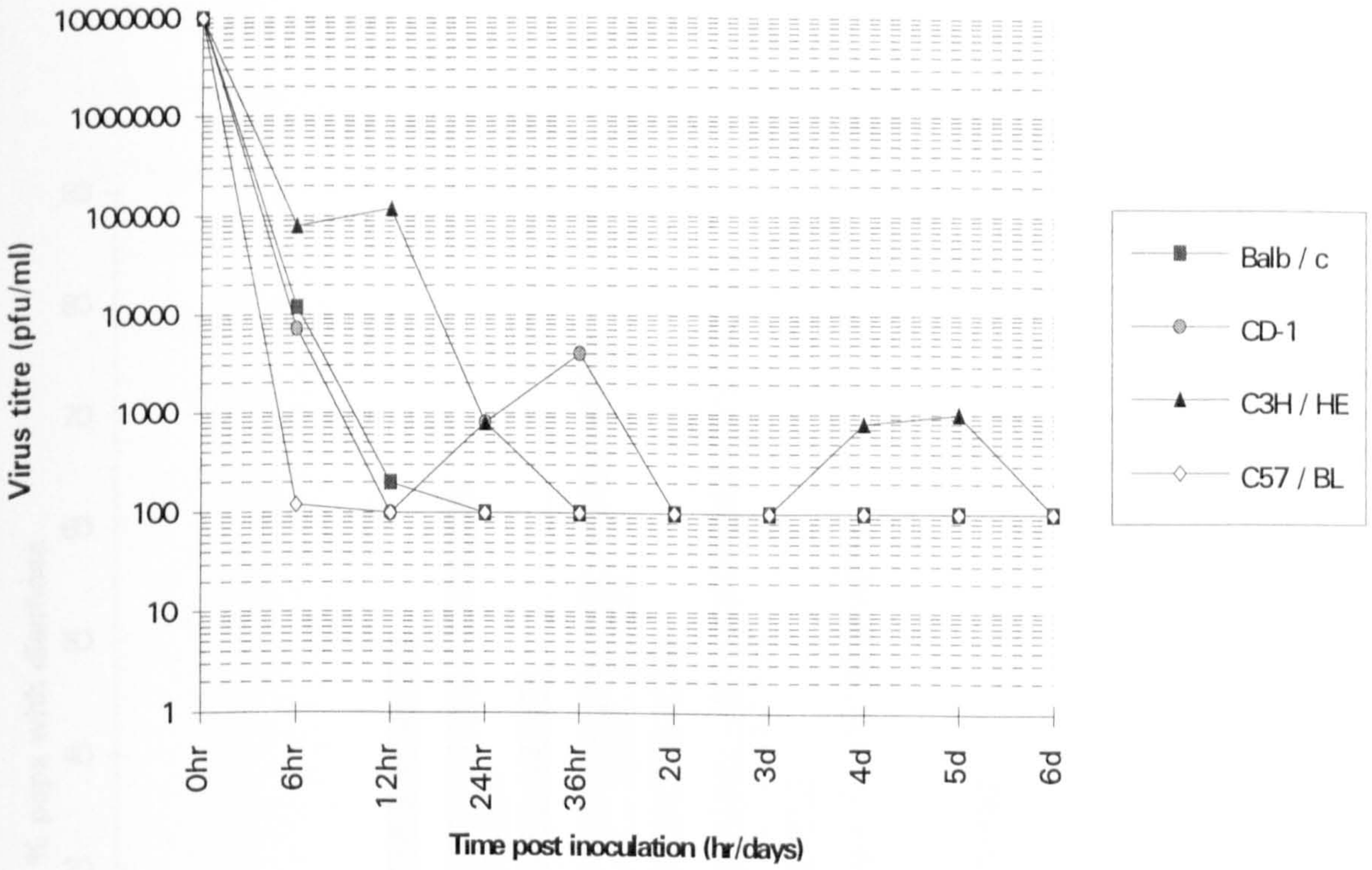


Figure 19 : Replication of B223 strain of rotavirus in 7 day old suckling pups of various mice strains.

Seronegative 7 day old suckling pups of various mice strains were orally inoculated with 10^7 pfu of B223 strain of rotavirus on day 0. At each time point, a pair of animals was sacrificed and entire gut was aseptically collected in cold PBS. Titres of the infectious virus in the intestinal homogenates were determined by plaque assay at various times post inoculation. Plotted are the mean from 2 experiments and in each experiment $n = 2$. The limit of virus detection in plaque assay was 1×10^2.

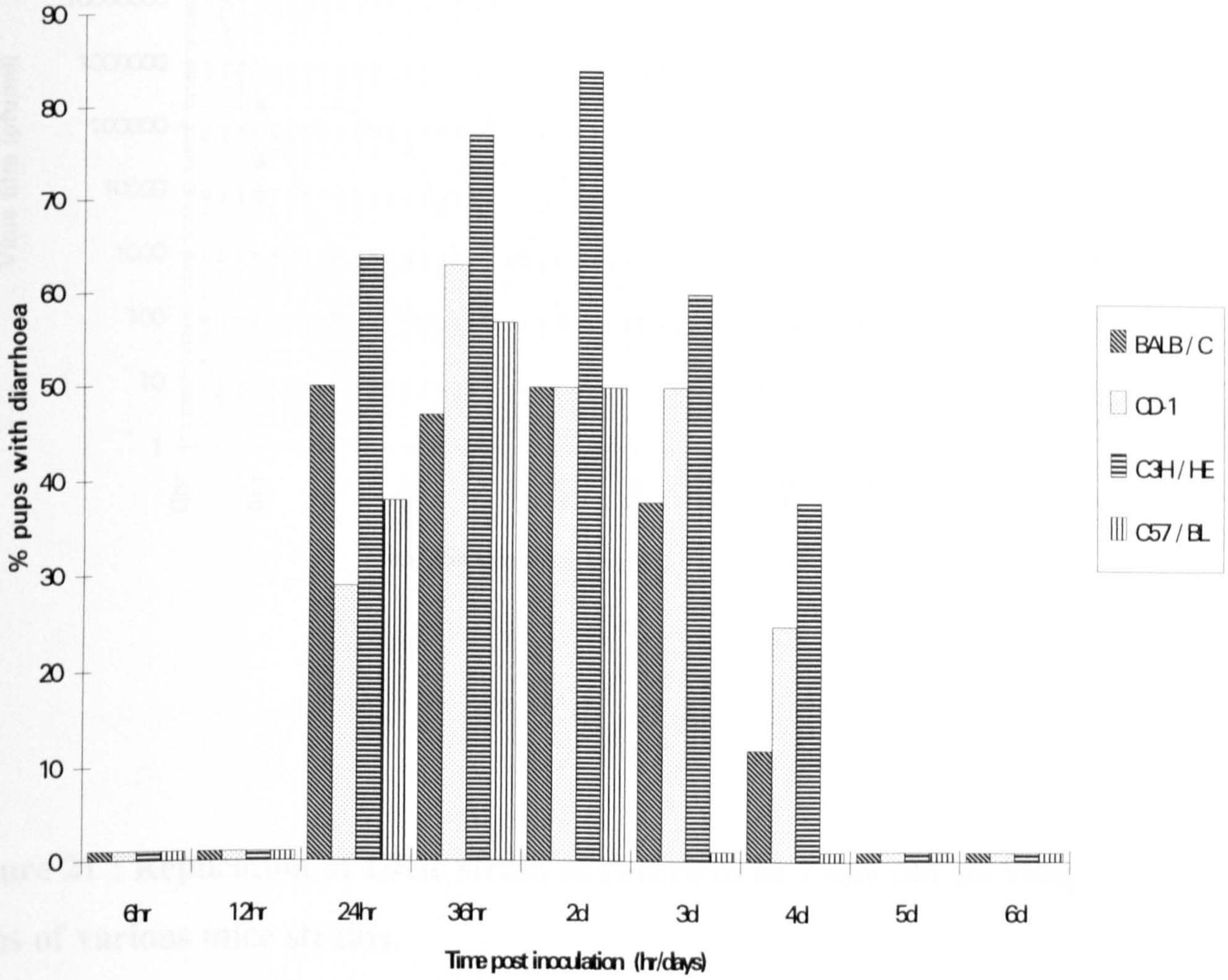


Figure 20 : Diarrhoea produced by UKtc strain of rotavirus in 7 day old suckling pups of various mice strains.

Seronegative 7 day old suckling pups of various mice strains were orally inoculated with 10^9 pfu of UKtc strain of rotavirus on day 0. At various times post inoculation the animals were examined for clinical signs of disease. At each time point, a pair of animals was sacrificed and observed for internal signs of disease. Plotted are the mean from 2 experiments and in each experiment n = 5-8. The standard deviation ranged from 0 to 7.7.

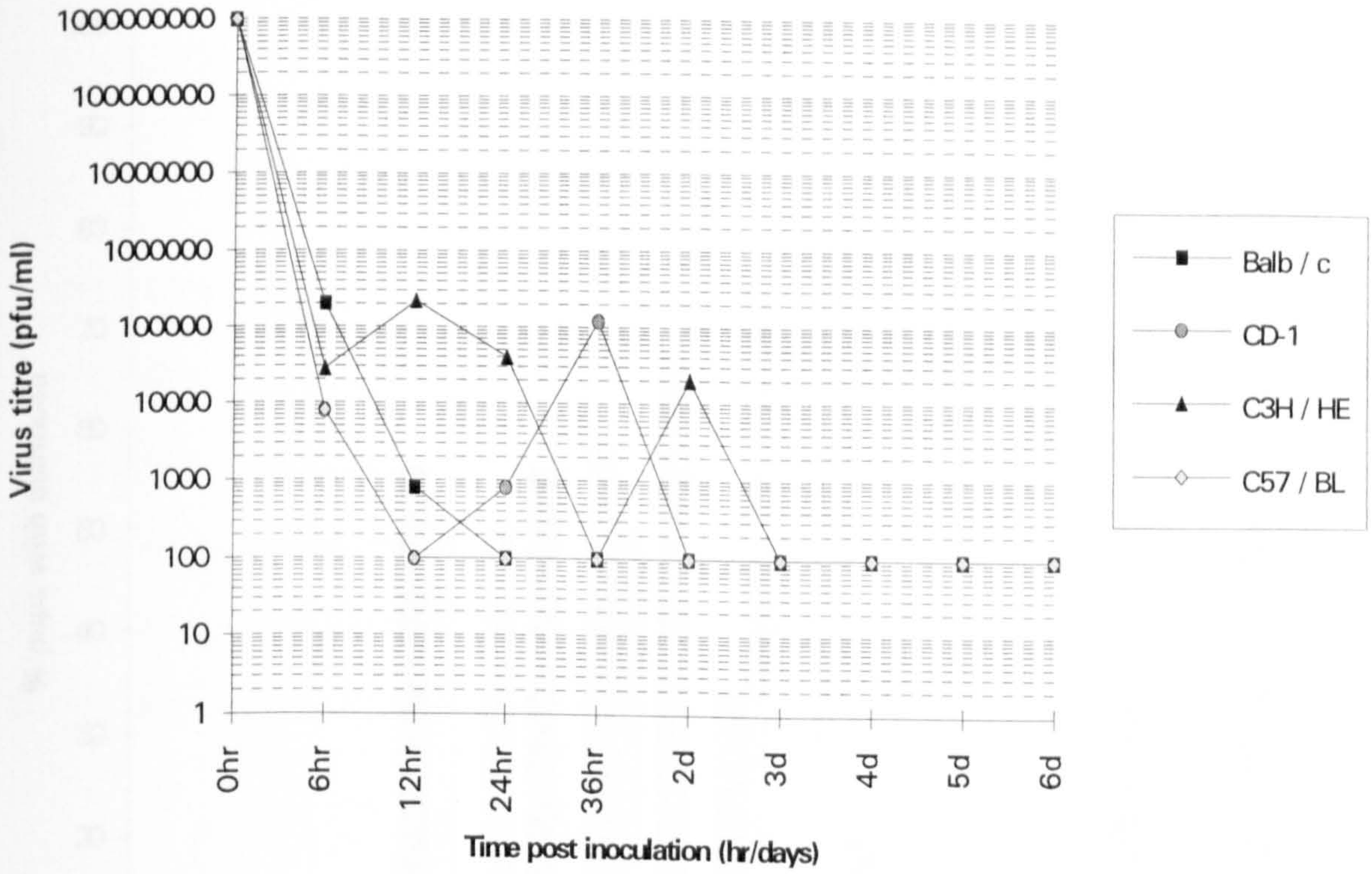


Figure 21 : Replication of UKtc strain of rotavirus in 7 day old suckling pups of various mice strains.

Seronegative 7 day old suckling pups of various mice strains were orally inoculated with 10^9 pfu of UKtc strain of rotavirus on day 0. At each time point, a pair of animals was sacrificed and entire gut was aseptically collected in cold PBS. Titres of the infectious virus in the intestinal homogenates were determined by plaque assay at various times post inoculation. Plotted are the mean from 2 experiments and in each experiment n = 2. The limit of virus detection in plaque assay was 1×10^2.

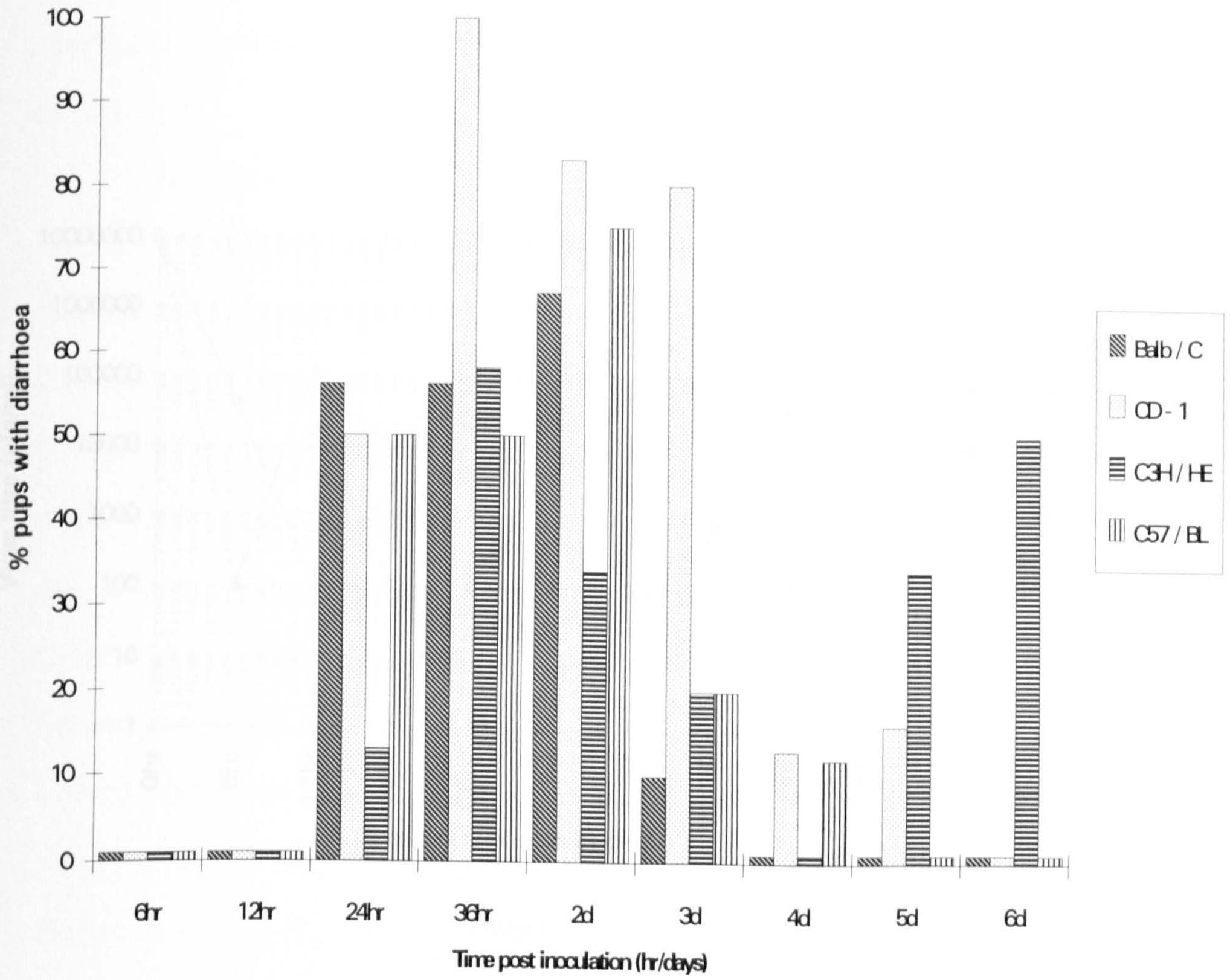


Figure 22 : Diarrhoea produced by OSU strain of rotavirus in 7 day old suckling pups of various mice strains.

Seronegative 7 day old suckling pups of various mice strains were orally inoculated with 10^7 pfu of OSU strain of rotavirus on day 0. At various times post inoculation the animals were examined for clinical signs of disease. At each time point, a pair of animals was sacrificed and observed for internal signs of disease. Plotted are the mean from 2 experiments and in each experiment n = 5-8. The standard deviation ranged from 0 to 5.7.

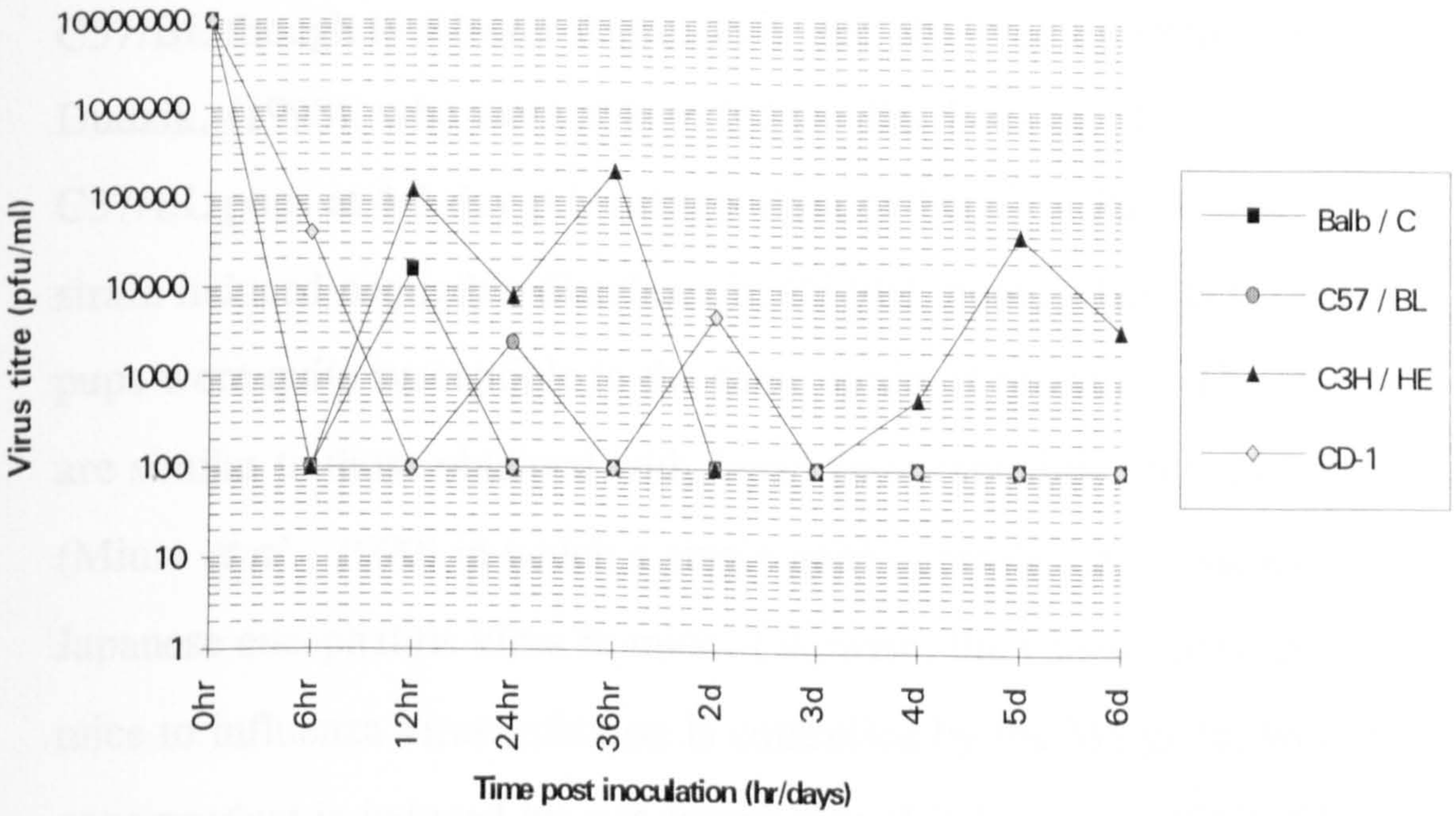


Figure 23 : Replication of OSU strain of rotavirus in 7 day old suckling pups of various mice strains.

Seronegative 7 day old suckling pups of various mice strains were orally inoculated with 10^7 pfu of OSU strain of rotavirus on day 0. At each time point, a pair of animals was sacrificed and entire gut was aseptically collected in cold PBS. Titres of the infectious virus in the intestinal homogenates were determined by plaque assay at various times post inoculation. Plotted are the mean from 2 experiments and in each experiment n = 2. The limit of virus detection in plaque assay was 1×10^2.

lethal for an unusually susceptible host. Susceptibility to viral infection is always influenced and sometimes determined by the genetic constitution of the host.

Heterologous OSU and UKtc rotavirus strains induced disease in C57/BL though to a lesser degree than that observed by Offit and Dudzik, (1990), who were able to induce diarrhoea in 100% of 7 day old C57/BL pups at 4-5 day p.i. using a dose of $5x10^5$ of RRV. The B223 strain induced negligible diarrhoea in C57/BL pups, whereas C3H/HE pups were quite susceptible to the same strain of rotavirus. The results are similar to those obtained with Japanese encephalitis virus in mice (Miura *et al.*, 1990) in which a single gene controls the resistance to Japanese encephalitis virus in mice. Likewise, the resistance of certain mice to influenza virus infection is controlled by the Mx gene, whose gene product is induced by α/β interferons (Lindenmann, 1962; 64; Haller *et al.*, 1979). Whether there is any genetic restriction of C57/BL on diarrhoea induction by B223 virus remains to be seen.

The three inbred strains of mice showed quite different patterns of disease. Whether MHC haplotype has any influence on the outcome of the infection requires further analysis. It also indicates that protection studies with rotavirus vaccines candidates should be done in the outbred CD-1 mice strain so that the results of these studies can be applied in a meaningful way for outbred animals and humans.

5.7.4 Conclusions

The CD-1 was found to be the most susceptible and C57/BL the least susceptible strain for infection with rotavirus. Productive replication usually leads to a second peak of disease which occurs 5-6 day post inoculation. The possibility of using Balb/c and C57/BL for conducting

virulence mapping studies was ruled out as DD_{50} could not be established in them.

5.8 Overall conclusions

A mouse model for monitoring rotavirus virulence has been developed. Virulence was not restricted to a given serotype(s). However, the porcine virus strain appeared to be more virulent than the bovine strains tested. Different host strains showed varying degrees of susceptibility to rotavirus induced disease.

The genetic and molecular studies with genetically manipulated rotaviruses would be conducted using the CD-1 strain of mouse where interpretation of the clinical signs was easy. Also, in CD-1 pups the differences in the DD_{50} and percentage of pups affected by OSU were significantly greater than those observed with either UKtc or B223. However, the difference was only $2\log_{10}$.

Chapter 6

Development of a simple and rapid method for nucleic acid extraction and genotyping

6.1 Introduction

Having developed the mouse model for monitoring rotavirus virulence (chapter 5), the next question to address was the identification of the viral gene(s) involved in this virulence. Such studies require a very large number of samples (genetically manipulated virus) to be screened (discussed in detail in chapter 7) to correlate phenotype with genotype. Consequently, a rapid screening assay needed to be developed, in which the manipulative steps are simple and minimum. In this chapter, the development of a simple and rapid nucleic acid extraction protocol and a simple assay for genotyping virus reassortants is described.

6.2 Nucleic acid extraction methods

The UKtc strain of rotavirus was used to develop a simple and rapid method for extraction of viral nucleic acid. When the method was optimised, it was used to test the extraction of nucleic acid from two other strains i.e. OSU and B223. During the course of development, the presence of rotavirus specific RNA in the extracted material was determined by amplification of the gene encoding VP7 in RT-PCR.

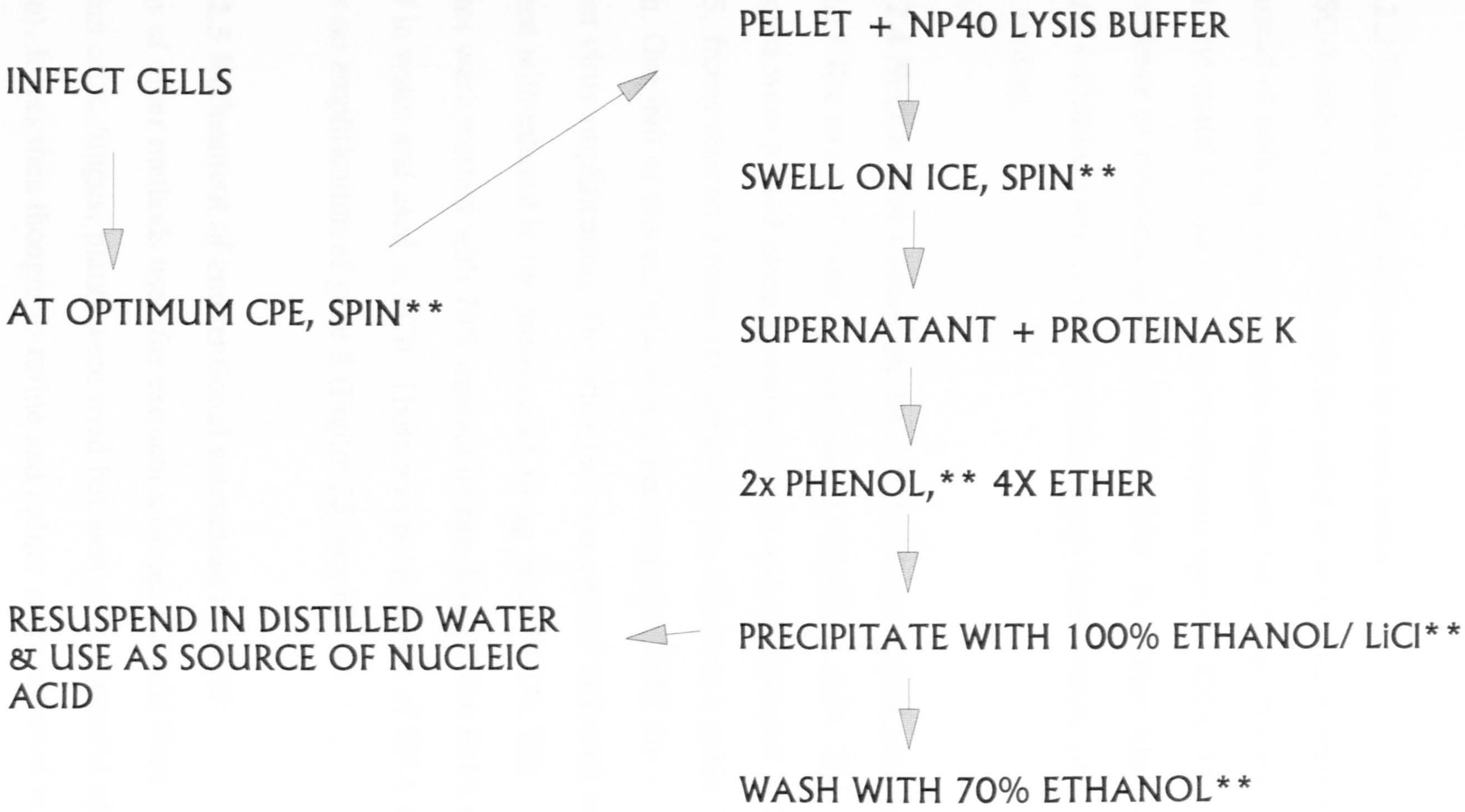
6.2.1 Conventional method of nucleic acid extraction

The RNA was extracted by conventional method, but there were 6 centrifugation steps: one for obtaining cell pellet, one for obtaining cytoplasmic extract after lysis with NP40, two at phenol extraction, and two during ethanol precipitation (see figure 24). This made the conventional method impractical for handling large number of samples.

6.2.2 Nucleic acid extraction by boiling

BSC-1 cells (10^6) were infected at a m.o.i. of 1 pfu/ cell. At about 50% CPE the cells were harvested, boiled for 2 min. After a brief

FIGURE 24 : A SCHEMATIC OF THE CONVENTIONAL METHOD OF NUCLEIC ACID EXTRACTION *



* From Clarke and McCrae, (1981) and Ballard et al., (1992). ** Centrifugation steps.

centrifugation the supernatant was tested for the presence of rotavirus specific RNA. RNA was amplified (figure 25, lane E) on some occasions and not reproducibly.

6.2.3 Nucleic acid extraction by sonication

BSC-1 cells were infected and harvested as described in section 6.2.2. Instead of boiling, the cells were sonicated for 30 sec in sonic waterbath and tested for the presence of rotavirus specific RNA. There was no evidence of rotavirus specific RNA in PCR. In another situation, boiling and sonication were combined without any improvement of results (data not shown).

6.2.4 Nucleic acid extraction directly from individual plaques

The UKtc strain of rotavirus was plaqued in BSC-1 cells. The virus plaques were picked using a pasteur pipette and resuspended in 100 µl of PBS, freeze thawed 3 times and sonicated for 30 sec in a sonic waterbath. One half of this material was stored at minus 70^{o}C for subsequent virus amplification. The other half was phenol extracted and precipitated with ethanol in the presence of 10 µg yeast tRNA. The precipitates were washed with 70% ethanol before drying. The RNA was dissolved in water and used in PCR. There was no evidence of RNA as there was no amplification of gene 8 (Figure 25 lane F).

6.2.5 Refinement of conventional extraction method

A number of other methods used for extraction of nucleic acid from mammalian cells, fungus, plants were tried but were not successful (data not shown). It was then thought to refine and reduce steps involved in the conventional method. At first ethanol precipitation steps were omitted without any deleterious effect on extracted RNA (Figure 25 lane

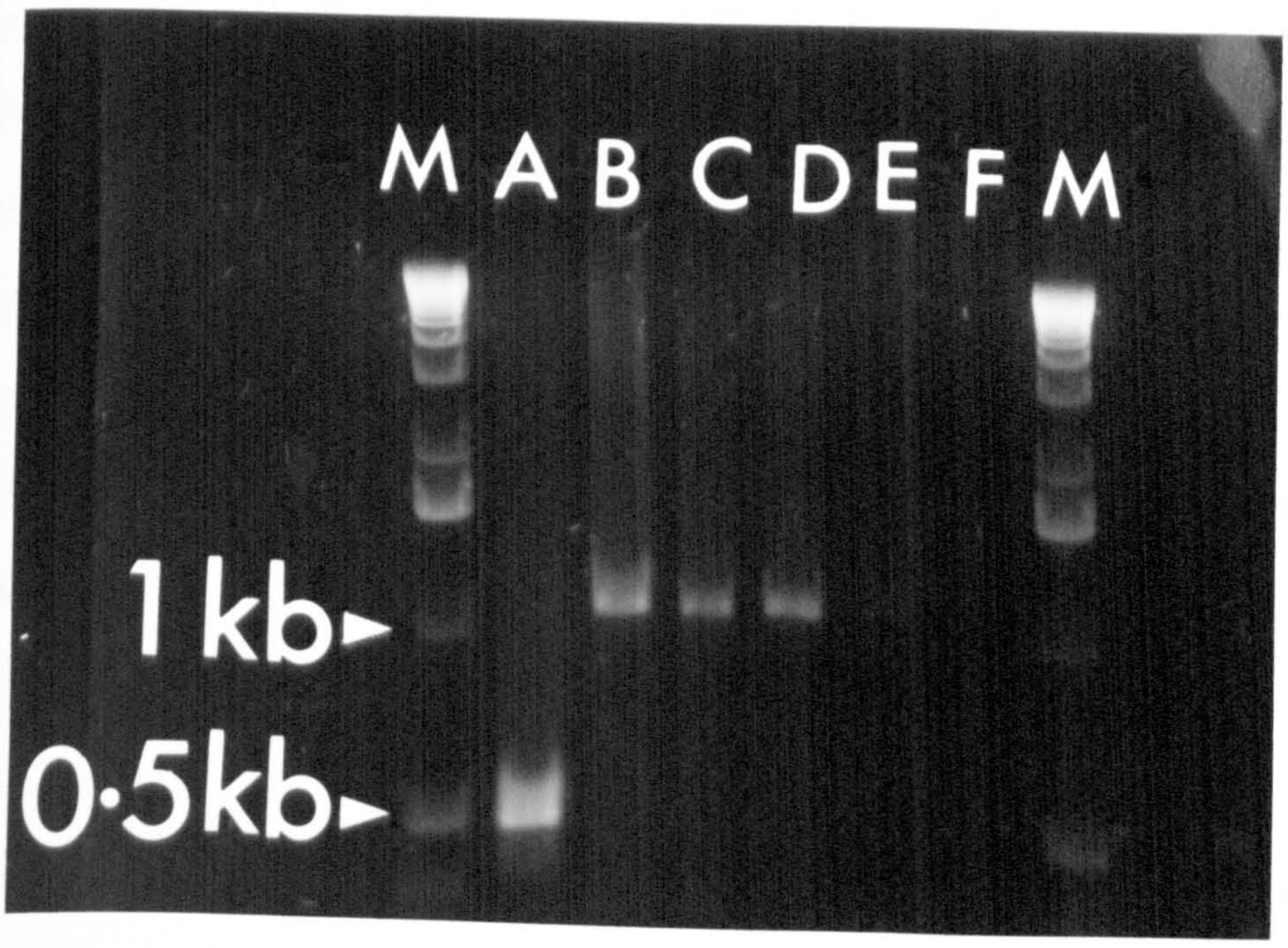


Figure 25 : Amplification of UKtc gene 8 in RT-PCR from RNA extracted by various methods.

BSC-1 cells were infected with UKtc strain of rotavirus. Viral RNA was extracted by various methods as described in Materials and Methods. Viral dsRNA was then detected by agarose gel electrophoresis and staining with ethidium bromide following RT-PCR amplification of gene 8.

Lane A = adeno control, lane B = RNA extracted by conventional method, lane C = no ethanol precipitation, lane D = no phenol step but deproteinisation with proteinase K followed by its inactivation at 80^{o}C/ 10 min, lane E = RNA extracted by boiling, lane F = RNA extracted directly from a plaque, lane M = ladder of DNA size markers.

C). No RNA could be extracted when the phenol/ether steps were omitted (data not shown). It was felt that the SDS and proteinase K interfered in the PCR. This problem was overcome by deleting the addition of SDS and by heat inactivating the proteinase K. A series of reconstruction experiments were carried out in which the RNA extracted by the conventional method was mixed with proteinase K in proteinase K buffer or just the buffer. The mixture was heat inactivated in a waterbath for various lengths of time prior to attempting amplification of UKtc gene 8 in RT-PCR. The mixture containing only the Proteinase K buffer left on ice prior to amplification in RT-PCR served as control to show that heating at 80^{o}C did not have any deleterious effect in PCR. And the mixture containing only the proteinase K buffer and heated at 80^{o}C for 30 min was included to show that the proteinase K buffer itself was not inhibiting the PCR. It was found that proteinase K could be inactivated at 80^{o}C in 10 min but not in 5 min, as determined by amplification of gene 8 of UKtc in PCR (Figure 26). Therefore, four centrifugation steps were reduced without any deleterious effect on RNA extraction.

In the conventional method, after the development of CPE the cells were scraped off and then centrifuged to obtain a cell pellet that was subsequently subjected to lysis with NP40. It was felt that this first centrifugation step could also be reduced by collecting the supernatant separately for further passage and adding the lysis buffer directly onto the monolayer in the tissue culture plates. This proved successful as there was sufficient RNA in the cytoplasmic extracts and the supernatant was found to contain sufficient virus to act as inoculum for subsequent passage (data not shown). Initially, plaques grown in 24-well plates were further amplified in 6-well plates before extraction of nucleic acid. However, it was found out that nucleic acid could directly be extracted

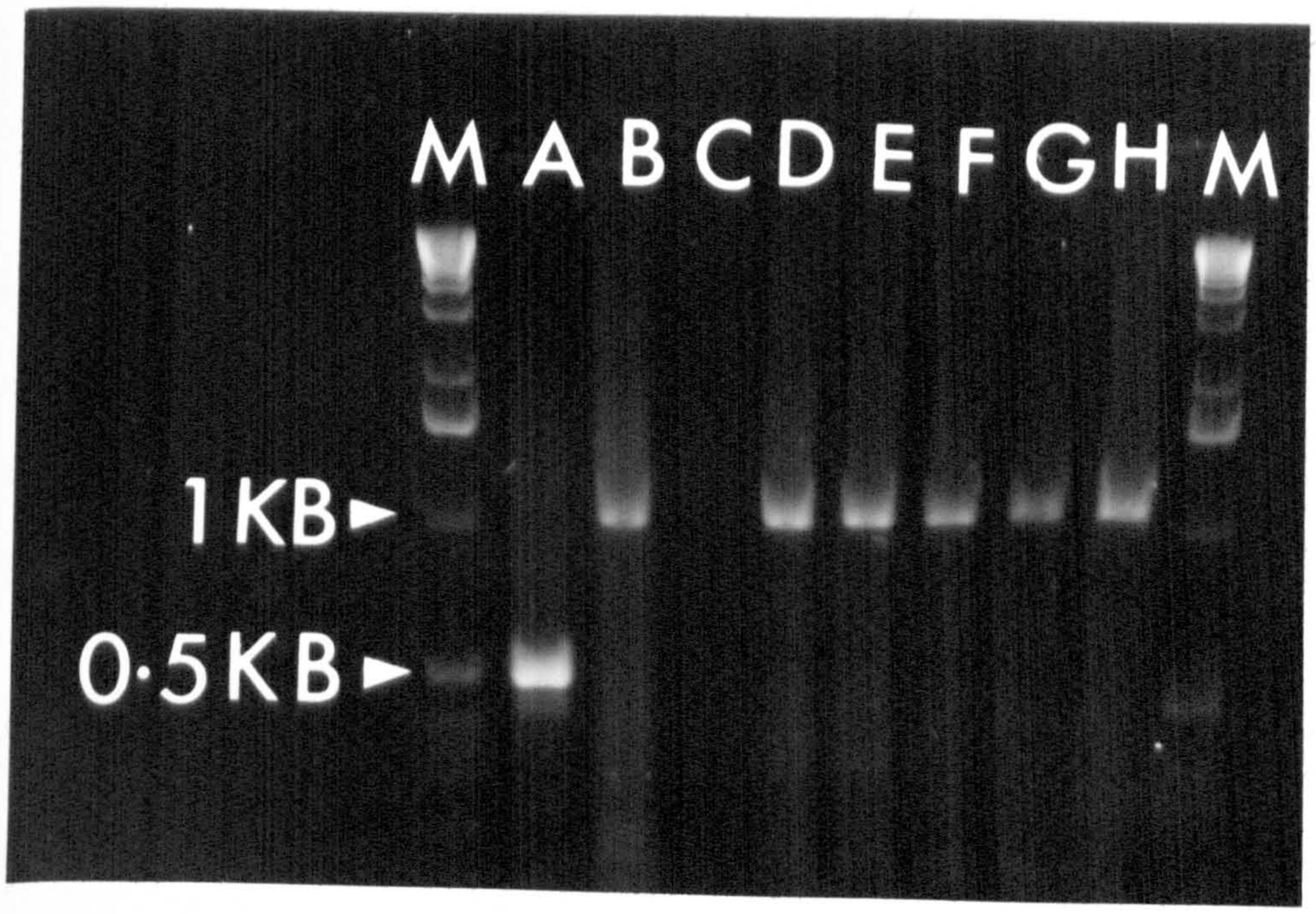


Figure 26 : Determination of the time required to inactivate proteinase K by heat.

BSC-1 cells were infected with UKtc strain of rotavirus. Uktc RNA extracted by conventional method was mixed with proteinase K or only proteinase K buffer and the proteinase K was inactivated by heating at 80^{o}C for various times. Then VP7 gene was amplified in RT-PCR. Products of RT-PCR showing full length cDNA copies of VP7 were separated on 1.5% agarose gel which was stained with ethidium bromide.

Lane A = adeno control, lane B = RT control of UKtc VP7 gene, lanes C-F shows amplification of VP7 gene after heat inactivation of proteinase K in 5 min (lane C), 10 min (lane D), 20 min (lane E), 30 min (lane F), or RNA+proteinase K buffer only, heated at 80^{o}C for 30 min (lane G), or kept on ice before performing RT-PCR (lane H), lane M = ladder of DNA size markers.

from the plaques grown in 24-well plates. This saved lot of time and labour.

6.2.6 Optimised extraction method

The nucleic acid extraction method optimised in the present study is described below. Rotaviruses were plaqued on BSC-1 cells. Individual plaques were picked and grown in 24-well dishes. Cytoplasmic extracts were prepared by NP40 lysis and deproteinisation was done by incubating the cytoplasmic extract with proteinase K.

Comparison of the optimised method (Figure 27) with the conventional method (Figure 24) will reveal that this does not require the use of any hazardous organic chemicals and requires just one centrifugation step, which not only reduces the manual labour involved in screening large number of samples but also reduces the chance of cross contamination.

This method was used to extract RNA from OSU and B223 strains of rotavirus as well as from genetically manipulated virus described in later studies.

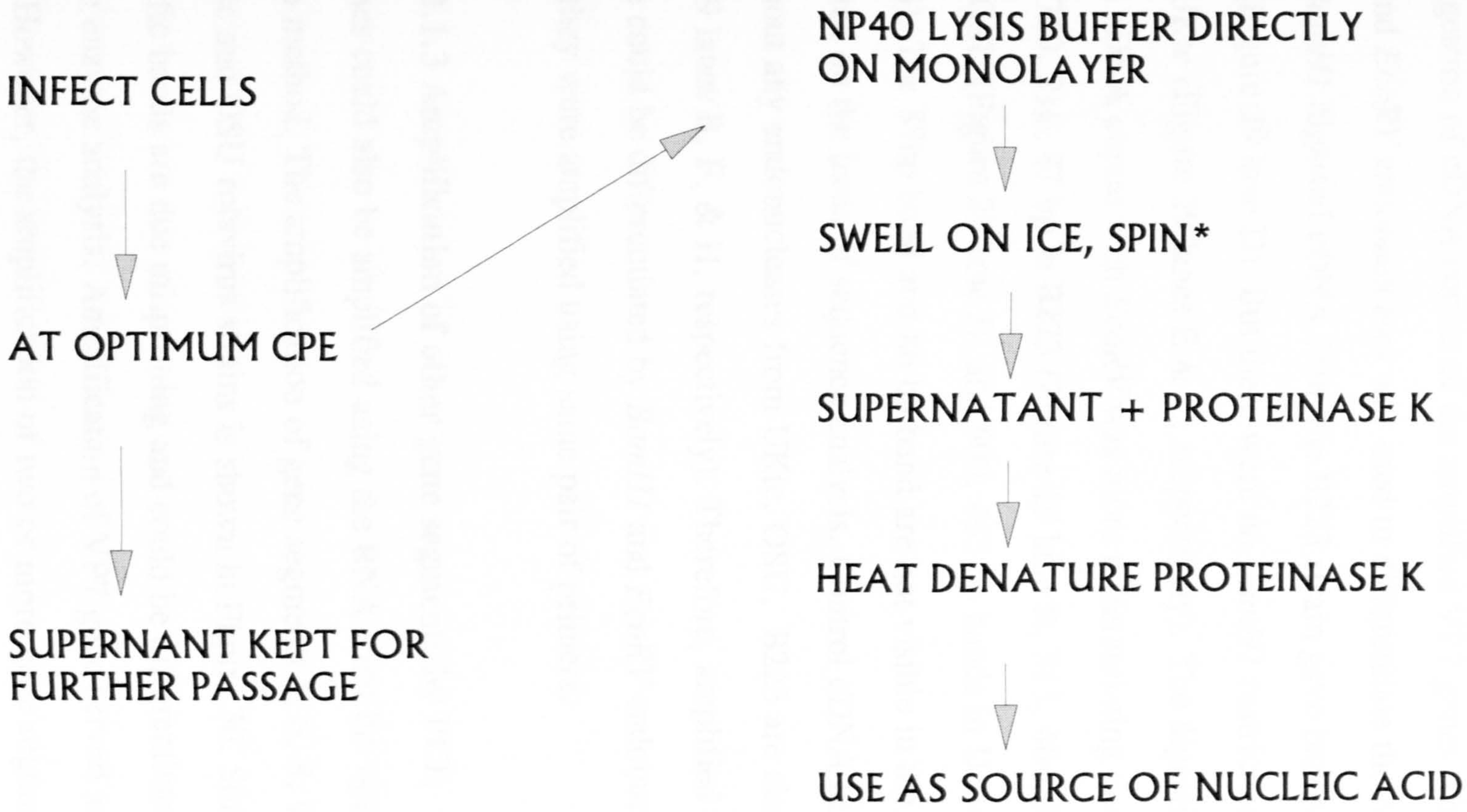
6.3 Genotyping assays

6.3.1 Polymerase chain reaction

6.3.1.1 Amplification of VP7 gene

The extracted RNA was used in a coupled reverse transcription RT-PCR using primers complementary to the termini of gene 8 as described previously (Xu *et al.*, 1990) with some modifications (described in materials and methods). Amplified DNA bands were fractionated on 1.5% agarose gels and visualised by staining with ethidium bromide (Sambrook *et al.*, 1989). The amplification of the VP7 gene (1062 bp band) from UKtc, OSU, and B223 strains of rotavirus is

FIGURE 27 : A SCHEMATIC OF THE OPTIMISED METHOD OF NUCLEIC ACID EXTRACTION



*This is the only centrifugation step involved.

shown in Figure 28. No band was detected in the material extracted from mock infected BSC-1 cells (Figure 28 lane E) or with UKtc RNA without gene 8 primers (Figure 28 lane F).

6.3.1.2 Restriction enzyme analysis of amplified VP7 gene

Digestion of cDNA copies of the amplified VP7 genes with *BamH1* and *EcoRV* endonucleases was used to differentiate the rotavirus strains. *BamH1* digested cDNA from the B223 strain gave bands of 628 and 434 (Figure 29 lane D). But there were no *BamH1* restriction sites in OSU or UKtc (Figure 29 lanes E & C, respectively). The digestion of amplified cDNA copies with *EcoRV* was more differentiating and gave bands of 760, 216, 87 bp in B223 (Figure 29 lane I), 513, 464, 86 bp bands in OSU (Figure 29 lane J), and 600, 463 bp bands in UKtc (Figure 29 lane G). The 87bp band and 86 bp band are not visible in the gel but are reported on the basis of sequence analysis. Control cDNA of VP7 gene without any endonucleases from UKtc, OSU, B223 are also shown (Figure 29 lanes B, F, & H, respectively). Therefore, amplified cDNA of VP7 gene could be differentiated by *BamH1* and *EcoRV* endonucleases, although they were amplified using same pair of primers.

6.3.1.3 Amplification of other gene segments by PCR

Other genes could also be amplified using the RNA from the optimised extraction method. The amplification of gene segments 5, 6, 8, 9, and 11 from UKtc and OSU rotavirus strains is shown in Figure 30. Some of the non-specific bands are due mispriming and could be differentiated by restriction enzyme analysis. Amplification of VP7 gene served as controls. However, the amplification of two or more gene segments from a strain in the same tube was not as successful as when the gene segments were amplified singly (data not shown).

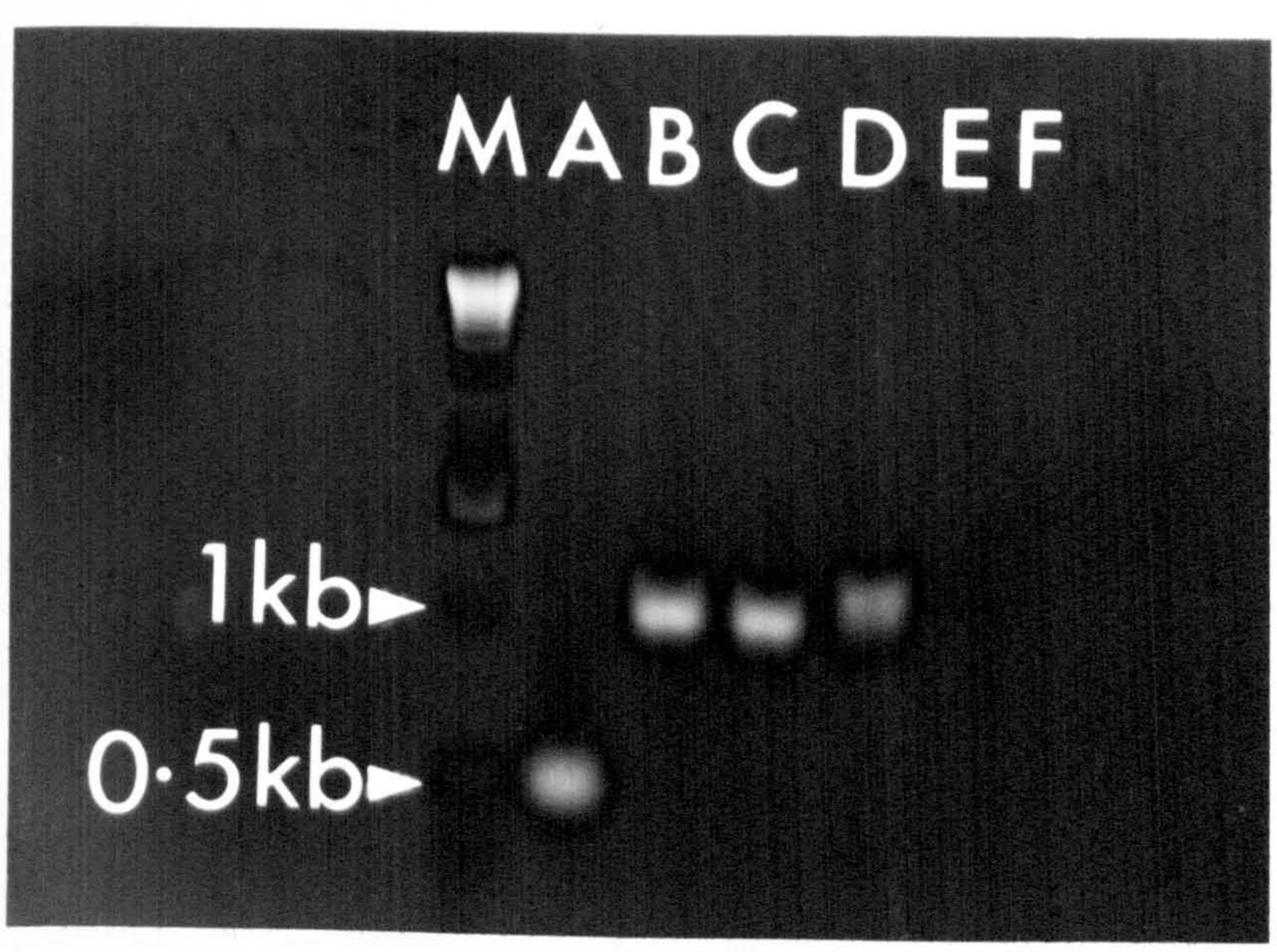


Figure 28 : Product of RT-PCR showing full length cDNA copies of VP7 gene.

BSC-1 cells were infected with various strains of rotavirus. Viral RNA was extracted by optimised method. The VP7 gene was amplified in RT-PCR. Products of RT-PCR showing full length cDNA copies of VP7 were separated on 1.5% agarose gel which was stained with ethidium bromide.

Positive adeno DNA control (lane A), UKtc (lane B), OSU (lane C), B223 (lane D), negative controls (lanes E & F) are also shown, lane M = ladder of DNA size markers.

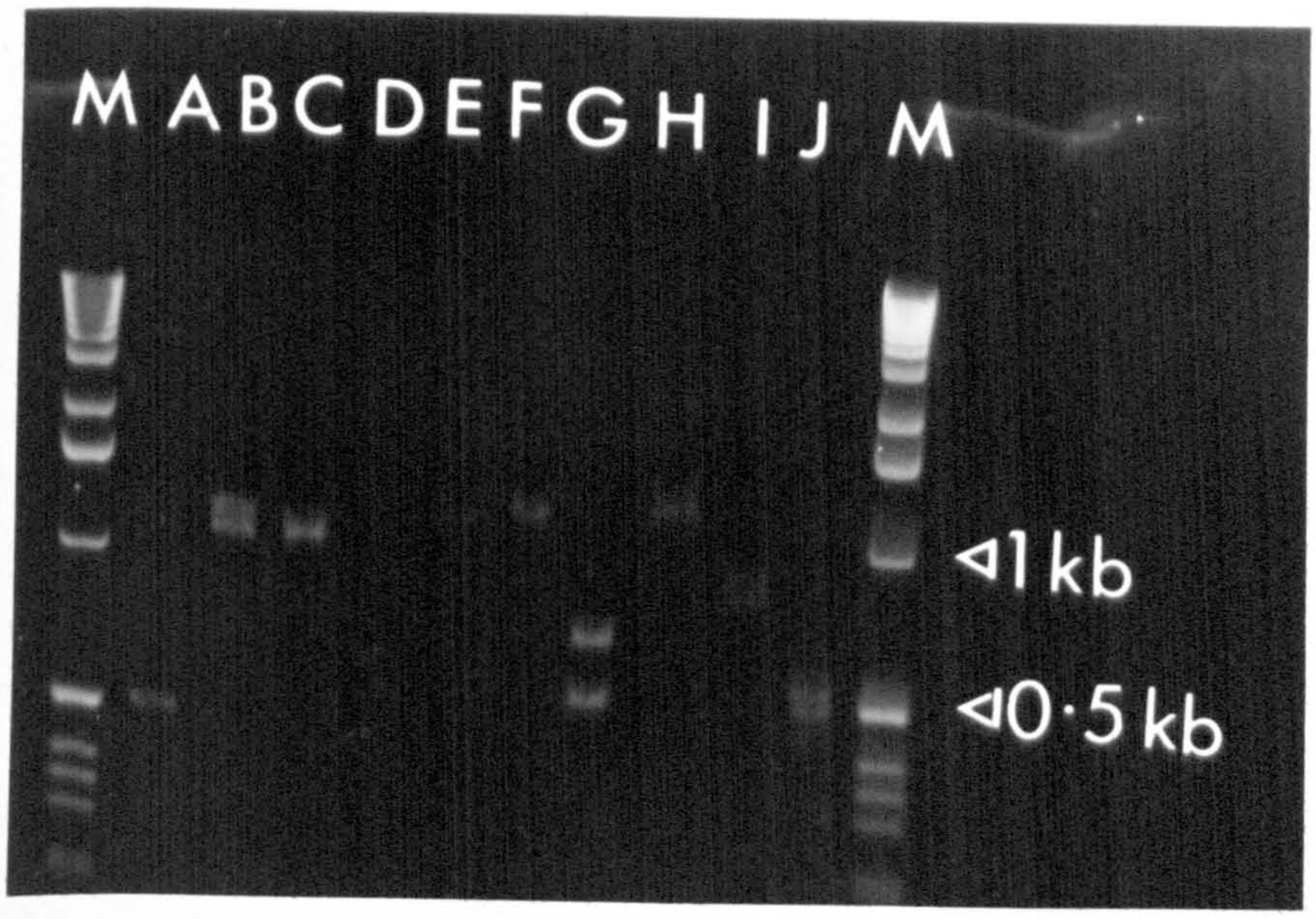


Figure 29 : Restriction patterns of VP7 gene copies of cDNA.

The VP7 gene amplified in RT-PCR from the RNA extracted by optimised method from the BSC-1 cells infected with various strains of rotavirus was digested by different restriction endonucleases. After digestion of cDNA with endonucleases, electrophoretic separation was done on 1.5% agarose gel which was stained with ethidium bromide. Lane A = Adeno control; lane C = UKtc+*BamH1*; lane D = B223+*BamH1*; lane E = OSU+*BamH1*; lane G = UKtc+*EcoRV*; lane I = B223+*EcoRV*; lane J = OSU+*EcoRV*; Control cDNA copies without any endonucleases are also shown in lane B = UKtc; lane F = OSU; lane H = B223; lane M = ladder of DNA size markers.

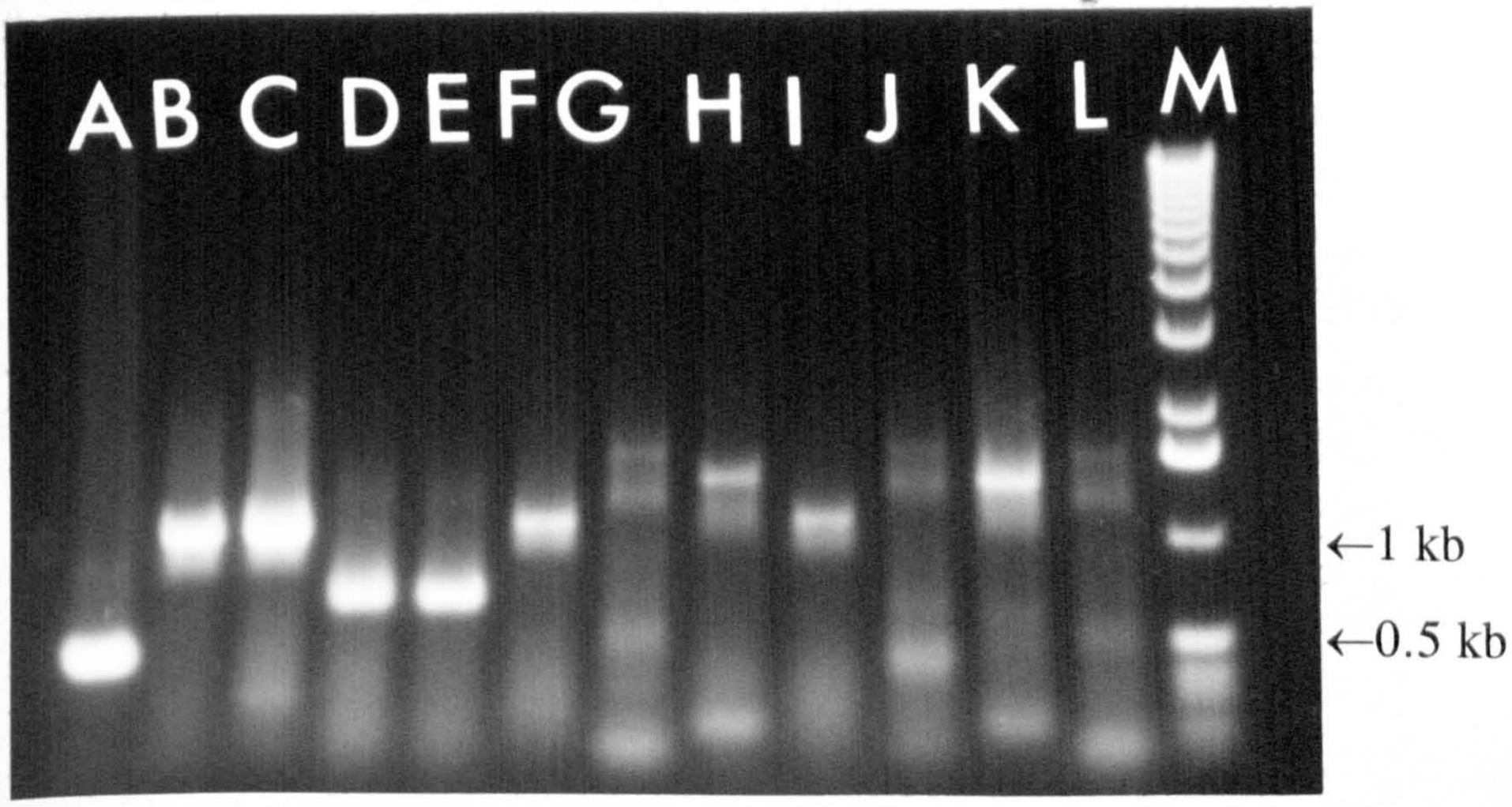


Figure 30 : Product of RT-PCR showing full length cDNA copies of various genes of UKtc and OSU.

BSC-1 cells were infected with UKtc or OSU strains of rotavirus. Viral RNA was extracted by optimised method. Various genes were amplified in RT-PCR. Electrophoretic separation was done on 1.5% agarose gel which was stained with ethidium bromide.

Lane A = positive adeno DNA control; lane B = UKtc gene 8; lane C = OSU gene 9; lane D = UKtc gene 11; lane E = OSU gene 11; lane F = UKtc gene 9; lane G = OSU gene 5; lane H = OSU gene 6; lane I = OSU gene 8; lane J = UKtc gene 5; lane K = B223 gene 6; lane L = UKtc gene 5; lane M = ladder of DNA size markers.

6.3.2 Polyacrylamide gel electrophoresis

The extracted RNA samples were also analysed by polyacrylamide gel electrophoresis. The fractionated dsRNA segments were visualised by silver staining. By employing a combination of coelectrophoresis and the running of parental strains on either side of the coelectrophoresed sample, the origin of about 6-7 segments could be identified. It was not possible to distinguish the parental origin of segments 2, 3, 8, and 11 between UKtc and B223 strains, and segments 2, 7, 8, and 11 between OSU and B223 strains. Also, segments 2, 7, 8, 9, and 11 could not be differentiated between UKtc and OSU strains of rotavirus (Figure 31).

6.4 Discussion

Two viral strains can be differentiated antigenically (by monoclonal antibodies or hyperimmune sera), biologically (e.g. haemagglutination, plaque formation), or genetically (nucleic acid probes, PCR, sequencing). However, in order to correlate phenotype with genotype, the knowledge about the genetic make-up of the organism is necessary, to show that the phenotypes are genetically determined and not due to some non-genetic mechanisms such as complementation or phenotypic mixing in *in vitro* studies or due to host factors in *in vivo* studies. The phenotype- genotype correlation studies often involve screening of a very large number of samples. Therefore, both the nucleic acid extraction and genotyping assays should not only be rapid and simple but have the minimum manipulative steps.

RNA has been isolated from purified rotavirus either by deproteinisation with phenol, or deproteinisation with proteinase K (Cash, 1982), deproteinisation with both proteinase K and phenol (Lourenco *et al.*, 1981), or simple lysis of the purified particles by boiling in buffer

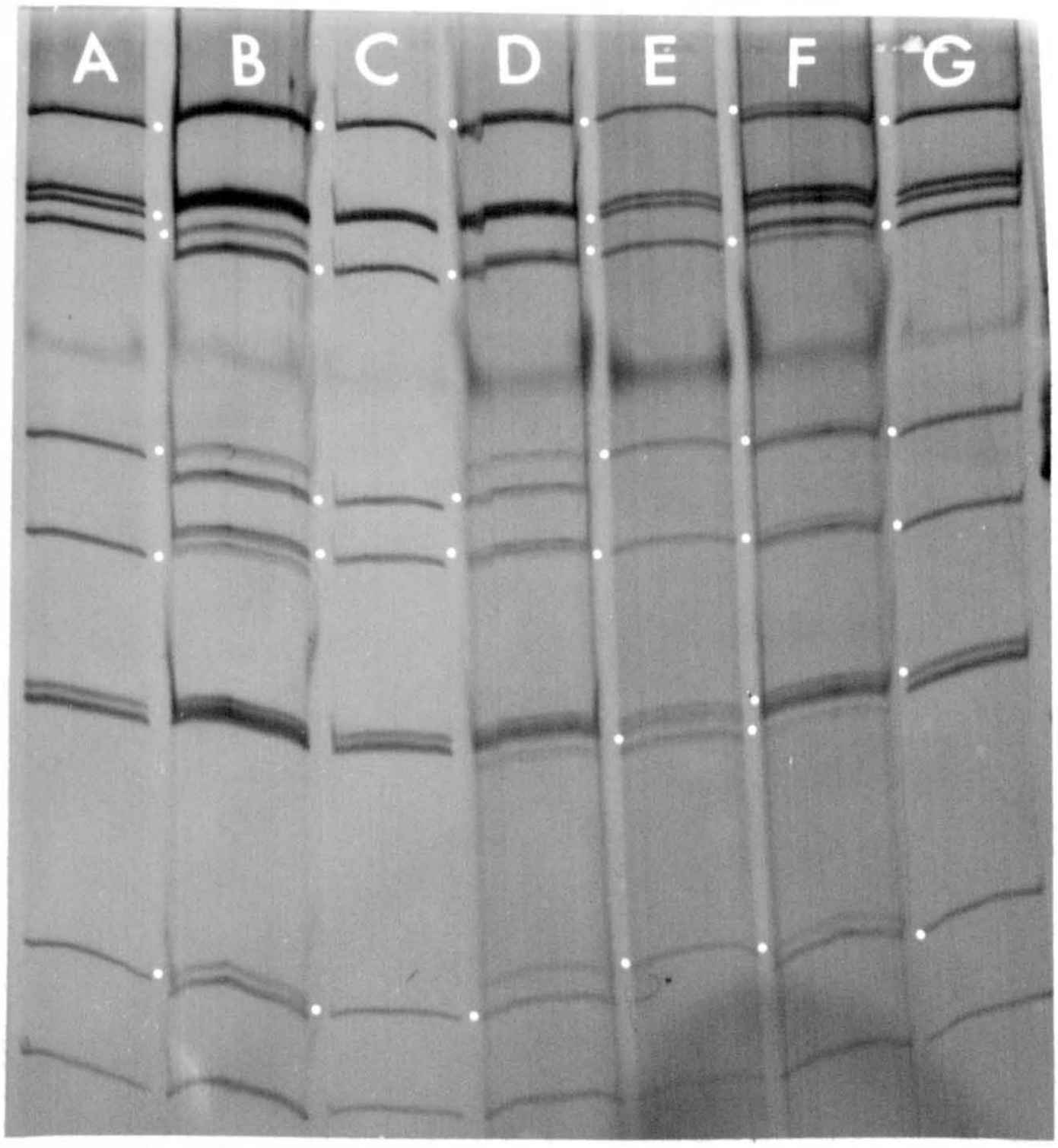


Figure 31 : Electrophoretic profile of genomic RNA of rotavirus strains separated on polyacrylamide gel and silver stained.

Viral RNA was extracted by optimised method from BSC-1 cells infected with various strains of rotavirus. Viral dsRNA were then detected by electrophoresis on a 6% polyacrylamide gel and silver staining as described in Materials and Methods.

Lane A = UKtc; lane B = UKtc+OSU; lane C = OSU; lane D = OSU+B223; lane E = B223; lane F = B223+UKtc; lane G = UKtc; lane M = ladder of DNA size markers. The parental origin of the gene segments in coelectrophoresed parents is indicated by a white dot on the side of parent. The gene segments are indicated on the left.

containing SDS and reducing agents, prior to analysis on gels (Croxson and Bellamy, 1981).

The conventional nucleic acid extraction method that was being employed for extracting rotavirus genomic RNA at the beginning of this study required six centrifugation steps. The use of boiling (Ferre and Garduno, 1989), or extraction of nucleic acid directly from viral plaques (Pasamontes *et al.*, 1991) have been described, but were unsuccessful for rotavirus RNA extraction in this study. Then it was thought to refine and reduce the steps involved in the conventional method. The method developed in present study does not require phenol extraction or ethanol precipitation steps. Bypass of ethanol precipitation following hydroxyapatite treatment of stools (Gouvea *et al.*, 1991), or phenol/ chloroform extraction after treatment of stools with glass powder (Gentsch *et al.*, 1992) for the extraction of rotavirus RNA have been reported. Bypass of phenol extraction (Edwards *et al.*, 1991; Laird *et al.*, 1991) or various centrifugation steps (Laird *et al.*, 1991) for DNA extraction from plants and mammalian cells have also been reported. Deproteinization by proteinase K followed by its inactivation by heating at 80^{o}C for 10 min was found to be sufficient for RNA extraction. Others have reported the inactivation of proteinase K in 10 min at 95^{o}C (Porter *et al.*, 1991) or by the use of BSA (Laird *et al.*, 1991).

The final method developed for nucleic acid extraction requires just one centrifugation step and does not require the use of any hazardous organic chemicals. The RNA extracted by the method developed in this study was used to develop genotyping assays. The PCR provides a technique by which the nucleic acid in a sample can be specifically amplified by up to 10^{6}- fold prior to its detection (Saiki *et al.*, 1985; Saiki *et al.*, 1988). RT-PCR has been used for the detection of rotavirus (Xu *et al.*, 1990; Gouvea *et al.*, 1990; 1991). The nucleotide sequence of

all the gene segments of UKtc has been determined (Desselberger and McCrae, 1994). Initially a strategy was planned in which the strain origin of each viral RNA segment could be identified by first amplifying the gene by RT-PCR followed by restriction enzyme analysis of the amplified product or by a second nested PCR using strain-specific primers. However, this strategy of solely genotyping by PCR had to be abandoned as it was found to be economically and practically not feasible. Because, sometimes the gene could not be amplified and in other situations the sequence was not available for many genes of OSU and B223 to construct the primers, as the primers constructed for UKtc did not work on OSU or B223 probably due to sequence heterogeneity. However, PCR amplification followed by restriction enzyme analysis was useful for some genes. Restriction enzyme analysis of VP7 gene of human and animal rotaviruses has recently been applied to divide more than 200 strains into 28 groups (Gouvea *et al.*, 1993). The strategy of amplifying various genes together in the same tube to reduce the cost was abandoned as it was observed that various primers interfered with each other. Because, gene segments that gave good amplification singly were found to be amplified poorly when it was tried to amplify them together with other gene segments in the same tube.

Rotavirus particles contain a genome of 11 segments of dsRNA that can be separated into distinct bands by gel electrophoresis and visualised by staining with either ethidium bromide or silver or alternatively by autoradiography after labelling the 3' end with ^{32}P (Clarke and McCrae, 1981; Herring *et al.*, 1982). The gel electrophoretic pattern of the segments (electropherotype) is a reproducible characteristic of a virus strain. It has been used for rotavirus detection (Newman *et al.*, 1975; Rodger *et al.*, 1975; Kalica *et al.*, 1976; Clarke and McCrae, 1981) as well as for genotyping rotavirus reassortants used in gene-coding

and gene function assignment studies (Greenberg *et al.*, 1981; 1983a; Kalica *et al.*, 1981; 1983; Offit *et al.*, 1986; Gombold and Ramig, 1986; Urasawa *et al.*, 1986; Ward *et al.*, 1988a; Liu *et al.*, 1988; Chen *et al.*, 1989; Snodgrass *et al.*, 1992; Broome *et al.*, 1993). One of the major advantage offered by PAGE-SS for genotyping is the simultaneous comparison of several gene segments between two strains of rotavirus. It was possible to differentiate about 6-7 segments between different strains used in this study by coelectrophoresis of their RNA followed by silver staining. Assignments of the gene segments by PAGE-SS were found to be correct when confirmed by RT-PCR on gene segment encoding VP7.

6.5 Conclusions

A very simple and rapid nucleic acid extraction method has been developed that can be applied to a very large number of samples saving time and labour. The nucleic acid extracted from genetically manipulated rotaviruses, constructed for conducting studies on virulence mapping, will be genotyped by using a combination of PAGE-SS and restriction enzyme analysis of RT-PCR amplified product.

Chapter 7

Construction and characterisation of rotavirus reassortants

7.1 Introduction

Until the last two decades the only approach to correlate a virulence phenotype observed in nature with a particular microbe was by proving the Koch's postulates, i.e. isolating the microbe from the diseased host, growing it in the laboratory, infecting the host to observe same kind of clinical signs, and isolating the same organism from the artificially infected host. But, with the advances made in molecular biology, virulence phenotypes are not only correlated to the microbe but also to its gene(s) by the use of variants, mutants, or recombinants.

The ultimate aim of this project was to identify the viral gene(s) determining rotavirus virulence. This could be achieved by the genetically manipulated rotaviruses. Theoretically, two categories of such viruses are possible. In the first category, an entire genome segment(s) is exchanged between two rotavirus strains by the process of reassortment. The second category involves the exchange of genomic segment(s) as such or modified by site-directed mutagenesis, between a rotavirus strain and cloned RNA segment derived from cDNA generated by reverse genetics. The second of these two possibilities remains at present only theoretical as strategies remain to be developed in rotavirus which allow gene rescue of cDNA's RNA in an infectious particle. This chapter describes the construction of rotavirus reassortants by the process of reassortment.

7.2 Rotavirus reassortants are essential for mapping virulence to gene(s)

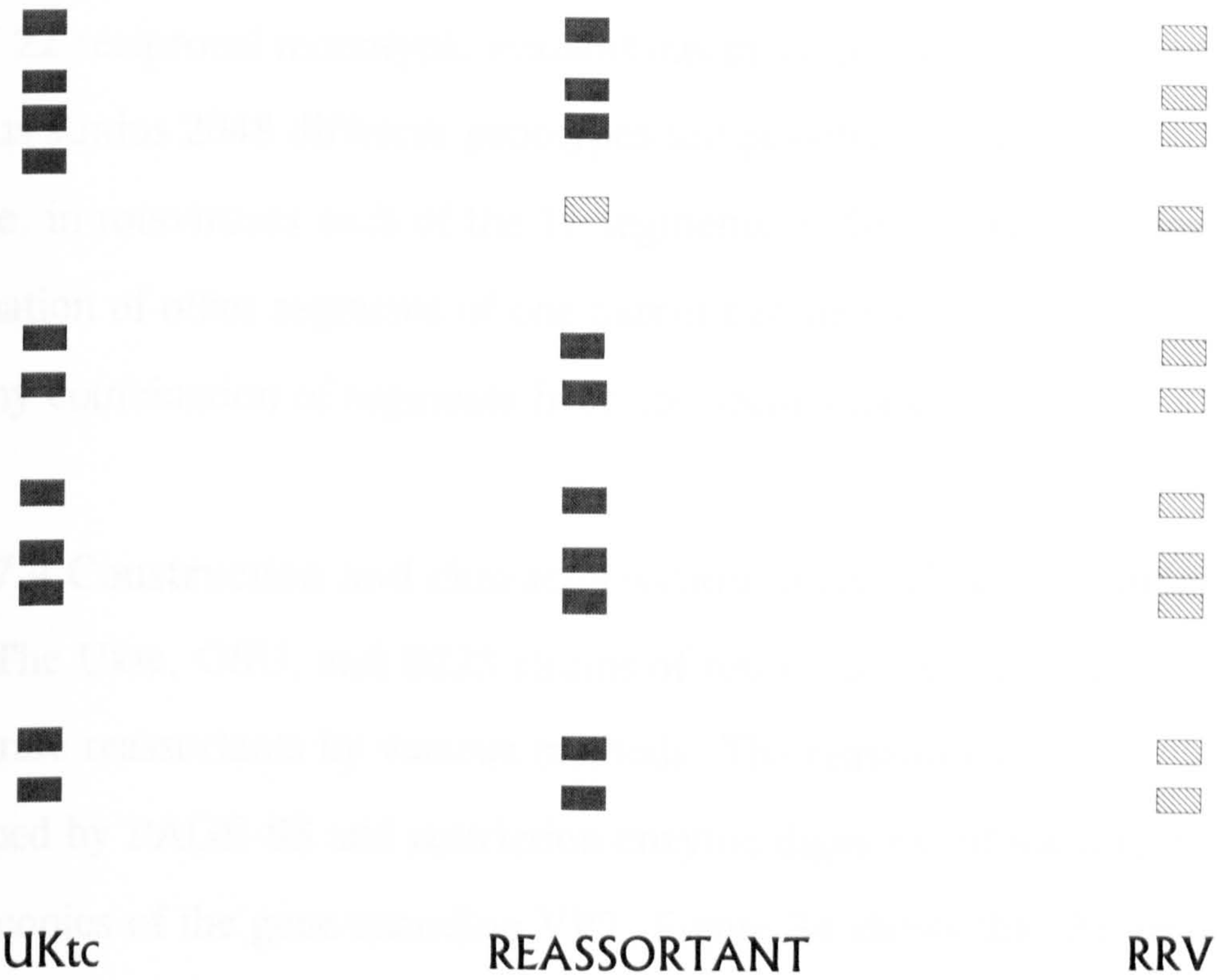
The phenotype of a gene of a RNA virus can be studied by making cDNA copies of the gene and then putting them into a vector to get a clone. After transformation or transfection of suitable cells with the clone, recombinants are obtained. Using the recombinant virus or the expressed protein the phenotype is then correlated with the genotype.

Reassortants generated by reassortment have proved to be the primary tool for the identification of gene function in rotaviruses and other segmented RNA viruses. When two closely related segmented viruses are allowed to infect mixedly either *in vivo* or *in vitro*, then some of the progeny viruses receiving genome segments from both the parents may be produced and are called reassortants (see section 2.6). An example of a reassortant is shown in Figure 32. In this figure UKtc and RRV represent two parents and in the middle is a reassortant which has derived its gene 4 from RRV and the remaining 10 segments from UKtc parent. A viral phenotype such as haemagglutination can be studied either by recombinants or by reassortants between these two parents. The recombinants or reassortants containing gene 4 from RRV parent will be haemagglutinating, as well as the RRV parent, whereas those recombinants or reassortants having the gene 4 from UKtc parent will not haemagglutinate. Because only RRV parent is haemagglutinating. Similarly, recombinants of all genes from rotavirus parents can be made. However, it is not yet possible to manipulate a gene or a part of the gene *in vitro* and then rescue it into an infectious particle in rotaviruses. The classical reverse genetics is not yet possible to rescue a gene into an infectious rotavirus and infectious virus is required for studying rotavirus virulence. In the absence of having a genetically manipulated infectious rotavirus by reverse genetics and recombinant DNA techniques, rotavirus reassortants are the only means available, at present, for studying rotavirus virulence.

7.3 Large number of reassortants need to be screened to map rotavirus virulence

The rotavirus genome consists of 11 gene segments of which all but one (gene 11) are monocistronic (see chapter 1). Assuming that virus

FIGURE 32 : A SCHEMATIC SHOWING A GENE 4 REASSORTANT BETWEEN UKtc AND RRV STRAINS OF ROTAVIRUS.



Shown here is a rotavirus reassortant containing segment 4 from RRV rotavirus and remaining 10 segments from UKtc rotavirus.

virulence is completely determined by a single viral gene then the exchange of one segment at a time from one parent on the background of a second parent can be used to segregate virulence to individual gene segments by infecting suitable host with 22 reciprocal monotypic reassortants from two rotavirus parents differing in their virulence phenotype. However, because of the random nature of the reassortment process, a very large number of samples need to be screened to obtain the desired 22 reciprocal monotypic reassortants since in a cross between two rotavirus strains 2048 different genotypes are possible (Figure 33). Because, in rotaviruses each of the 11 segments, individually or in any combination of other segments of one parent can reassort with individual or in any combination of segments from the second parent.

7.4 Construction and characterisation of rotavirus reassortants

The Uktc, OSU, and B223 strains of rotavirus were used in pairs to generate reassortants by various methods. The reassortants were genotyped by PAGE-SS and restriction enzyme digestion of the amplified cDNA copies of the gene encoding VP7. Figure 34 shows that the results obtained with coelectrophoresis of the parental strains in PAGE-SS (see figure 31; chapter 6) can also be applied on rotavirus reassortants.

7.4.1 Reassortant construction without any external selection pressure

Putative reassortants were generated by coinfection of BSC-1 cells with different pairs of rotavirus strains (see Materials and Methods). The progeny clones were characterised by PAGE-SS of viral genomic RNA and restriction enzyme analysis of the amplified cDNA copies of VP7 gene obtained in RT-PCR. Two reassortants (RS11, and RS7) were isolated in the cross between UKtc and B223 out of 97 plaques, whereas

FIGURE 33 : A SCHEMATIC SHOWING THE NUMBER OF GENOTYPES POSSIBLE DURING CONSTRUCTION OF RECOMBINANTS AND REASSORTANTS

RECOMBINANTS

TRANSFECT cDNA

INFECT wt VIRUS

3 GENOTYPES POSSIBLE

REASSORTANTS OF

BISEGMENTED VIRUS

1ST PARENT X 2ND PARENT

A a

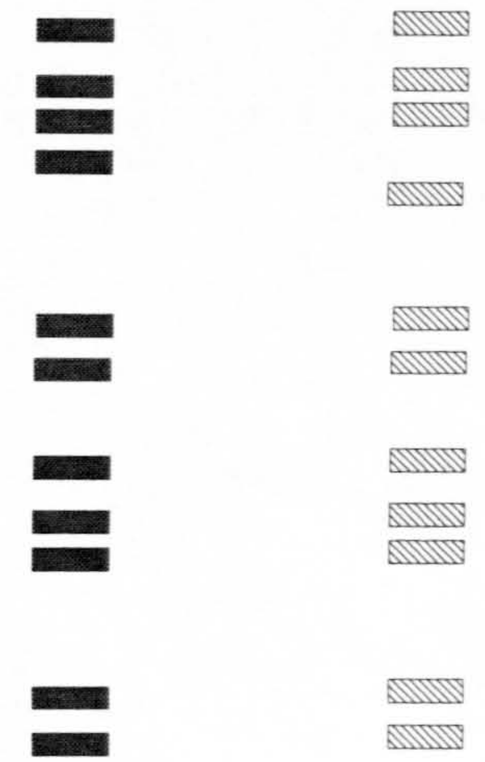
B b

2^2 = 4 GENOTYPES POSSIBLE

(AB ab Ab aB)

ROTAVIRUS REASSORTANTS

1ST PARENT X 2ND PARENT



2^{11} = 2048

GENOTYPES POSSIBLE

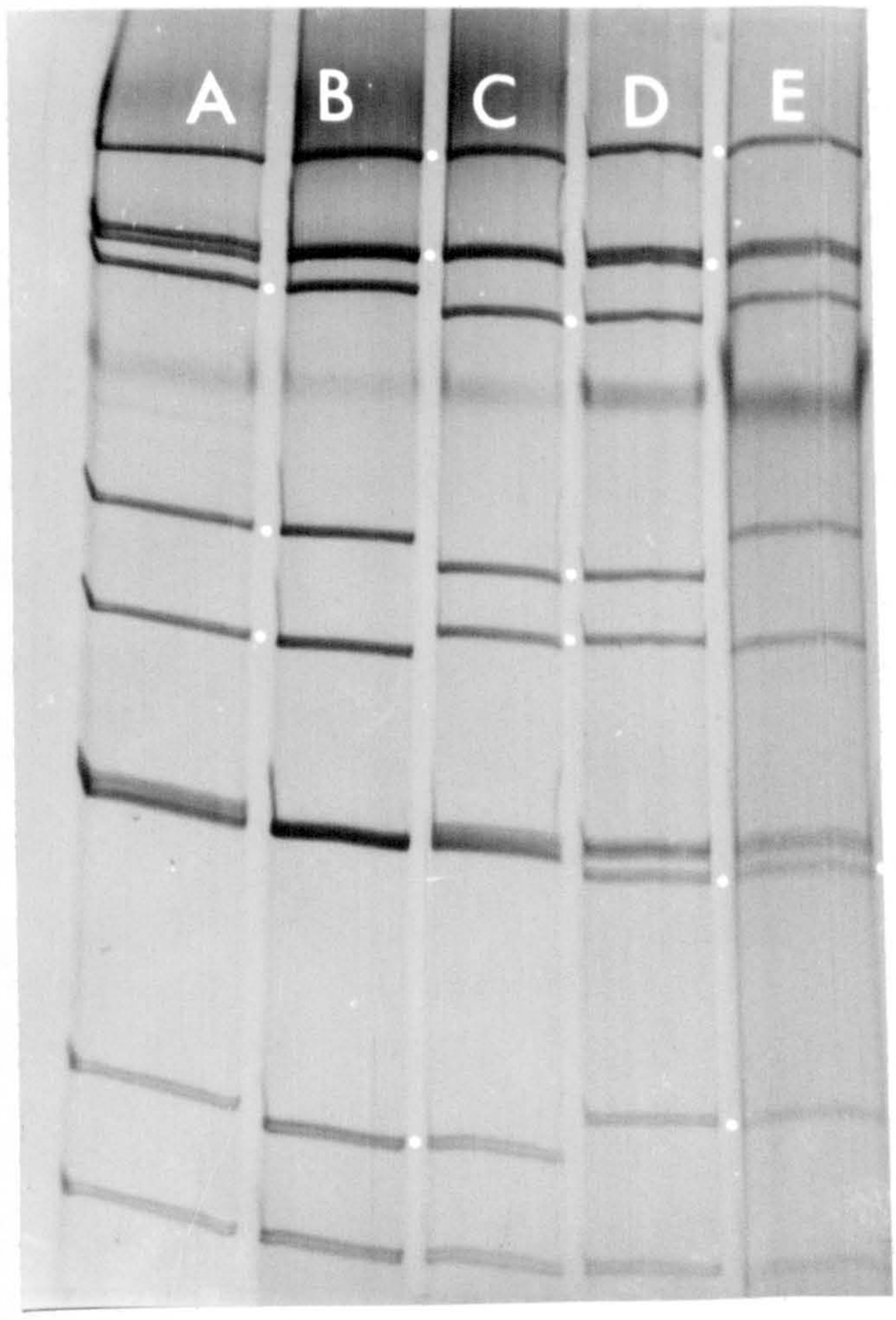


Figure 34 : Genotyping of rotavirus reassortants by polyacrylamide gel electrophoresis and silver staining.

BSC-1 cells were infected with various rotavirus strains or reassortants. Viral RNA was extracted by the optimised method from infected BSC-1. Viral dsRNA was then detected by silver staining following fractionation by electrophoresis on a 6% polyacrylamide gel as described in Materials and Methods. The parental origin of the gene segments in reassortants is indicated by a white dot on the side of parent. The gene segments are indicated on the left.

Lane A = UKtc; lane B = RS3; lane C = OSU; lane D = RS1; lane E = B223.

of 110 plaques picked in a cross between UKtc and OSU, 4 reassortants were isolated (Table 8). All the reassortants obtained from UKtc and OSU cross had the same profile (RS3). The genotype of the reassortants is shown in Table 9, and Figure 35. Most of the progeny clones showed UKtc RNA profile. Out of 43 progeny clones between OSU and B223 cross, 32 progeny clones showed OSU RNA profile, 7 showed B223 profile, while the remaining 4 were reassortants (Table 8). The genotype of three of these reassortants (RS1, RS4, and RS5) is shown in Table 9, and figure 35, fourth having a genotype identical to RS1.

Multiple passages of coinfected cultures have been shown to result in large scale production of reassortants in the progeny by replacement of at least one segment from each parent (Ward *et al.*, 1988a). Another experiment was conducted in which the coinfected material from first passage was given six further blind passages. Then plaques were picked, propagated, and characterised as described above. No reassortant virus was isolated in any of the crosses. Again, UKtc was found in majority of the clones when it was one of the coinfecting parent, whereas OSU was dominant in the cross between OSU and B223 (Table 8). Since only a few reassortants could be isolated, it was considered necessary to apply some sort of external pressure for the generation of reassortants.

7.4.2 Reassortant construction by differing multiplicity of infection

Multiplicity of infection (m.o.i.) has been shown to affect the frequency of reassortment (Gombold and Ramig, 1989). Also, changes in the relative m.o.i. of the coinfecting viruses have been found to alter the selection of certain segments in reassortants (Ward *et al.*, 1988a). In the

Table 8 : Summary of electropherotypes of progeny clones obtained by various methods.

			No. of clones with following electropherotypes			
Methods	Parents	No. of clones	UKtc	OSU	B223	Reassortant
No selection	UKtc x OSU[a]	110	104	2		4
	UKtc x B223[a]	97	95		0	2
	B223 x OSU[a]	43		32	7	4
	UKtc x OSU[b]	20	19	1		
	UKtc x B223[b]	20	20		0	
	B223 x OSU[b]	20		18	2	
Differing m.o.i.	UKtc x OSU	58	51	7		
	UKtc x B223	46	45		1	
	B223 x OSU	23		18	5	
Superinfection	UKtc x OSU	42	31	11		
	UKtc x B223	33	29		4	
	B223 x OSU	18		13	5	
Plaque on MA104 cells	UKtc x OSU	30	29	1		
	UKtc x B223	30	30		0	
In vivo	UKtc x OSU	24	22	2		
	UKtc x B223	26	26		0	
	B223 x OSU	18		18	0	
Reassortant as parent	RS6 x OSU	19				19[c]

[a]passage one used for genotyping; [b]passage seven used for genotyping
[c]preformed reassortant parent

Table 9 : Genotypes of rotavirus reassortants

			Parental origin of gene segments[a]										
Reassortant	Lab number	Parents	1	2	3	4	5	6	7	8	9	10	11
RS1	1OB1.A5	OSU x B223	B	X	B	O	O	O	X	B	B	B	X
RS2	UB.A4.L4	UKtc x B223	U	X	X	U	U	U	S	U	S	U	X
RS3	UO.P2.E8	UKtc x OSU	O	X	O	U	U	U	S	U	S	O	X
RS4	1OB1.D6	OSU x B223	B	X	B	O	O	B	X	B	B	B	X
RS5	1OB1.D7	OSU x B223	B	X	B	O	?	B	X	B	B	B	X
RS6	UB.B3.F1	UKtc x B223	U	X	X	U	U	U	U	B	B	U	X
RS7	UB.A1.8	UKtc x B223	U	X	X	U	U	U	U	U	B	U	X
RS8	UO.M.A8	UKtc x OSU	U	X	U	O	U	U	X	U	X	U	X
RS9	OB.M.H1	OSU x B223	B	X	B	O	O	B	X	B	B	O	X
RS10	UO.M.A7.3	UKtc x OSU	U	X	U	O	U	U	S	U	S	O	X
RS11	UB.A1.4	UKtc x B223	U	X	X	U	U	U	B	U	U	U	X
RS12	UO.M.A4.1	UKtc x OSU	O	X	O	O	O	U	S	U	S	O	X
RS13	UO.M.A4.3	UKtc x OSU	U	X	U	O	U	U	X	U	X	U	X
RS14	OB.M.H11.2	OSU x B223	B	X	B	O	O	B	X	B	B	O	X
RS15	UO.M.B4.6	UKtc x OSU	U	X	U	O	O	U	X	U	?	O	X

[a] Reassortant gene segment assignments were determined by comparison of gene segment migration in PAGE-SS and restriction enzyme digestion analysis of amplified RT-PCR product. B = B223; O = OSU; U = UKtc; S = single band triplet; ? = migration of segment like neither parent; X = indistinguishable in PAGE-SS

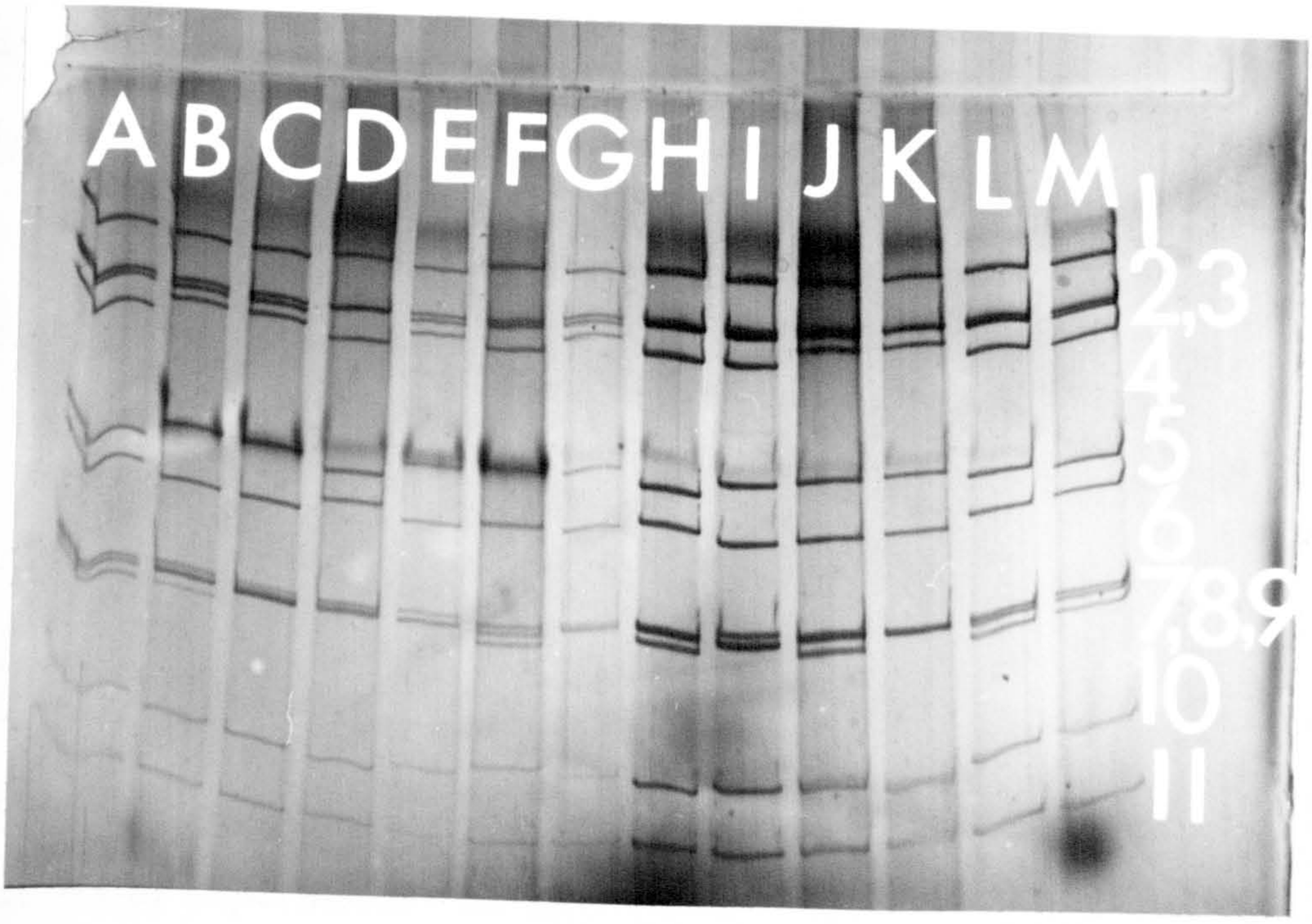


Figure 35 : Electrophoretic profile of genomic RNA of rotavirus reassortants separated on polyacrylamide gel and silver stained.

BSC-1 cells were infected with various rotavirus strains or reassortants. Viral RNA were extracted by optimised method from BSC-1 cells infected with various strains of rotavirus. Viral dsRNA were then detected by electrophoresis on a 6% polyacrylamide gel and silver staining as described in Materials and Methods. The gene segments are indicated on the right.

Lane A = RS1; lane B = RS7; lane C = RS12; lane D = OSU; lane E = UKtc; lane F = B223; lane G = RS2; lane H = RS9; lane I = RS8; lane J = RS6; lane K = RS3; lane L = RS5; lane M = RS4.

previous experiments, coinfections were performed using equal m.o.i. for each virus strain within a pair. In this set of experiments, the m.o.i. of UKtc was reduced to 0.1 pfu per cell involving crosses where UKtc was one of the coinfecting parent. The m.o.i. of 0.1 pfu per cell of OSU was used in crosses between OSU and B223 strains. No reassortants were identified among 127 plaques picked between various pairs of coinfecting viruses (Table 8). The UKtc was still found in most of the progeny clones when it was one of the coinfecting parent. However, there was slight increase in percentage of the progeny showing B223 RNA profile in OSU x B223 cross.

7.4.3 Reassortant construction by superinfection

Non-dominant parent in a coinfecting pair was superinfected to give it growth advantages to increase the probability of dual infection of the same cell, thereby increasing the chances of reassortants formation. In another set of experiments, the parents were coinfected at equal m.o.i. of 5 pfu per cell. However, after incubation at 37°C for 3 hr, the BSC-1 cells were infected again with 10^6 pfu of B223 in crosses involving B223 as one of the parent, and with 10^6 pfu of OSU in UKtc x OSU cross. No reassortant was identified out of 93 progeny clones derived from various crosses. However, following superinfection the relative % OSU and B223 detected in the progeny clones slightly increased compared to the construction method where no selection pressure was applied (Table 8).

7.4.4 Reassortant construction in MA104 cells

The results from the above studies showed that reducing the m.o.i. of UKtc or superinfecting the non-dominant parent can slightly increase the % progeny showing their genotypes. It indicated that UKtc had some growth advantages in BSC-1 cells. Because, UKtc had routinely been

grown in BSC-1 cells in our lab for the last 14 years, whereas the OSU or B223 strains were not grown that frequently. Assuming this to be the case, a different cell line, MA104, was chosen for coinfection as well as for picking progeny clones. MA104 cells were coinfected by different pairs of viruses at m.o.i. of 5 pfu per cell. The plaques were picked, propagated, and characterised. The UKtc was found in almost all the progeny clones (Table 8).

7.4.5 *In vivo* reassortment

Reassortants have been constructed by coinfecting mice with two different rotavirus strains (Gombold and Ramig, 1986; 1989; Broome *et al.*, 1993) and then picking the plaques from a cell line that has been inoculated with intestinal homogenate from the coinfected mice. Seven day old seronegative suckling CD-1 pups were orally inoculated with $5x10^6$ pfu of each virus in a pair. At 24 hr p.i. all the pups were sacrificed and the entire intestines were removed. The intestines were frozen, thawed, minced, homogenised and sonicated. The intestinal homogenates from pups mixedly infected with various pairs of rotavirus were inoculated onto BSC-1 cell monolayer in a dilution to give well isolated plaques. The plaques were picked, propagated, and characterised as described in section 7.4.1. No reassortant was identified in any of the crosses. UKtc was found in the majority of the progeny clones (Table 8).

7.4.6 Reassortant construction by using a preformed reassortant as one parent

The reassortant virus RS6 was used as one of the parents in a cross with OSU strain. The progeny plaques all belonged to the reassortant parent genotype (Table 8).

7.4.7 Reassortant construction under antigenic selection pressure

The passage one coinfected material (described in section 7.4.1) was incubated with 1:100 dilution of monoclonal antibody or monospecific antiserum, either singly or in a mixture as shown in Table 10 before being inoculated onto BSC-1 cell monolayers. In addition the monoclonal antibody or the antiserum was also added in the agar overlay. The monoclonal antibody #5632 (α UKtc VP7) had a neutralisation titre (reciprocal) of >2560 against homologous UKtc, whereas the monoclonal antibody # 4907 (α OSU VP7) had a neutralisation titre (reciprocal) of 1280-2560 against homologous OSU strain of rotavirus. The antiserum # 5925 (α UKtc VP4) had a neutralisation titre (reciprocal) of 160 against homologous UKtc. A few reassortants were identified. The number of various electropherotypes obtained is shown in Table 10.

All of the above experiments were conducted after activating the viruses with trypsin and also incorporating trypsin in the medium. Another set of experiments was conducted in which the trypsin was present only in the agar overlay medium. The coinfected virus inoculum was incubated with appropriate monoclonal antibodies (α UKtc VP4), which were also incorporated in the medium. The rationale was that only UKtc can form plaques in the absence of trypsin (McCrae and Faulkner-Valle, 1981) and the use of antibodies against VP4 of the UKtc would increase the chances of reassortant formation, as VP4 has been shown to be responsible for protease enhanced plaque formation (Kalica *et al.*, 1983). The progeny clones were picked, propagated, and characterised by usual methods. No reassortant was isolated. The summary of the various electropherotypes obtained is shown in table 10.

Table 10 : Summary of electropherotypes of clones derived under antigenic selection pressure[a]

Parents	Antibodies	No. of clones	No. of clones with following electropherotypes			
			UKtc	OSU	B223	Reassortant
UKtc x OSU	α UKtc VP7	75	21	53		1
UKtc x OSU	α UKtc VP4	84	25	57		2
UKtc x B223	α UKtc VP7	67	62		2	3
UKtc x B223	α UKtc VP4	48	43		5	
UKtc x OSU	α OSU VP7	38	35	1		2
OSU x B223	α OSU VP7	25		19	4	2
UKtc x OSU	α UKtc VP7 + α UKtc VP4	26	9	17		
UKtc x OSU	α OSU VP7 + α UKtc VP4	27	20	5		2
UKtc x OSU[b]	α UKtc VP4	20	20	0		

[a]The passage one coinfected material was incubated with 1:100 dilution of monoclonal antibody or monospecific antiserum, either singly or in a mixture before being plated onto BSC-1 cells. The monoclonal antibody or monospecific antiserum were also added in the agar overlay. Plaques were randomly picked and the RNA profile determined by PAGE-SS.

[b] trypsin was not used at any stage

7.5 Genotypes of various reassortants

A total of 22 reassortants were identified out of 1068 progeny clones picked by various methods. The 22 reassortants belonged to 15 different genotypes (Table 9, Figure 35). Four different genotypes, resembling the genotypes of the reassortants, RS1, RS3, RS6, and RS8 were isolated more than once. The reassortants RS2, RS3, RS10, and RS12 had a single band at the triplet. The origin of the VP7 gene of these reassortants was determined by restriction enzyme analysis of amplified cDNA copies in RT-PCR.

In addition to the electropherotypes of the clones shown in tables 8 and 10; 56 progeny showed 12 or more segments and 63 progeny clones did not show any band in the PAGE-SS.

7.6 Discussion

Three different rotavirus strains UKtc, B223, and OSU were used in pairs to construct rotavirus reassortants. Initially, no external pressure was exerted for the generation of reassortants. Ten reassortants were identified in more than 300 progeny clones. Four of these had identical genotype indicating that they may be the product of the same cell. Majority of the progeny showed UKtc parental genotype when it was one of the parents in the cross. OSU parental genotype was isolated in majority of the progeny in crosses between OSU and B223. Even after six blind passages, no more reassortants were identified in the progeny clones. The UKtc was dominant over OSU and B223, whereas OSU was dominant over B223 parent. It was therefore, thought to apply some kind of external antigenic or non-antigenic selection pressure to suppress the growth of the dominant parent. Temperature-sensitive mutants have been used in some of the early rotavirus reassortant experiments (Greenberg *et*

al., 1981; Kalica *et al.*, 1983). The genetic selection pressure, in the form of using a ts mutant of the dominant parent and then selecting the progeny at non-permissive temperature, was not used in the present study because of three reasons. First, it has been reported that the ts mutant interferes with the growth of wild-type virus (Ramig, 1983). Second, because all rotavirus ts mutants have been selected following mutagenesis of wild-type virus, the potential exists for the presence of a non-temperature-sensitive mutation in these mutants and the associated problems of unexpected phenotypes with non-temperature-sensitive mutation (Rubin and Fields, 1980). Third, non-availability of ts mutants for many of the gene segments of OSU, and B223 strains.

Reducing the m.o.i. of the dominant parent slightly increased the percentage of the other parent genotype in the progeny clones. However, percentage of the progeny showing the genotype of the dominant parent was still very high. Moreover, no reassortants could be identified in the progeny clones. Superinfection with the non-dominant parent in a cross slightly increased progeny showing its genotype but did not tilt the percentage in its favour. However, no reassortant could be identified and the progeny clones showing the genotype of the dominant parent was still high. Rotavirus reassortants have been isolated by superinfecting with a second virus 24 hr after the first infection (Ramig, 1990).

UKtc was still found in all the progeny clones even in MA104 cells. Host cell factors have been shown to play a significant role in selecting reassortants and parents even under conditions of single growth cycle and high m.o.i. of both parents (Graham *et al.*, 1987). In crosses between UKtc and a human rotavirus with rearranged segments, UKtc was isolated in 7.4% plaques on BSC-1 cells and 34.3% on MA104 cells in the above studies conducted by Graham *et al.*, (1987). The human rotavirus did not form any plaques on any cell lines and the remaining

were reassortants. They routinely used MA104 cells for growing their rotaviruses. Host cells were also shown to influence the origin of the segments from the parents in the reassortants.

No reassortants could be isolated in *in vivo* experiments. Others have been successful in isolating reassortants in *in vivo* experiments (Gombold and Ramig, 1986; 1989; Broome *et al.*, 1993). During the course of development of an animal model in the present study, very little infectious virus was isolated on most occasions 12 hr p.i. Gombold and Ramig, (1986) have reported that all clones were reassortants at 96 hr p.i. even though very little infectious virus was detected at that time by them.

A method used to force selection of reassortants with desired genome segments which code for specific VP4 or VP7 proteins has been the treatment of the progeny of coinfection with monoclonal or polyclonal antibodies directed at the neutralisation proteins of coinfecting strains (Greenberg *et al.*,, 1981; Midthun *et al.*, 1985; 1986; Urasawa *et al.*, 1986; Hoshino *et al.*, 1987b; Broome *et al.*, 1993). In the present study, the antigenic selection pressure resulted in the production of a few more reassortants. The percentage of the OSU genotype found in the progeny clones was higher than UKtc in the coinfection which were treated with antibodies specific for VP4 and/ or VP7 of UKtc and which were also included during plaque picking. However, percentage of the progeny clones showing B223 genotype did not increase dramatically when antibodies against the other coinfecting parent were used. The basis of this is not known. The progeny clones showing parental genotype against which the antibodies were used appear to be the breakthrough plaques and not neutralisation escape mutants. Because, some of them when tested by PRN were neutralised by the homologous antibodies. The titre of antibodies used for giving antigenic selection pressure may not have been high enough.

In some experiments where antibodies to VP4 and VP7 of UKtc were used in combination also gave similar results to those obtained when they were used singly i.e. percentage of the progeny showing OSU genotype was found to be higher than UKtc. However, when antibodies to OSU VP7 and UKtc VP4 were used simultaneously in OSU and UKtc crosses, more UKtc genotype was found in the progeny clones.

The UKtc strain can form plaques even in the absence of trypsin (McCrae and Faulkner-Valle, 1981) whereas B223 and OSU strains can not form plaques in its absence. The UKtc has been reported not to form plaques in the absence of trypsin (Kalica *et al.*, 1983). In all of the above experiments in the present study, the viruses were treated with trypsin prior to inoculation onto the cells and trypsin was also incorporated in the overlay medium. Another set of experiments was conducted in which trypsin was not used at any stage except in the agar overlay medium during plaque picking. However, the coinfected material was incubated with the antibodies to VP4 of UKtc which were also incorporated in the overlay medium. It was hoped that all the progeny plaques would contain VP4 of OSU. However, no reassortants were isolated.

In another experiment, one preformed reassortant from the present study was used as one of the parents in a cross with OSU. However, only the reassortant parent genotype was observed in the progeny clones indicating that it is a stable reassortant.

In the present study, only about 2% progeny clones out of more than 1000 plaques, picked from different methods of reassortant construction, were reassortants. Reassortant frequency varying from 0% to 100% in rotaviruses has been documented. Formation and detection of reassortants is probably most efficient when the coinfecting strains are closely related. This is expected since the genome segments or the proteins they encode must function together. Studies with influenza

viruses have indicated that more closely the gene products are related, the more likely a replacement will be tolerated (Klenk and Rott, 1988). In rotaviruses, no reassortant has been isolated between rotaviruses belonging to different rotavirus groups (Yolken *et al.*, 1988). The progeny of coinfection between subgroup I and subgroup II rotavirus strains yielded comparatively smaller % reassortants, <1% (Urasawa *et al.*, 1986), <3.5% (Garbarg-Chenon *et al.*, 1984), or 14% (Ward and Knowlton, 1989). In contrast, coinfection with cultured cells with different pairs of subgroup II human rotaviruses resulted in high % (33-48%) of plaque picked colony being a reassortant (Ward *et al.*, 1988a). The reassortant frequency observed in the present study is very low even though the pairs of viruses used belonged to subgroup I.

Reassortant frequency, between two serotype G3 strains, of 5% (Broome *et al.*, 1993), and 38-100% (Gombold and Ramig, 1986) have been reported from *in vivo* crosses. No reassortant could be isolated from the *in vivo* crosses. It has been reported that no reassortant was detected among progeny clones examined from mixedly infected, homotypic immune mice. Whereas about 11% were identified among progeny clones from mixedly infected, heterotypically immune mice. Reassortment was reduced more than 50-fold by homotypic immunity and approximately three fold by heterotypic immunity relative to the frequency observed in non-immune mice (Gombold and Ramig, 1986; 1989). The immune status of the pups cannot be the reason for not getting any reassortant, as the *in vivo* crosses were performed in seronegative animals. Reduced m.o.i. has been shown to affect the frequency of reassortment *in vivo* (Gombold and Ramig, 1989). However, sufficiently high m.o.i. of each parent was used in the present study in our *in vivo* crosses.

Multiple blind passages have been shown to increase the reassortant frequency (Ward *et al.*, 1988a) or replace the preformed

reassortants by one or the other parent when passaged in the presence of their parental viruses (Ward and Knowlton, 1989). However, the results of passage one and passage seven were not different in the present study.

The reason for finding very less number of reassortants even under antigenic selection pressure could be due to the low neutralisation titre of the antibodies used. In the present study antibodies had been used both prior to inoculation onto the cells and in the overlay medium. It has been reported that addition of antibody to the progeny of coinfection prior to inoculation could result in the loss of most desired reassortants (Ward *et al.*, 1988b). It is not known if that could be the reason for low reassortant frequency. It is also not known if reassortant exclusion occurred between the parents used. Superinfection exclusion and prevention of reassortment has been reported for blue tongue virus (Ramig *et al.*, 1989; El Hussein *et al.*, 1989). Finally, the author is not aware of any report in which pairs used in the present study have been used by others for construction of reassortants. Although they have been used as a parent independently with some other parent not used in the present study for reassortant construction e.g. B223 (Chen *et al.*, 1989), OSU (Hoshino *et al.*, 1987), and UKtc (Greenberg *et al.*, 1981; Kalica *et al.*, 1983; Midthun *et al.*, 1985; 1986).

Some of the other reasons of getting only 2% reassortants may be the inability to pick up the right kind of plaque, or the reassortants were never formed or they were formed but did not form a plaque because they were non-infectious or segments from one of the parents were selected because they are dominant as a group.

The plaque size and morphology has been used for characterisation of rotaviruses (Kalica *et al.*, 1983; Chen *et al.*, 1989). However, different strains could not be differentiated on that basis, though the plaques formed by UKtc were more sharp.

In addition to the electropherotypes of the clones shown in tables 8 and 10; 56 progeny showed 12 or more segments and 63 progeny clones did not show any band in the PAGE-SS. However, they were excluded while calculating the reassortant frequency. Most of the PAGE-SS negative clones belonged to the category where no CPE or very little CPE was observed at the end of 5 day incubation at 37^{o}C in 24 wells. One reason can be that the plaques were missed while picking from the agar overlay. However, some of these PAGE-SS negative clones were positive for VP7 amplification in RT-PCR. The RT-PCR has been reported to be 10^5 times more sensitive than PAGE-SS for detection of rotavirus (Xu *et al.*, 1990). A few of them showed rotavirus specific RNA profile in PAGE-SS after three passages in BSC-1 cells. However, no attempt was made to propagate the remaining PAGE-SS negative clones as the main aim was to screen large number of clones. Therefore, there is a chance that some of these PAGE-SS negative clones might have turned out to be a reassortant when tested later after growing them to higher titres. PAGE-SS negative clones have been reported by others also (Gombold and Ramig, 1986).

Isolates containing more than 11 segments have been reported (Lourenco *et al.*, 1981; Spencer *et al.*, 1983; Garbarg-Chenon *et al.*, 1984; Nicolas *et al.*, 1984; Gombold and Ramig, 1986). These may represent progeny of two different clones or an intermediate step in the establishment of a stable reassortant (Garbarg-Chenon *et al.*, 1984). Some of the progeny clones containing more than 11 segments were used to pick progeny clones. Some of the reassortants were obtained in this way.

The number of reassortants obtained is very small to apply some statistics. However, a few points are obvious. Although gene 4 has been related to growth restriction of rotaviruses in cultured cells (Greenberg *et*

al., 1983a), it did not always come from the better growing parent. The gene 4 from OSU was present in all the reassortants except one, in UKtc x OSU cross, although UKtc was observed to be the dominant and better growing parent. Similar findings have been reported by others (Ward *et al.*, 1988a). Gene segment 4, and 5 never came from B223, however, gene 5 was derived almost equally from UKtc or OSU. The VP7 gene never came from OSU. Among the remaining separable segments, the segments from all three parents were represented at least once. The origin of the VP7 gene of reassortants that had a single band at the triplet was determined by restriction enzyme analysis of amplified cDNA copies in RT-PCR. The reasons of migration of the segments 7 and 9 different than their parents could be due to the rearrangement phenomenon (Tian *et al.*, 1993) and needs further investigations. Reassortment is a random process. However, non-random selection of single gene as well as gene association have been reported (Gombold and Ramig, 1986; Graham *et al.*, 1987; Ward *et al.*, 1988a).

7.7 Conclusions

Very few rotavirus reassortants could be identified between the pairs of rotaviruses used to generate them indicating that reassortment is a random process and the reassortment frequency depends upon the parents used.

Chapter 8

Mapping of rotavirus virulence to its gene(s)

8.1 Introduction

The 22 monotypic reciprocal reassortants that had been sought as a first step to segregate rotavirus virulence to individual gene(s) in animal studies could not be obtained, from any of the crosses with three different pairs of rotavirus parents. Despite genotyping more than 1100 progeny clones, it was possible to identify only 22 reassortants, and these belonged to 15 different genotypes (Table 9; chapter 7). Published reports on rotavirus virulence are scanty, the VP4, or VP5 are considered to be important candidates in those reports. Therefore, reassortants containing VP4, VP5, or VP7 genes were selected for virulence mapping studies, which are described in this chapter.

8.2 Results

The reassortants, RS1, RS2, RS3, RS4, RS5, RS6, RS8, RS10, and RS12, were chosen to conduct the virulence mapping studies. The genotype of these reassortants can be seen in Table 9 of chapter 7. The reassortants were purified plaque to plaque three times in BSC-1 cells before being grown into high titre stocks for use in the virulence studies. Seronegative litters of 7 day old suckling CD-1 pups were orally inoculated with 10^7 pfu per pup of the reassortant viruses or control preparations derived from uninfected cells administered in an equal volume. At various times post inoculation the animals were examined for the clinical signs of the diarrhoea. At each time point a pair of pups was sacrificed and examined for internal signs of disease. Also, the entire gut was aseptically collected in PBS for infectivity titration and determination of RNA profile.

The induction of the diarrhoea by three different reassortants RS1, RS4, and RS5 obtained from the OSU x B223 cross, was similar to that of OSU parent. These three reassortants had the gene 4 from OSU parent

on a B223 background. In addition to the OSU gene 4, the RS4 had the OSU gene 5, and reassortant RS1 had both genes 5 and 6 from OSU on B223 background. The onset of diarrhoea occurred at 24 hr p.i. and reached a peak at 36 hr p.i. in RS1, and on day 2 in other two reassortants (Figure 36), when 100% pups showed overt (+3) diarrhoea. The diarrhoea observed was bright yellow and opaque. Post mortem revealed distension of the colon and yellow coloured liquid faeces. The duration of diarrhoea observed with all three reassortants was four days, i.e. from day 1 through day 4 p.i. The RNA profile of all the three reassortants determined from the intestinal homogenates of pups sacrificed on day 2 either directly or after passaging once in BSC-1 cells, was identical to the respective inoculated reassortant profile (data not shown).

The induction of diarrhoea by reassortants derived from OSU x UKtc crosses is shown in Figure 37. The diarrhoea induced by the reassortants containing gene 4 of OSU on UKtc background (RS8, RS10) or OSU gene 4 on OSU background with VP6 and VP7 from UKtc (RS12) was similar to the OSU strain. The diarrhoea started at 24 hr p.i. and reached a peak at 36 hr p.i. The duration of diarrhoea was three days in RS10 or four days (in RS8, and RS12). Overt diarrhoea (+3) was observed on day 2 and day 3, and diarrhoea of considerable severity (+2) was observed on day 1 and day 4. The colon was distended containing liquid faeces. However, in the reassortant RS3 which had VP4, VP7 and gene 5 from UKtc on the OSU background behaved like UKtc parent in its induction of diarrhoea in CD-1 suckling pups. The colon was less distended and contained semiformed faeces. The RNA profile of the inoculated material and of the RNA obtained from day 2 intestinal homogenates was identical for each reassortant virus (data not shown).

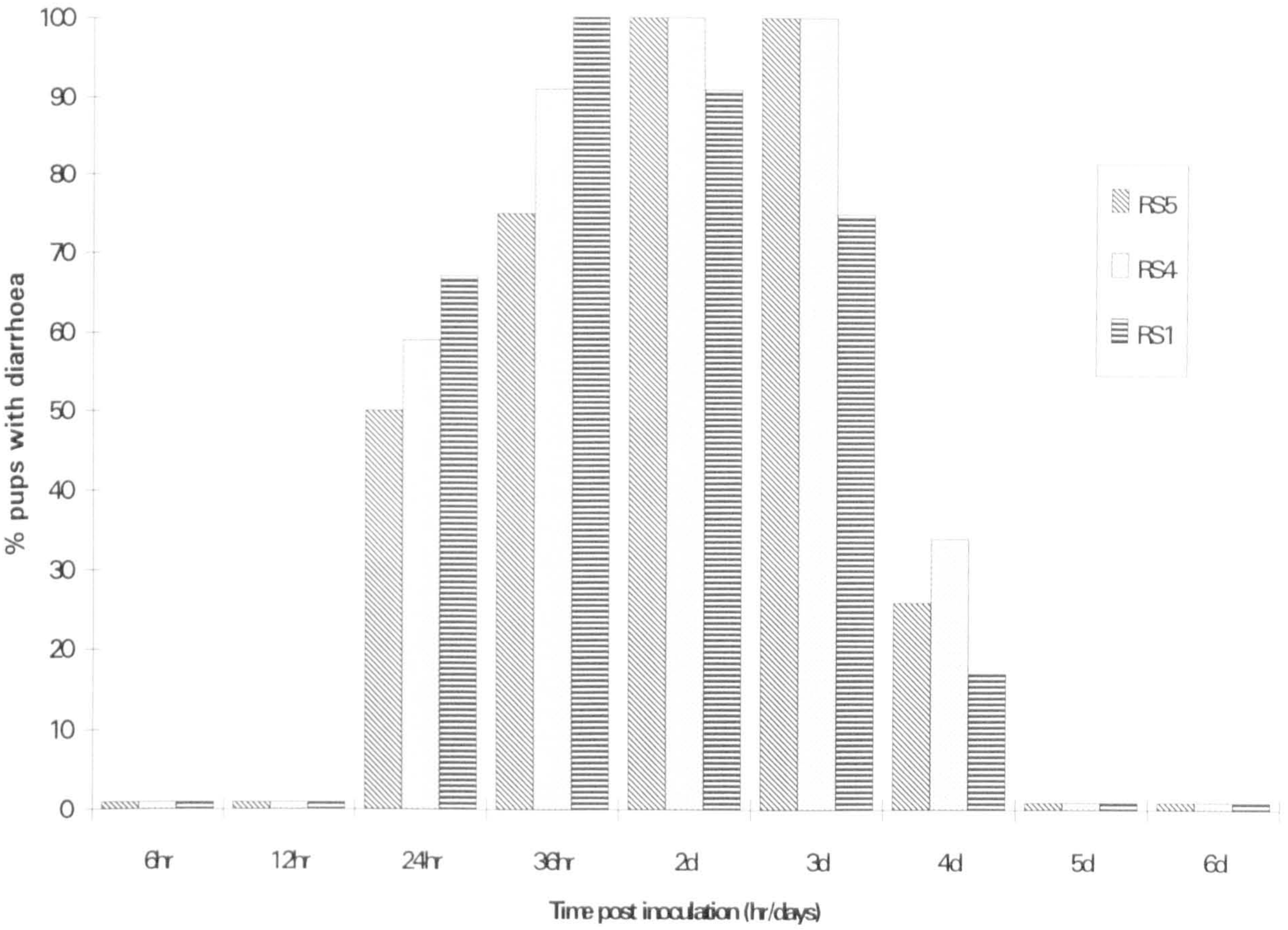


Figure 36 : Diarrhoea produced by rotavirus reassortants derived from OSU x B223 cross.

Seronegative 7 day old suckling CD-1 pups were orally inoculated with 10^7 pfu of various rotavirus reassortants derived from OSU x B223 cross on day 0. At various times post inoculation over the next 6 day period, the animals were examined for the clinical signs of diarrhoea. At each time point post inoculation a pair of pups was sacrificed and examined for internal signs of disease. Plotted are the mean from 2 experiments and in each experiment n = 5-8. The standard deviation for various values ranged from 0-7.6.

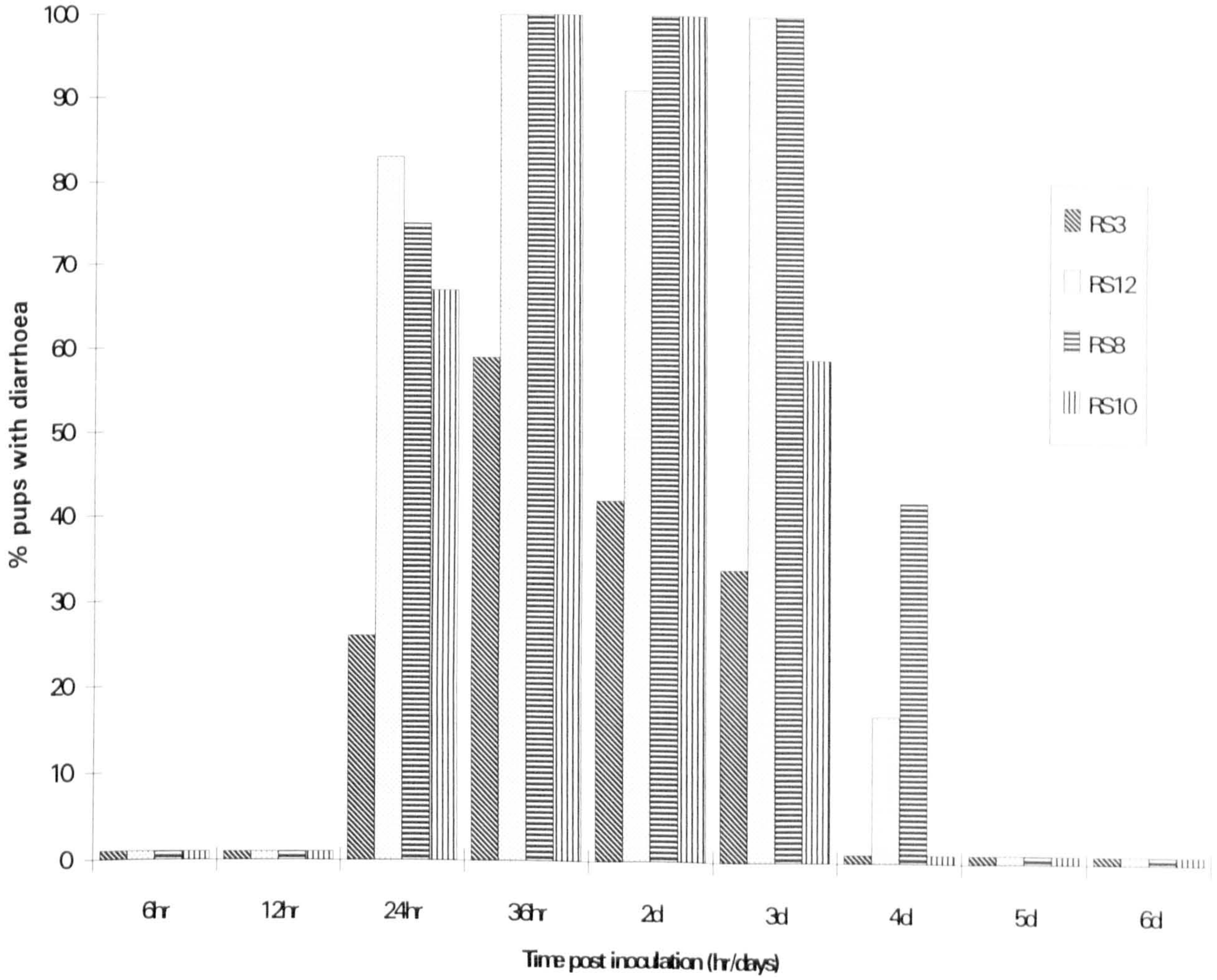


Figure 37 : Diarrhoea produced by rotavirus reassortants derived from OSU x UKtc cross.

Seronegative 7 day old suckling CD-1 pups were orally inoculated with 10^7 pfu of various rotavirus reassortants derived from OSU x UKtc cross on day 0. At various times post inoculation over the next 6 day period, the animals were examined for the clinical signs of diarrhoea. At each time point post inoculation a pair of pups was sacrificed and examined for internal signs of disease. Plotted are the mean from 2 experiments and in each experiment n = 5-8. The standard deviation for various values ranged from 0- 6.4.

The diarrhoea induced by the reassortants from the B223 x UKtc cross was similar to the UKtc parent (Figure 38). The RNA profile of the inoculated material and that obtained from the intestinal homogenates at day 2 was identical to respective reassortant (data not shown).

The infectivity titration with all the reassortants revealed that the inoculated material rapidly disappeared from the gut. No replication of the virus was observed though a minor increase was observed at 12 hr p.i. or 24 hr p.i. similar to that observed with parent strains (data not shown).

8.3 Discussion

Analysis of co-segregation of genome segments with biological phenotype in reassortants has been the method of choice for mapping gene function in viruses with segmented genomes. In these studies the phenotype has generally been seen to segregate with one segment or some constellations of genome segments. Reassortants are generated between two parent viruses by coinfection and the parental origin of gene is determined. If a group of reassortants are examined that all possess the phenotype of interest, the genome segment responsible for that phenotype must be present in all the reassortant progeny clones and the parent which show that phenotype. Conversely, a group of reassortants lacking that phenotype will always derive one segment from the parental virus that did not display that phenotype. Two requirements must be met if the segregation analysis is to successful. First, the phenotype of the parental viruses must be distinguishable. This was established in chapter 5. Second, the genotype of the parents be different. This was established in chapter 6.

The desired 22 monotypic reciprocal reassortants could not be obtained, from crosses with three different pairs of viruses, for carrying

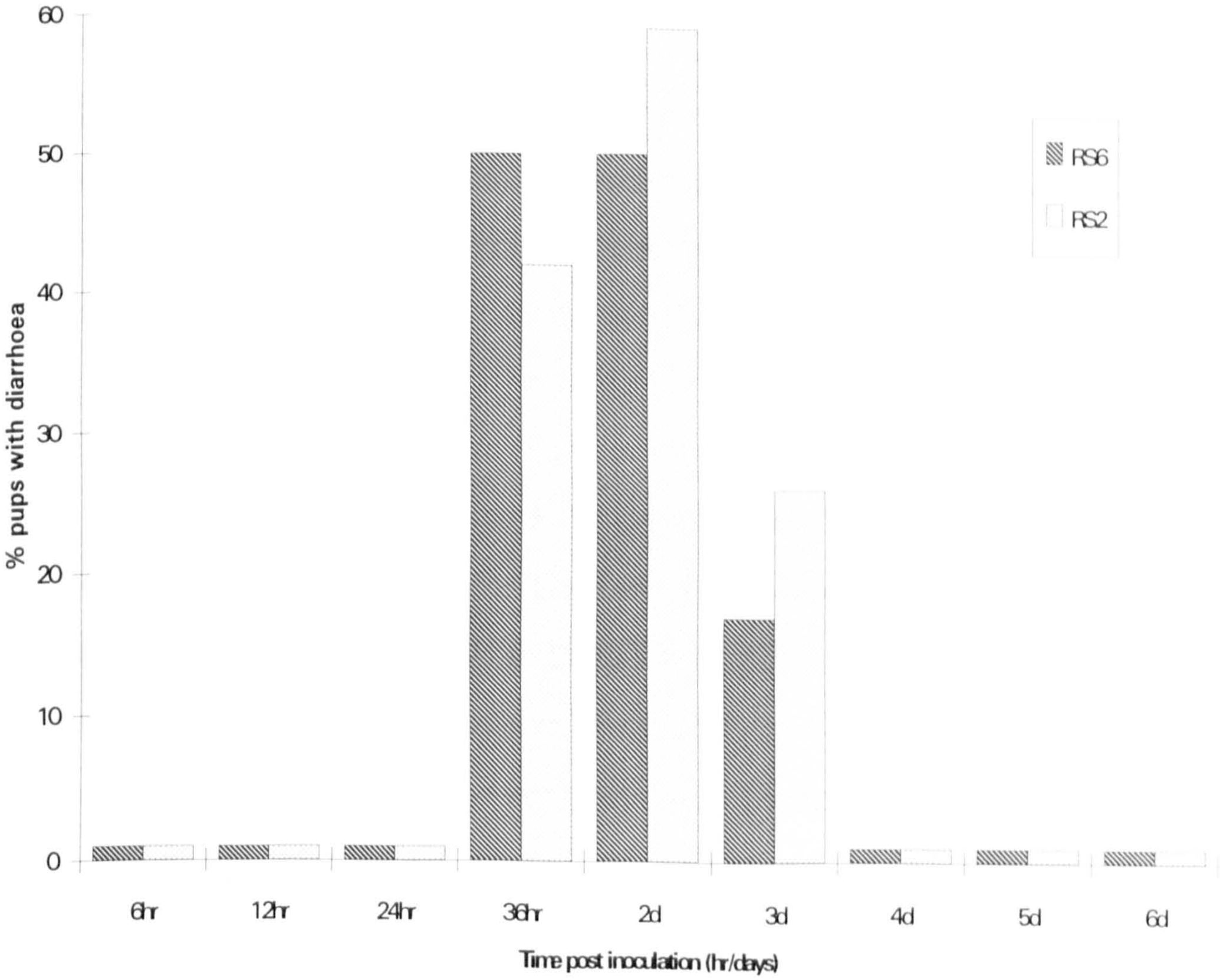


Figure 38 : Diarrhoea produced by rotavirus reassortants derived from UKtc x B223 cross.

Seronegative 7 day old suckling CD-1 pups were orally inoculated with 10^7 pfu of various rotavirus reassortants derived from UKtc x B223 cross on day 0. At various times post inoculation over the next 6 day period, the animals were examined for the clinical signs of diarrhoea. At each time point post inoculation a pair of pups was sacrificed and examined for internal signs of disease. Plotted are the mean from 2 experiments and in each experiment n = 5-8. The standarddeviation for various values ranged from 0-4.2.

out a complete segregation analysis of virulence phenotype of rotaviruses. However, the construction of one set of 11 monotypic reassortants by substitution of one segment at a time from the avirulent DS-1 strain on the background of the virulent SB-1A rotavirus strain for virulence and immune response studies has recently been reported (Hoshino and Kapikian, 1994). In the absence of the desired 22 monotypic reciprocal reassortants, the reassortants carrying VP4, VP5, or VP7 substitutions were chosen for conducting virulence mapping studies.

The OSU strain was found to be more virulent than UKtc or B223 strains of rotaviruses (chapter 5). The OSU gene 4 was found to confer the virulence phenotype of OSU to B223 strain (Figure 36). The presence of gene 5 did not affect the virulence. Similarly, the OSU gene 4 on UKtc background segregated with the virulence phenotype of OSU, whereas the reassortant containing gene 4 from UKtc on OSU background behaved like UKtc parent (Figure 37). This reassortant also had gene 5, VP7 gene from UKtc. Another reassortant (RS12) which had only VP6 and VP7 from UKtc on OSU background behaved like OSU parent. This indicates that gene 4 of the OSU strain is heavily involved, if not wholly responsible for determining its virulence phenotype.

Rotavirus gene 4 has been associated with its virulence in animal studies (Offit *et al.*, 1986; Bridger *et al.*, 1992; Burke *et al.*, 1994a; 1994b; Hoshino and Kapikian, 1994; Ijaz *et al.*, 1994). The gene 4 has also been implicated with rotavirus virulence indirectly due to the observed differences in the gene 4 of rotaviruses isolated from symptomatic and asymptomatic children by electropherotyping (Parez-Schael *et al.*, 1984), RNA:RNA hybridisation (Flores *et al.*, 1986), and sequence analysis (Gorziglia *et al.*, 1986; 1988b). VP4 has also been found to have several biologically important properties that might be linked to virulence. The cleavage of VP4 by trypsin into VP5* and VP8*

enhances infectivity *in vitro* (Clark *et al.*, 1981; Espejo *et al.*, 1981; Estes *et al.*, 1981) by facilitating the entry of rotaviruses into the cells (Fukuhara *et al.*, 1988; Kaljot *et al.*, 1988). VP4 is the viral haemagglutinin, involved in neutralisation, growth restriction in cell culture, and is important in protease-enhanced plaque formation (Greenberg *et al.*, 1983a; Kalica *et al.*, 1983; Offit and Blavat, 1986).

However, genes other than gene 4 have also been shown to be associated with rotavirus virulence. The gene 3, gene 10, and the gene encoding VP7 have all been shown to play an independent role in virulence of a porcine rotavirus in its natural host (Hoshino and Kapikian, 1994). The VP4 and VP7 genes were not found to be primarily responsible for virulence in the mouse model using homologous murine rotavirus. Rather, gene 5 followed by gene 7 (encoding NS 35) were found to be the main determinants of murine rotavirus virulence. However, this association was not absolute, as many reassortants had gene 5 or gene 7 from murine rotavirus and yet behaved like simian parent used in these crosses (Broome *et al.*, 1993). This is in contrast to another recent study in which the murine rotavirus VP4 was reported to be associated with virulence (Ijaz *et al.*, 1994). Also, though the virulence was seen to segregate with four different genes independently in porcine rotavirus in its natural host, neither gene 5 nor gene 7 were found to be associated with virulence (Hoshino and Kapikian, 1994). Similarly, whereas single monotypic reciprocal reassortants of gene 4 from SA11 and NCDV rotaviruses were observed to be associated with virulence phenotypes of their respective parents, single monotypic reassortant containing gene 5 of NCDV did not confer it the virulence phenotype of NCDV rotavirus in mouse (Offit *et al.*, 1986). However, Broome *et al.*, (1993) observed that the reassortant containing gene 4 and gene 5 of RRV on murine rotavirus (EDIM-RW) background behaved like RRV, whereas

the reassortant containing gene 4 only from RRV behaved like murine rotavirus parent. Besides the association of single genes, a combination of two genes has also been reported to be statistically associated with virulence. Broome *et al.*, (1993) observed that the gene pair 5+2 to be strongly associated with virulence. This pair was found to be present in all fully virulent parents but was found to be absent in the intermediate reassortant which showed DD_{50} similar to EDIM-RW but did not spread as EDIM-RW. Another pair 5+7 was also found to be strongly associated with virulence. However, this pair was also found in the intermediate reassortant as well as many non-virulent reassortants.

Recently, while implicating gene 4 in the pathogenicity of a porcine rotavirus, it was reported that a cell culture grown porcine rotavirus became highly pathogenic during serial pig to pig passage due to a single aa change from a hydrophilic residue, glutamine to a hydrophobic residue, leucine at residue 469 (Burke *et al.*, 1994a). This virus was reported to be adapted to cell culture from the faeces of a diarrhoeic pig. The residue at 469 of gene 4 from the original faeces would have shown if it became attenuated during initial adaptation in cell culture. This is important because after pig to pig passage, the virus was readapted to cell culture and still retained the mutation at residue 469. It would be interesting to see if the readapted cell culture virus after retaining the mutation is still pathogenic to the same extent in piglets. This point mutation at residue 469 also implies that rotavirus causing the asymptomatic infection should have glutamine at residue 469 instead of leucine. However, this does not appear to be the case. Moreover, it has been reported that the VP4 of the standard strain of NCDV (Cody) which is virulent for cows differs in only one aa (23, Gln to Lys) from the VP4 of a NCDV mutant (RIT 4237, Lincoln) which is attenuated for both cows and children (Nishikawa *et al.*, 1988).

The reassortant containing VP7 from B223 on UKtc background (Figure 38) behaved like the UKtc parent. The VP7 of B223 has been shown to give replication advantages *in vitro* (Xu and Woode, 1994) and may affect the phenotype (Chen *et al.*, 1989), and antigenicity of VP4 (Chen *et al.*, 1992). However, the VP7 of B223 was not found to affect the segregation of virulence phenotype with VP4 on either UKtc background (see reassortant RS6, Figure 38), or OSU (see reassortant RS4, Figure 36) in the present study.

The host range restriction which is associated with the spread of the virus amongst the population, has been suggested not to be entirely related with virulence. The gene 5, and not the genes encoding VP4 or VP7, was indicated to be involved in host range restriction (Broome *et al.*, 1993). The support for this observation also comes from the recent identification and characterisation of asymptomatic human rotavirus strains isolated from neonates in India, which had sequence homology with B223 VP4 and VP7 (Sukumaran *et al.*, 1992; Das *et al.*, 1993; Dunn *et al.*, 1993) but with gene 5 representative of human rotavirus. The gene 5 selection of human rotavirus type over bovine rotavirus type presumably helped the Indian strains to survive and circulate in the population, albeit asymptomatically without causing disease. In the present study OSU gene 4 which segregated with virulence was not found to be the determinant of host range restriction as the reassortants with OSU gene 4 did not spread to the uninoculated pups housed in the same cages.

8.4 Conclusions

Gene 4 of OSU and UKtc strains was found to cosegregate with rotavirus virulence among the separable segments. Segment 5 of OSU and segments 5, and 8 of UKtc strains may also be involved in virulence.

Chapter 9

Studies on murine rotaviruses

9.1 Introduction

When this project was started, it was planned to conduct mapping studies on rotavirus virulence with murine rotaviruses in a homologous system. However, murine rotaviruses could be obtained only after a considerable length of time. This chapter describes various experiments conducted with them.

9.2 Results

Murine rotavirus, EDIM, obtained from Professor R. Ward, James N. Gamble Institute of Medical Research, Cincinnati, Ohio, USA, was grown in BSC-1 cells. Very little CPE was evident. Amplification of the VP7 gene on RNA extracted from passage one in BSC-1 cells by RT-PCR gave a very weak band. The RNA profile determined by PAGE-SS also gave very weak bands. Five additional blind passages were given before testing again for the presence of rotavirus specific RNA. The amplification of VP7 gene in RT-PCR and RNA profile of the RNA extracted at fifth passage became negative for rotavirus specific RNA. Adaptation of EDIM strain in MA104 cells also was negative as evident by CPE, RT-PCR amplification, and PAGE-SS. The EDIM strain also did not form any plaques in either BSC-1 or MA104 cells.

The original material obtained was also orally inoculated (100 µl) into a 7 day old suckling CD-1 pup. The pup was sacrificed 48 hr p.i. and gut collected in 2 ml of PBS. After freeze-thawing, homogenisation, sonication, centrifugation, this intestinal homogenate was used for a number of experiments. It was used for two additional mice to mice passages, as inoculum to infect BSC-1 and MA104 cells, as well as for extracting viral RNA.

The RNA extracted from intestinal homogenates was used to determine the RNA profile (Figure 39) and for amplification of the gene

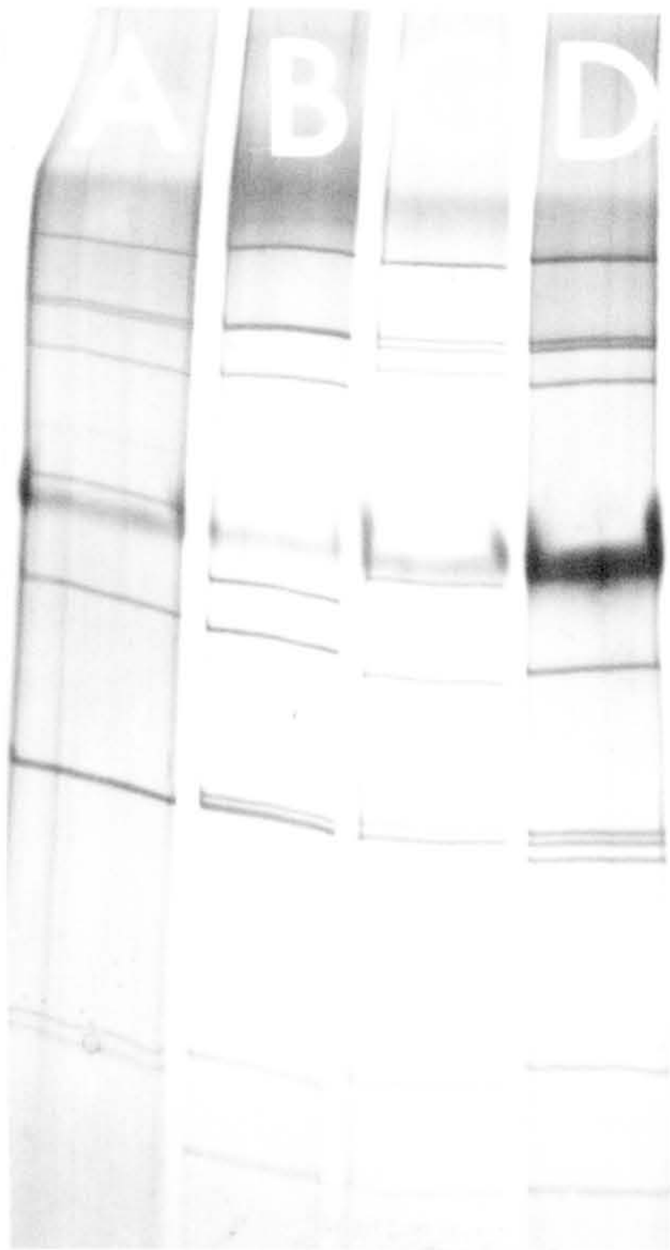


Figure 39 : Electrophoretic profile of genomic RNA of EDIM separated on polyacrylamide gel and silver stained.

Viral RNA was extracted by the optimised method from BSC-1 cells (i) infected with various strains of rotavirus or (ii) lipofected with EDIM strain. Viral dsRNA was then fractionated by electrophoresis on 6% polyacrylamide gel and detected by silver staining as described in Materials and Methods. Segment numbers are shown on right.

Lane A = EDIM; lane B = OSU; lane C = UKtc; lane D = B223.

encoding VP7 in RT-PCR (Figure 40). By comparison of the RNA profile between EDIM and other non-murine rotavirus strains, 7-8 gene segments could be differentiated (Figure 39). It was not possible to differentiate the parental origin of segments 2, 3, and 8 between EDIM and UKtc or B223 strains. In addition to above segments, parental origin of gene segment 9 could not be distinguished between EDIM and UKtc. All segments except 2, 8, 9, and 10 between EDIM and OSU strains could be differentiated using PAGE-SS (figure 39). The amplified VP7 gene cDNA of EDIM was found to contain an *EcoRV* and *HaeIII* endonuclease site but not a *BamHI* endonuclease site. PAGE-SS, and RT-PCR followed by restriction enzyme digestion of amplified cDNA copies of the gene encoding VP7, were used to genotype the progeny clones obtained from EDIM x UKtc cross for reassortant construction. The BSC-1 or MA104 cells inoculated with the intestinal homogenates from passage one in mice did not show any CPE or rotavirus specific RNA in either PAGE-SS or RT-PCR. The intestinal homogenate from passage three (M3) in mice was inoculated at various dilutions into 7 day old suckling CD-1 pups which were observed for the development of clinical signs of disease. The onset of diarrhoea in pups inoculated with M3 occurred on day 2 p.i. and reached a peak on day 4 when 100% pups were found to be suffering from overt (+3) diarrhoea (Figure 41). Diarrhoea induced in pups inoculated with various 10-fold dilutions of M3 started on 3 day p.i., reached a peak on day 4 p.i., and could be observed on day 5 p.i. in all dilutions except 10^{-3} dilution of M3 (Figure 41).

Liposome mediated transfection of rotavirus (Bass *et al.*, 1992a) to remove the block at the level of plasma membrane penetration in non-permissive cells has been described. Because the EDIM was found not to replicate in BSC-1 or MA104 cells when inoculated, a strategy was

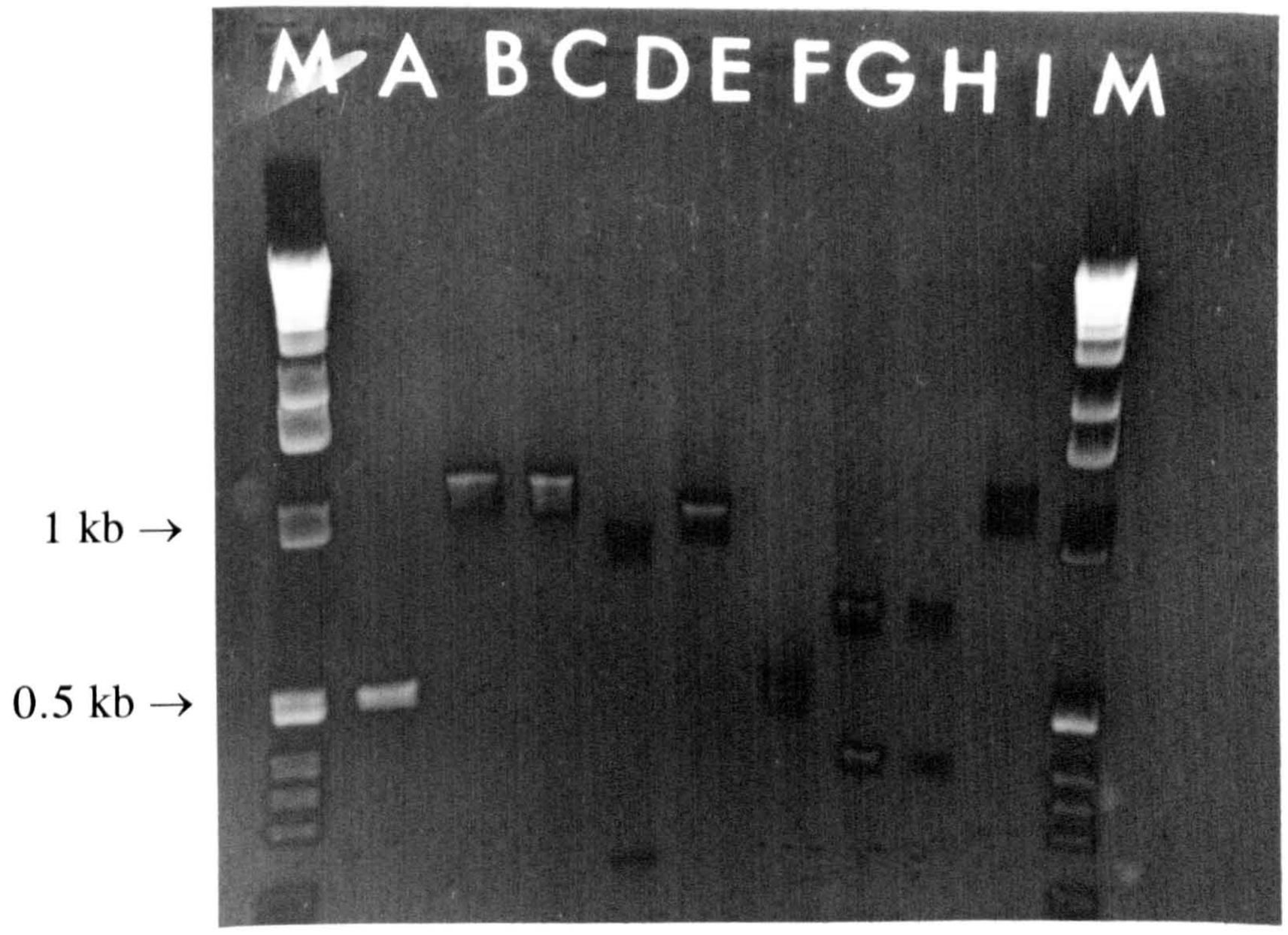


Figure 40 : Restriction enzyme digestion patterns of cDNA copies of the gene encoding VP7 of EDIM and EBR strains of rotavirus.

The gene encoding VP7 was amplified using RT-PCR on RNA extracted by optimised method from BSC-1 cells lipofected with either EDIM or EBR strains of rotavirus and digested by different restriction endonucleases. Following digestion, restriction fragments were seperated by electrophoresis on a 1.5% agarose gel which was then stained with ethidium bromide.

Lane A = Adeno control; lane B= EDIM VP7 gene control; lane C = EDIM+*BamH1*; lane D = EBR+*BamH1*; lane E = EDIM+*EcoRV*; lane F = EBR+*EcoRV*; lane H = EDIM+*HaeIII*; lane G = EBR+*HaeIII*; lane I = EBR VP7 gene control; lane M =ladder of DNA size markers.

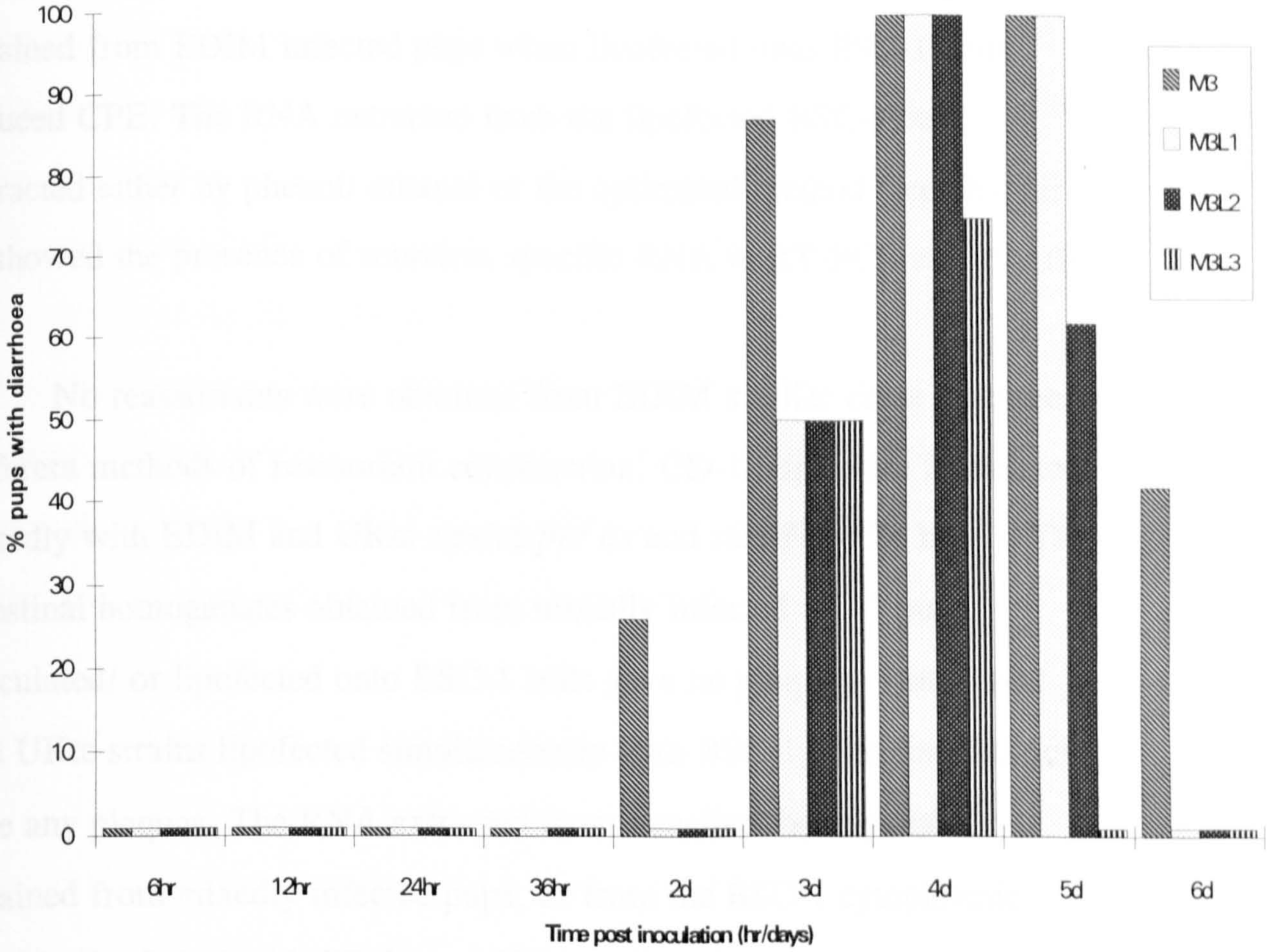


Figure 41 : Diarrhoea produced by EDIM in 7 day old suckling CD-1 pups.

Seronegative 7 day old suckling CD-1 pups were orally inoculated with various dilutions of 3rd mouse passage EDIM on day 0. At various times post inoculation over the next 6 day period, the animals were examined for clinical signs of diarrhoea. At each time point post inoculation a pair of pups was sacrificed and examined for internal signs of disease. Plotted are the mean from 2 experiments and in each experiment n = 5-8.

M3 = EDIM passage 3 in 7 day old suckling CD-1 pups; M3L1 = 10^{-1} dilution of M3; M3L2 = 10^{-2} dilution of M3; M3L3 = 10^{-3} dilution of M3. The standard deviation for various values ranged from 0-2.1.

planned for liposome mediated transfection. The intestinal homogenates obtained from EDIM infected pups when lipofected onto BSC-1 cells induced CPE. The RNA extracted from the lipofected BSC-1 cells extracted either by phenol/ ethanol or the optimised method (see chapter 5) showed the presence of rotavirus specific RNA in RT-PCR and PAGE-SS.

No reassortants were obtained from EDIM x UKtc cross by three different methods of reassortant construction. CD-1 pups were inoculated mixedly with EDIM and UKtc strains *per os* and sacrificed 24 hr p.i. The intestinal homogenates obtained from mixedly infected CD-1 pups inoculated/ or lipofected onto BSC-1 cells gave no plaques. The EDIM and UKtc strains lipofected simultaneously onto BSC-1 cells also did not give any plaques. The RNA extracted from intestinal homogenates obtained from mixedly infected pups, or from the BSC-1 cytoplasmic extracts lipofected with EDIM and UKtc strains, showed only EDIM RNA profile. In another experiment, 34 plaques picked from the BSC-1 cells coinfected with EDIM and UKtc strains showed only UKtc RNA profile.

Another murine rotavirus (EBR) obtained from Birmingham, UK, was inoculated into CD-1 pups. The intestinal homogenate obtained was lipofected onto the BSC-1 cells and RNA was extracted. The RNA profile of EBR strain was found to be different from EDIM strain in that the EBR had two bands at 7-8-9 triplet whereas EDIM had only a single band. Also the gene segment 2 and 3 were closely migrating in EBR as compared to EDIM strain (data not shown). The VP7 gene of EBR was found to be different to EDIM strain as revealed by endonuclease digestion of the PCR amplified product. The amplified VP7 gene copies of cDNA has *BamHI* and *EcoRV* sites which were different to EDIM, but had an identical *HaeIII* restriction enzyme digestion pattern (Figure 40).

genes encoding VP7 of EDIM and EBR strains were sequenced and the deduced amino acid sequences determined. Comparison of the deduced amino acid sequence of the first 130 residues of VP7 of EDIM and EBR strains showed 41 to 67% and 41 to 65% identity, respectively with the 14 G serotypes (Table 11). The amino acid identity between EDIM and EBR strains in the first 130 residues of VP7 was 72%. When deduced amino acid sequence of the strains used in the present study were compared with previously published five murine rotavirus strains, or prototype G3 serotype - SA11 strain, differences were observed over the entire sequence as well antigenically important region A (Figure 42).

9.3 Discussion

The RNA extracted from passage one in BSC-1 cells showed a very weak rotavirus specific RNA in RT-PCR and PAGE-SS, whereas the subsequent passages in BSC-1 or MA104 cells became negative for rotavirus specific RNA. The carryover RNA present in the original material used as inoculum might be responsible for the positive reaction in the first passage and there might be some block at the level of plasma membrane penetration in subsequent passages in the cultured cells. The RNA extracted from the intestinal homogenates from passage one in mice showed the presence of rotavirus specific RNA. However, when the intestinal homogenate was inoculated onto the BSC-1 or MA104 cells no rotavirus specific RNA was evident. The block of EDIM at the plasma membrane penetration in cultured cells proved to be the case as the lipofected intestinal homogenate gave CPE as well as the extracted RNA showed the presence of rotavirus specific RNA in PAGE-SS and RT-PCR. Rotaviruses have been shown to replicate in non-permissive cells as efficiently as in permissive cells when the block at the plasma membrane

Table 11 : Amino acid sequence identities between the first 130 amino acids of VP7 of murine rotavirus strains and strains representing various G serotypes

	% identity to VP7 of murine rotavirus strains		
G Type	Strain	EBR	EDIM
1	Wa	56 (75)[a]	58 (78)[a]
2	hu5	52 (75)	54 (74)
3	SA11	65 (83)	67 (85)
4	St3	55 (77)	59 (78)
5	OSU	64 (83)	65 (82)
6	UKtc	61 (79)	61 (81)
7	Ch2	41 (60)	41 (65)
8	69M	57 (78)	61 (78)
9	Wi61	62 (82)	65 (81)
10	B223	58 (76)	58 (75)
11	YM1	60 (79)	64 (80)
12	L26	57 (76)	59 (78)
13	L338	57 (77)	58 (79)
14	F123	58 (82)	63 (83)
	EBR		72 (87)

a numbers in parentheses indicate % similarity

The sequence data necessary to construct this Table was taken from relevant enteries in the EMBL database. Sequence analysis was done by Genetics Computer Group set of programmes (Devereux *et al.*, 1984)

	1				50
EW	MYGIEYTTAL	TFLISFLLLR	YILKSVVKIM	DFIVYRFLFV	ILILSPCIKA
EB	----------	----------	----L-----	----------	----------
EHP	----------	----------	-M------M-	-----K--L-	------Y---
EC	----------	----------	----T---M-	--------L-	----------
EL	----------	----------	---------M-	--------L-	----------
EDIM	-C-M----V-	--------WG	----------	--M----GV-	M--WA-F---
EBR	***M----A-	--------WG	-V-----RML	--V----GVG	V-----W-RG
SA11	--------V-	-----II--N	-----LTR--	-C-I--L--I	-V--FLR---

	51				100
EW	QNYGINLPIT	GSMDTAYANS	TQPETFLTST	LCLYYPTEAA	TEIKDNSWKD
EB	----------	----------	--S-------	----------	---N------
EHP	----------	----------	---S-------	----------	---S------
EC	----------	----------	---SD-------	----------	--MN------
EL	----------	----------	--S-------	----------	--MN------
EDIM	-----K----	-A---GFE--	--------A-	------K---	-----S----
EBR	-S------M-	---E-GCV-L	--S-----A-	------A---	-----T----
SA11	----------	----------	--E-------	----------	---K------

	101		130
EW	TLSQLFLTKG	WPIGSVYFKE	YTDIAAFSID
EB	------M---	--T-------	-N---V----
EHP	-------M--	--T-------	AN--------
EC	----------	----------	A---------
EL	----------	--T-------	-A--------
EDIM	-----C----	----AGC---	C---GG----
EBR	-----C----	-----A-VR-	-AG-----AG
SA11	----------	--T-------	--N--S--V-

Figure 42 : Comparison of the deduced amino acid sequence (first 130 residues) of VP7 of EDIM and EBR murine rotaviruses.
Murine rotaviruses EDIM and EBR are compared to murine rotavirus strains EW, EB, EHP, EC, and EL and the G3 prototype - SA11. Amino acid residues that are identical to that of EW are shown as dashes (-). Gaps in the sequence of EBR are shown by asterisk (*).

penetration was removed by liposome mediated transfection (Bass *et al.*, 1992a).

The onset and peak of diarrhoea induced by EDIM was found to be delayed by 24 hr, when compared with heterologous rotavirus strains. However, the induction of diarrhoea by EDIM strain in pups was similar to that reported by other investigators (Sheridan *et al.*, 1983; Eydelloth *et al.*, 1984; Starkey *et al.*, 1986). In the absence of plaque formation in cultured cells (Greenberg *et al.*, 1986), the virus was titrated by mouse infectious dose 50% as described by Broome *et al.*, (1993). The EDIM strain was found to be infectious even at 1000-fold dilution (Figure 41).

The attempts to map rotavirus virulence using EDIM in a homologous system were not successful as no reasortants could be obtained in crosses between EDIM and UKtc. Broome *et al.*, (1993) were also unable to identify any reassortant between RRV x EW cross *in vitro*. However, they were able to identify about 5% reassortants from 450 progeny clones in RRV x EW cross *in vivo*. In the present study, all the 34 progeny clones obtained by coinfection of BSC-1 cells with EDIM and UKtc strains showed the UKtc genotype, when analysed by PAGE-SS and RT-PCR. No plaques were obtained either when the intestinal homogenates obtained from mice mixedly infected with UKtc and EDIM strains were inoculated onto the BSC-1 cells or when BSC-1 cells were mixedly lipofected with UKtc and EDIM strains. The RNA profile determined from the intestinal homogenates from mixedly infected pups or cytoplasmic extracts of BSC-1 cells mixedly lipofected with EDIM and UKtc strains did not show mixed profile, but only EDIM profile. The reason for this is not known. The murine rotavirus somehow suppressed the replication of UKtc.

In the absence of getting any reassortants from a cross between EDIM and UKtc strains for mapping rotavirus virulence, it was

considered to correlate rotavirus virulence indirectly to its genes by sequence analysis. Conflicting reports about virulence of murine rotavirus have recently been published (Broome *et al.*, 1993; Ijaz *et al.*, 1994). The VP7 gene of EDIM and another murine rotavirus strain EBR were amplified by RT-PCR. Their amplified VP7 gene appeared to be different in *BamHI* and *EcoRV* restriction endonuclease pattern. However, their *HaeIII* restriction pattern was same. Similar *HaeIII* endonuclease pattern of the VP7 gene of a murine strain has recently been documented (Gouvea *et al.*, 1993). The comparison of the deduced amino acid sequence of the first 130 residues of VP7 of EDIM and EBR strains showed only 72% identity. This region contains one of the three regions (region A; aa 87-101) that is known to be involved in neutralisation based on mapping the sequence changes in neutralisation escape mutants (Dyall-Smith *et al.*, 1986; Mackow *et al.*, 1988b; Taniguchi *et al.*, 1988b). Both the sequences were also found to be different from the recently published sequences (Dunn *et al.*, 1994) of various murine rotavirus strains. Differences were also observed in region A (aa 87-101) among murine strains used in the present study and the previously published five murine strains (Dunn *et al.*, 1994) as well as G3 prototype (SA11 strain).

Chapter 10

General discussion

10.1 Rotavirus virulence : a perspective

Rotaviruses are the single most important etiological agents of severe diarrhoea in infants and young children, as well as in the neonates of most mammalian and avian species, in both developed and developing countries. The World Health Organisation has identified the development of a rotavirus vaccine as a priority area for routine childhood immunisation to control rotavirus infections. However, the candidate vaccine strains employed to-date have not been very successful due to their inability to induce heterotypic immunity. This may be because of the multiplicity of serotypes (Hoshino and Kapikian, 1994), resulting from the independent segregation of VP4 and VP7 genes (Hoshino *et al.*, 1985; Offit and Blavat, 1986) responsible for determining serotype specificity in rotaviruses. The number of potential serotypes predicted by reassortment and cross-neutralisation studies may increase due to masking and unmasking of some of the neutralising epitopes (Chen *et al.*, 1992; Kool *et al.*, 1992).

There has been a recent resurgence of interest in the pathogenesis of viral infections. The application of molecular methods has made it fruitful to re-examine old questions and to ask new ones about the determinants of the outcome of the virus-host encounter. The role of virus virulence has been one of the major questions explored during this renaissance of work on viral pathogenesis. Studies on rotavirus virulence will not only help in developing better vaccines, but may also provide a direction for developing antivirals as an alternative strategy for the control of rotavirus induced diarrhoea.

At the start of this project, there was only single report (Offit *et al.*, 1986) of work conducted directly on animals. The lack of a suitable animal model and the inability to manipulate a gene *in vitro* and then to rescue it into an infectious particle were both identified as major

limitations to studies on virulence. The aims of this project were designed to overcome these limitations to obtain a clearer picture of rotavirus virulence. A three step approach was undertaken (i) development of an animal model, (ii) construction and characterisation of reassortants between rotavirus strains of different virulence, (iii) mapping of virulence to specific rotavirus gene segments.

The mouse model developed in the present study (chapter 5) revealed that virus virulence is dependent upon viral dose and viral strain in addition to host age and host strain. Mouse models for studying rotavirus disease and immune response has been used previously for both homologous (Wolf *et al.*, 1981; Little *et al.*, 1982; Sheridan *et al.*, 1983; Eydelloth *et al.*, 1984; Greenberg *et al.*, 1986; Starkey *et al.*, 1986), and heterologous rotavirus strains (Offit *et al.*, 1984; Gouvea *et al.*, 1986; Ramig, 1988; Ward *et al.*, 1990). However, with the exception of one study (Ramig, 1988), the other studies were not carried out in a very systematic and/or comprehensive fashion. In majority of the cases, rotavirus antigen detection in the intestines, and seroconversion were taken to represent disease. These parameters only represent infection rather than disease. It was clearly demonstrated in the present report that adult mice can be infected (adult mice seroconverted) without getting any disease. Consequently, observations of the clinical signs of the disease should be taken as the end point in virulence studies on rotavirus in animal models, which may be supported by seroconversion, virus shedding, detection of antigen in intestines, and histopathology.

Two sets of experiments, one in which different rotavirus strains were studied in the same strain of mouse, and another in which the same rotavirus strain was studied in different strains of mouse, revealed that virulence in this virus system is not tightly linked to host susceptibility. The outcome of rotavirus infection was found to depend on the interaction

of virus virulence and host resistance/ susceptibility. Also, the duration of disease, the percentage of pups affected, and the severity of disease were all found to be independent variables of rotavirus virulence. For example, the bovine rotavirus B223 strain failed to produce diarrhoea in C57/BL pups, whereas the porcine rotavirus OSU strain was able to produce diarrhoea in them. By contrast in C3H/HE pups, the B223 strain appeared to affect a higher percentage of pups than the OSU strain. However, the severity of the disease was only +1 in majority of the pups infected with the B223 strain, whereas with the OSU strain the diarrhoea induced varied between +2 to +3 (overt diarrhoea), and was observed for longer duration.

The disease induced by the rotavirus strains employed was quite different in the three inbred mice strains that were studied in the present project. It is not known at present whether MHC haplotype has any influence on the outcome of rotavirus infection. The role of MHC haplotype is very important in CMI (Zinkernagel and Doherty, 1974). The present study also indicates that protection studies with rotavirus vaccine candidates should be done or at least compared in the outbred CD-1 mice strain so that the results of these experiments can be applied in a meaningful way for human and animals.

The inability to rescue a genetically manipulated gene into an infectious virus particle or to swap a specific gene between two rotavirus strains 'at will' underscores that reassortants are the only means available, at present, to conduct virulence mapping studies on rotaviruses. The viral genome consists of 11 gene segments, most of which are monocistronic (Mason *et al.*, 1980; Smith *et al.*, 1980; McCrae and McCorquodale, 1982). Assuming that virus virulence is completely determined by a single gene then virulence in rotaviruses has the possibility of being mapped to individual gene segments using segregation

analysis, in which a suitable host is infected with 22 monotypic reciprocal reassortants derived from two rotavirus parents differing in their virulence phenotype. However, reassortment being a random process, a very large number of progeny clones need to be genotyped in the absence of suitable selection protocols to obtain the desired 22 monotypic reciprocal reassortants derived from two rotavirus parents as 2048 different genotypes are possible. Consequently, both the nucleic acid extraction method and genotyping assays have to be both simple and rapid with a minimum number of manipulative steps. The nucleic acid extraction method developed in the present study (chapter 6) fulfils these criteria, as it requires only one centrifugation step and does not require the use of any hazardous organic chemicals.

Putative reassortants were generated using a variety of methods and genotyped by determining the relative migration of their gene segments on polyacrylamide gels, coupled in the case of genomic segment 8/9 with restriction enzyme digestion of the PCR amplified products. This revealed that only 2% out of more than 1100 progeny clones examined were reassortants. Possible reasons for obtaining this low number of reassortants are discussed in detail in chapter 7. The general points that emerged can be summarised as : (i) other (than the 22 identified) reassortants were never formed (ii) the right kind of progeny clones were missed during plaque picking (iii) the reassortants were formed but they did not form the plaques because they were non-infectious (iv) the reassortants were formed but they were not able to form the plaques on the BSC-1 or MA104 cells (v) reassortants were formed in which exchange occurred in those gene segments where it was not possible to differentiate the parental origin (vi) segments from one of the parents are selected *in toto* because they are dominant as a group.

The observation that the majority of the progeny clones showed a UKtc electropherotype when it was one of the coinfecting parent, or an OSU electropherotype in the cross between OSU x B223 can be explained in two ways either (i) their genomes are selected *in toto* because they are dominant as a group (ii) or interference between two rotavirus strains.

The selection of segments *in toto* in reassortment experiments has been reported by others (Ward and Knowlton, 1989). Interference between mutant and wild-type animal viruses following mixed infection is a well described phenomenon (Whitaker-Dowling and Youngner, 1987). Recently, interference between two wild-type reoviruses following mixed infection has been reported (Rozinov and Fields, 1994). Two possible explanations for these observations were made. First, the wild-type may in fact be a mutant. The definition of wild-type largely refers to its growth as an isolate that has not 'intentionally' been mutagenised or otherwise selected as a mutant. The passage of viruses in cell culture can select mutations that cause the virus to differ from the original virus in nature. In fact, the high rate of mutations in RNA viruses provides an opportunity for mutations to appear in putative wild-type stocks very rapidly (Holland *et al.*, 1982). Thus, interference may be a general property of a number of 'wild-type' viruses. Second, viruses that are separated in nature are constantly evolving and may change in ways that lead to interference. Virion assembly might be quite permissive for individual virions, however, the combinations of proteins from two parents could lead to inefficient virion assembly or no assembly at all. Phenotypic mixing has been suggested (Rozinov and Fields, 1994) as one situation which allows the proteins from two different parents to be present in some progeny virions, to be responsible for interference. A high degree of phenotypic mixing during coinfection of cells with two rotavirus strains has been reported (Ward *et al.*, 1988b). The strains used

to construct rotavirus reassortants in the present study are laboratory strains that are constantly being passaged in cells. These strains may in fact be mutants or may have evolved to such an extent where protein-protein interactions between the two parents might not allow normal assembly as a result of modified conformation. Whether interference was responsible for obtaining the UKtc elecropherotype in the majority of clones requires further investigation.

The production of reassortants between the highly virulent EDIM strain and other less virulent heterologous rotavirus strains would have been highly desirable for virulence mapping attempted in this work. However, no reassortants could be identified in crosses which employed EDIM as one of the parent viruses. The murine rotavirus strains were probably blocked at the level of plasma membrane penetration as no infectious virus could be recovered following infection of BSC-1 or MA104 cells. However, when they were transfected by Lipofectin^{TM}, infectious rotavirus could be recovered (chapter 9). When the EDIM and UKtc strains were coinfected progeny clones showed only UKtc electropherotype. When UKtc and EDIM strains were transfected together or coinfected *in vivo*, no virus plaques were obtained upon plating out progeny. However, the nucleic acid extracted from coinfected cells or from intestinal homogenates prepared from coinfected mouse, showed only the EDIM electropherotype in PAGE-SS. The reasons for this suppression of UKtc are not known.

Preliminary experiments indicated that the VP7 gene sequences of EDIM and EBR strains were different than published reports (Dunn *et al.*, 1994). Differences were also found in region A which is known to be involved in virus neutralisation, based on mapping the sequence changes in neutralisation escape mutants (Dyall-Smith *et al.*, 1986; Mackow *et al.*, 1988b; Taniguchi *et al.*, 1988b).

The 22 reassortants obtained in the present study belonged to 15 genotypes (chapter 7). The application of statistics to such a small number of reassortants is difficult. However, a few points are obvious. Thus, although gene 4 has been related to growth restriction of rotaviruses in cultured cells (Greenberg *et al.*, 1983a), it did not always come from the better growing parent in the reassortants examined in this study. The gene 4 from the OSU parent was present in all the reassortants except one, in UKtc x OSU cross, although UKtc was observed to be the dominant and better growing parent. Similar findings have been reported by others (Ward *et al.*, 1988a). Gene segment 4, and 5 never came from B223, however, gene 5 was derived almost equally from UKtc or OSU. The VP7 gene never came from OSU. Among the remaining separable segments, the segments from all three parents were represented at least once. Reassortment is a random process. However, non-random selection of single gene as well as gene association have been reported (Gombold and Ramig, 1986; Graham *et al.*, 1987; Ward *et al.*, 1988a).

The analysis of cosegregation of genome segment with phenotype in reassortants has been the method of choice for mapping gene function in viruses with segmented genomes. Despite considerable efforts, the generation of the desired 22 monotypic reciprocal reassortants that were sought in the present study, as a first step to segregate rotavirus virulence to its genes, was not successful. The virulence mapping studies conducted in mice with the reassortants available in the present study indicated that gene 4 of the OSU and UKtc strains of rotavirus is involved in virulence (chapter 8). Segment 5 of OSU strain and segments 5, and 8 of UKtc strain may also be involved in rotavirus virulence.

10.2 Reverse genetics in influenza A virus

10.2.1 Rescue of synthetic gene into influenza A virus genome

Influenza A viruses are negative stranded viruses that contain eight different genome segments (Palese, 1977). These RNAs are tightly associated with the viral polymerases and nucleoprotein in the ribonucleoprotein (RNP) complex (Lamb, 1989) both in virions and infected cells. The genomic RNA needs to be transcribed into mRNA in order to direct the synthesis of viral proteins in the host cell. This function is controlled by a virally coded RNA-dependent-RNA-polymerase, which is also responsible for the replication of the genome. In contrast to DNA and positive-strand RNA viruses, influenza viruses had until recently been refractory to genetic manipulation.

The amplification, transfection and rescue of synthetic RNA molecules from a RNA virus was first achieved in 1989 (Luytjes *et al.*, 1989). A biologically active influenza virus RNP complex was reconstituted using synthetic RNA and purified viral proteins, and amplification and expression of the reporter gene was driven by an influenza helper virus.

Viral RNP cores containing transcriptional activity can be isolated from disrupted viruses or from infected cells. The templates for this activity are endogenous viral RNA molecules that are tightly associated with the purified RNPs. In order to use synthetic RNAs as templates, a procedure to separate the viral proteins and RNA from the RNP cores was needed. The purification of the three polymerase(P) proteins and/ or nucleoprotein (NP) from native RNAs (Honda *et al.*, 1988; Szewczyk *et al.*, (1988) helped Parvin *et al.*, (1989) to demonstrate functional reconstitution using synthetic RNA and purified NP-P protein complex. These reconstituted RNP complexes were able to copy synthetic RNA molecules whose 15 3'-terminal nucleotides were identical to those

present in the viral RNAs. This reconstitution system was then used (Luytjes *et al.*, 1989) for introducing synthetic RNA sequences into the genomes of influenza virus particles.

The RNP transfection method was further improved by coupling the *in vitro* transcription from plasmid derived cDNA with the reconstitution (Enami and Palese, 1991) or the use of native RNP cores themselves (Yamanka *et al.*, 1990), or coexpression of the P and NP proteins in the same cell using vaccinia virus recombinants (Huang *et al.*, 1990), or the use of NP-P complexes isolated from influenza virus infected cells rather than from purified virus (Martin *et al.*, 1992).

10.2.2 Genetic manipulation of influenza A virus

The first influenza virus gene exchanged by an *in vitro* synthesised RNA was the neuraminidase (Enami *et al.*, 1990). Recent developments have enabled the exchange of HA and NS genes of influenza A virus by cDNA-derived RNAs (Enami and Palese, 1991). Transfectant influenza A viruses have been constructed that contain chimeric HAs (Li *et al.*, 1992) from two different subtypes, or foreign epitopes (Li *et al.*, 1993b) from HIV-1.

10.2. 3 Reverse genetics : a powerful tool to study influenza A virus virulence

The cleavage HA of influenza A virus has important role in virulence (see chapter 3). Using reverse genetics, it has recently been demonstrated directly that influenza virus virulence depends on the cleavability of haemagglutinin (Horimoto and Kawaoka, 1994). A transfectant virus containing the wild-type HA with Arg-Arg-Arg-Lys-Lys-Arg at the cleavage site, which was readily cleaved by endogenous proteases in chicken embryo fibroblasts (CEF), was highly virulent in

chickens after intramuscular or intranasal/ oral inoculation . In contrast, a mutant containing HA of an avirulent- like sequence (Arg-Glu-Thr-Arg) at the cleavage site, which was not cleaved by the proteases in CEF, was avirulent in chickens, indicating that a genetic alteration confined to the HA cleavage site can affect cleavability and hence virulence. It has been further proposed that viruses with the Arg-Arg/Lys-Arg-Lys-Thr-Arg motif (Wood *et al.*, 1993) should be considered in the same category as virulent viruses with xxx-xxx-Arg/Lys-xxx-Arg/Lys-Arg motif based on similarity in the virulence for chickens of field isolates and laboratory mutants produced by reverse genetics.

The neurovirulence observed in some influenza A viruses is associated with its neuraminidase (see chapter 3). Recently, the absence of the glycosylation site at position 130 of the neuraminidase has been shown to play a key role in the neurovirulence of influenza virus strain WSN in mice (Li *et al.*, 1993a). A glycosylation positive (Glyc+) mutant virus was generated using reverse genetics methods, in which the glycosylation site at 130 position was introduced into the WSN virus neuraminidase. In contrast to wild-type or revertant viruses, the Glyc+ mutant neither underwent multiple cycles of replication in MDBK cells nor was able to multiply in mouse brain.

10.3 Application of reverse genetics in rotaviruses : a prospective

Although the 'rescue' of a rotavirus gene was first described (Greenberg *et al.*, 1981) 13 years ago, it was only a reassortant experiment in which the gene segment was exchanged by reassortment using a combination of ts mutant and antibody selection pressure. A more recent report described conditions under which reovirus RNA was infectious (Roner *et al.*, 1990). However, the molecular mechanisms

involved are poorly understood and the rescue of a single reovirus gene, from a cloned DNA copy into virus particle has yet to be achieved. The expression of a synthetic gene flanked by NCR of a rotavirus gene has been reported (Gorziglia and Collins, 1992), but the result remains to be confirmed by other investigators. Moreover, they were not able to rescue it into an infectious rotavirus particle.

In order to rescue a rotavirus gene in its 'true sense', two things need to be achieved i.e. (i) to force an exogenous RNA to enter into the pool of RNA molecules available for (re)assortment into infectious progeny virus, (ii) to be able to identify the rescued infectious particles by appropriate selection systems.

The first steps should be to identify the genes encoding the proteins that form the polymerase complex and then techniques need to be developed to be able to separate them. Further experiments may follow on the lines of ribonucleoprotein transfection method to allow the exogenous RNA to enter into the pool of RNAs available for replication, i.e. the synthetic or altered rotavirus genes be reconstituted with rotavirus RNA polymerase complex and transfected into appropriate cells.

Identification and selection of the rescued virus particles is equally important, if not more important, to devise methods to introduce exogenous RNA to enter into the pool of RNAs available for replication. The rescued virus particles can be identified with some of the following selection systems (i) CAT expression of synthetic rotavirus-chloramphenicol acetyltransferase gene (ii) growth in the presence or absence of trypsin (iii) haemagglutination or haemadsorption of the haemagglutinating parent (iv) use of ts mutant and selection at non-permissive temperature (v) selection in the presence of specific neutralising antibodies.

The majority of the investigators trying to rescue a rotavirus gene are following the strategy described below : A transcription vector under the control of T7 promotor is constructed by RT-PCR using appropriate primers to generate a rotavirus segment specific modified cDNA. This cDNA is then linearised and transcribed using T7 RNA polymerase. This cDNA derived *in vitro* transcript is transfected into appropriate cells which have simultaneously been infected with another rotavirus strain. Progeny clones are then screened for virions containing the rescued gene.

A method combining the transfection and selection of the exogenous RNA containing rotavirus would be highly desirable. Ribozymes acting in *cis* or *trans* may be helpful in such situations. Simultaneous transfection of an exogenous rotavirus transcript, together with a ribozyme specific for the equivalent segment from a helper virus would greatly enhance the chances of getting virus particles with rescued gene. The above experiment may be combined with another experiment in which a ribozyme may be ligated into a rotavirus transcript which is to be selected against. The simultaneous transfection of this construct together with the desired exogenous equivalent transcript derived from cloned DNA would allow the amplification of both type of gene segments, but only the virion that contained the desired gene without the ligated ribozyme would be able to assemble.

Reverse genetics will open the flood-gates for devising chimeric rotaviruses to be used as vaccine candidates. This can be done by epitope grafting using T- cell epitopes from different rotavirus strains on the lines of influenza A virus (Li *et al.*, 1992). However, the insertion of foreign epitope into the aa sequence of a viral protein can sometimes alter the properties of the protein sufficiently to prevent the generation of viable viruses. Rescue of bicistronic rotavirus genes constructed by the use of internal ribosomal entry site elements (Garcia-Sastre *et al.*, 1994), or by

inserting in frame self cleaving 2A protease sequences (Percy *et al.*, 1994) on the lines of influenza A virus, will allow the insertion of an entire gene. Reverse genetics will also allow rotaviruses to be used as expression vector for other pathogens in which mucosal immunity is important.

10.4 Concluding remarks

The findings of the present project are summarised below : Studies on rotavirus virulence can help in achieving the goal set by WHO to control rotavirus infections in young children. Rotavirus reassortants are the only means available, at present, to conduct studies on virulence. However, attempting to map rotavirus virulence by reassortants has its own limitations. The generation of desired reassortants for conducting virulence studies can be both labour intensive and time consuming. Also in the event of getting the desired reassortants by reassortment, the experiments with such reassortants will map the virulence phenotype with a precision not greater than assigning it to a segment(s). The specific domains in general and nucleotides in particular that are involved in rotavirus virulence cannot be determined in this type of study. A detailed and clearer picture of virulence can only emerge when the techniques of reverse genetics to rescue a cloned gene, and genes modified by site-directed mutagenesis become available for applying to rotaviruses.

The reverse genetics techniques, once available for rotaviruses, can be used to swap a rotavirus gene 'at will' between the equivalent gene segments from two rotavirus strains differing in their virulence for identifying the gene segments involved. The modifications of the gene segments by site-directed mutagenesis and their subsequent rescue will help to identify the amino acid(s) involved in virulence/attenuation.

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