

STUDIES ON SEROLOGICAL VARIATION
AMONG BOVINE ROTAVIRUSES

by

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'The fear of the Lord is the beginning of wisdom...'

Psalms 111 v 10

To

Sola, Emeka, Uche, Ada & Olisa
with love and deep appreciation.

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DECLARATION

I certify that the work presented in this thesis is my own and that areas of collaboration and assistance from colleagues have been fully identified in the appropriate sections of the text.

Clement Kanyidinma Ojeh

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ABBREVIATIONS

BSA	Bovine serum albumin
CF	Complement-fixation
cpe	cytopathic effect
CRV	Calf rotavirus
DEAE	Diethylaminoethyl
E '59	Eagles 59 medium
EBK	Embryonic bovine kidney
EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EOK	Embryonic ovine kidney
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
HA	Haemagglutination
HAI	Haemagglutination-inhibition
H&E	Haematoxylin and eosin
HEHA	Haemadsorption-elution haemagglutination assay
HEPES	Hydroxypiperazine-N'-2-ethanesulfonic acid
hr.	hour(s)
IAHA	Immune adherence haemagglutination assay
ID	Immunodiffusion
IEM	Immune electron microscopy
IEOP	Immune electro-osmophoresis
IF	Indirect immunofluorescence
min.	minute(s)
NCDV	Neonatal calf diarrhoea virus

NT	Neutralisation tests
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS/T	Phosphate buffered saline with 0.05% Tween 20
p.i.	post-infection
RIA	Radioimmunoassay
rpm	revolutions per minute
sec	seconds
TCID ₅₀	50% tissue culture infective dose
u.v.	ultra violet
v	volume
VP	Viral polypeptide
w	weight

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ABSTRACT OF THESIS (Regulation ^{7.9}~~6.9~~)

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Title of Thesis Studies on serological variation among bovine rotaviruses

All 12 field strains of rotavirus used initially to provide virus stock and convalescent antisera were capable of multiplying in gnotobiotic lambs but without inducing diarrhoea. None grew on primary bovine and bovine embryonic kidney cell cultures nor on MDBK, LLC-MK2 and Vero cell lines but all adapted well to growth in roller cultures of MA104 cells created with 10 μ g/ml of trypsin. Pre-treatment of virus inocula was essential for further propagation of the virus strains on MA104 cells.

Because 4 of 12 original strains were found to contain more than one rotavirus electrophoretype, the remaining 8 strains were selected for more detailed serological investigations. Neutralisation tests using convalescent lamb antisera and hyperimmune rabbit antisera against faecal and tissue culture viruses confirmed the presence of two distinct serotypes; in that they showed more than a 20-fold reciprocal difference in titre and an antigenic relatedness value of less than 5%. When samples of infected calf faeces were titrated in hyperimmune antisera to these two serotypes, four untypable rotaviruses were isolated of which three belonged to a hitherto unknown third serotype, while the fourth strain showed a partial relationship to this new serotype.

In summary of the 108 calf rotavirus strains examined, no fewer than 80 (74%) including the UK(Compton), Northern Ireland, and Lincoln strains belonged to serotype 1, one was identified as serotype 2 and three as serotype 3. Of the remainder, one had a partial relationship to serotype 3 while 23 produced insufficient fluorescing foci to enable them to be characterised fully. Attempts to develop ELISA and HAI tests for the detection of rotaviruses of serotypes 1 and 2 in diagnostic faecal samples proved unsuccessful.

Examination of the electrophoretotypes of rotavirus involved in 30 outbreaks of diarrhoea in calves revealed that one strain usually predominated in each individual herd, although infection with multiple strains occurred on 5 of the farms examined. There was no evidence that genomic and polypeptide variations exhibited by the calf rotaviruses were related to their serotypic differences.

In experimental calf, infection of ligated gut loops showed that variations in virulence were obtained with different strains of rotavirus, although no correlation occurred between virulence and the serotypes used. There was no appreciable difference between faecal and tissue culture virus in the ability to infect intestinal epithelial cells and most tended to favour the ileal (middle and distal) rather than the jejunal (proximal) parts of the small intestine for replication.

The practical importance of indentifying rotavirus serotypes was confirmed by passive cross-protection immunisation experiments in gnotobiotic lambs. Thus lambs fed with antiserum for three successive days and challenged with rotavirus between the first and second feeds showed good protection against the homologous virus; but there was no cross-protection; affected lambs excreted the challenge virus at similar titres and over the same period as did unprotected control lambs.

CHAPTER 1INTRODUCTION

Diarrhoea has long been recognised as an important disease in young animals (Mills, 1776, cited by Woode and Bridger, 1975). The problem is world-wide and is a major cause of economic loss in the farming industry. The losses are not only due to mortality which ranges between zero and eighty per cent, but also due to high medical costs, labour and poor growth gains, particularly in herds in which the morbidity can be up to 100%. The disease is usually associated with a wide range of factors including infectious agents, environment, nutrition and management, as well as a variety of immunological and genetic factors. Micro-organisms are capable of causing diarrhoea either on their own or by interacting with each other. For example, there are numerous reports of outbreaks of diarrhoea in calves which have been attributed to rotavirus (Flewett and Woode, 1978; McNulty, 1978; McNulty, McFerran, Bryson, Logan and Curran, 1976; Woode, Bridger, Hall and Dennis, 1974), enterotoxigenic Escherichia coli (Moon, McClurkin, Isaacson, Pohlenz, Skartvedt, Gillette and Baetz, 1978) or cryptosporidium (Panciera, Thomassen and Garner, 1971). Similarly, but to a lesser extent, there are also reports which show the involvement of viruses, bacteria and parasitic agents in mixed diarrhoeic infections of animals and children (Morin, Lariviere and Lallier, 1976; Morin, Larviere, Lallier, Begin, Ray and Ethier, 1978; Acres, Laing, x

Saunders and Radostits, 1975; Black, Merson, Rahman, Yunus, Alim, Huq, Yolken and Curlin, 1980; Snodgrass, Sherwood, Terzolo and Synge, 1982).

Early surveys of calf mortality in Britain carried out by Jordan (1933) on 26 farms revealed that 22% of heifer calves died "within a comparatively short time". These findings were confirmed when a similar survey was carried out a year later on 52 farms and an annual death rate of 20% was reported (Smith, 1934). A survey of 335 herds in England, Wales and Scotland showed that 5.5% of female calves in England and Wales and 11.4% in Scotland died during the first six months of life (Lovell and Bradford-Hill, 1940). Withers (1952; 1953) reported similar findings of 6.8% for England and Wales and 11.9% for Scotland in calves under 6 months, when he surveyed calf mortality in 44 farms in England, Wales and Scotland. Forty-three per cent of these deaths were attributed to infectious agents, mainly bacteria. Lovell and Hughes (1935) showed that in 100 calves studied, 37% died from colibacillosis within the first fortnight of the calf's life. Kilkeny and Rutter (1975) estimated that enteric diseases and septicaemic conditions caused by E. coli and Salmonella species in calves accounted for about 70% of the diagnosis during the first 2 months of life. Thus while accurate estimates of calf scour mortality are impossible to obtain, and probably have varied over the years, there is general agreement that the problem is one of the major

diseases in cattle. The old diagnosis of 'colibacillosis' should be taken to indicate enteritis of unknown aetiology, rather than, specifically, diarrhoea caused by E. coli.

In this present review, viral causes of neonatal diarrhoea will be discussed, with emphasis on rotavirus, while other enteropathogens such as bacteria and protozoa, will be mentioned briefly.

REVIEW OF THE LITERATURE

VIRAL CAUSES OF NEONATAL DIARRHOEA

A. ROTAVIRUSES

Rotaviruses have been the subject of major reviews either as journal articles (Flewett and Woode, 1978; Holmes, 1979; McNulty, 1978) or as chapters of books (Tyrrell and Kapikian, 1982; Kapikian, Greenberg, Wyatt, Kalica, Kim, Brandt, Rodriguez, Parrott and Chanock, 1982). Consequently, only those aspects which are relevant to the present study will be presented here.

Mebus, Underdahl, Rhodes and Twiehaus (1969) were the first to report the reproduction of diarrhoea in colostrum-deprived calves by oral administration of a bacteria-free faecal filtrate from scouring calves. On examination by EM, the filtrate was shown to contain numerous viral particles which measured approximately 65 nm in diameter. Since then, similar viruses have been shown to be very important in neonatal diarrhoea in all mammalian and

avian species that have been studied so far (Flewett and Woode, 1978; McNulty, 1978; McNulty, Allan and Stuart, 1978). Morphologically, all the virus isolates from these various species are indistinguishable, having an icosahedral core with two layers of capsid polypeptides. As a result of the morphological similarity with orbivirus and reovirus, the rotaviruses were initially referred to as the 'orbivirus group' or 'reovirus-like agent' (Middleton, Szymanski, Abbott, Bortolussi and Hamilton, 1974; Mebus, Wyatt, Sharpee, Sereno, Kalica, Kapikian and Twiehaus, 1976; Kapikian, Kim, Wyatt, Cline, Arrobio, Brandt, Rodriguez, Sack, Chanock and Parrott, 1976; Konno, Suzuki, Imai and Ishida, 1977). However, it was Flewett, Bryden, Davies, Woode, Bridger and Derrick (1974) who, on studying the relation between viruses from acute gastroenteritis of children and newborn calves, first suggested the name 'rotavirus'. In 1978 the International Committee for the Nomenclature of Viruses recognised the name, classified them in the family Reoviridae, and assigned them to a new third genus: 'rotavirus' along with reovirus and orbivirus (Derbyshire and Woode, 1978).

Morphology

Preparations of negatively stained faeces show that there are two types of rotavirus particles. The intact particle appears as a smooth double-shelled capsid structure measuring approximately 70nm in diameter and is believed to be the infective form (Bridger and Woode, 1976). It consists

of a centre (36-38 nm in diameter) which is bounded by a membrane from which the inner capsomeres radiate out as short spokes. Attached to these capsomeres is the outer capsid layer giving the whole structure the appearance of a sharply defined rim attached to short spokes on a wide hub; while the second type is the rough or single-shelled particle, measuring about 60nm in diameter.

Viral replication takes place in the cytoplasm where complete and incomplete particles are seen (Chasey, 1977; Elias, 1977). The morphogenesis of the virus takes place in association with the cisternae of the endoplasmic reticulum. Granular or fibrillar masses considered as precursor material of the virus or viroplasm are found exterior to the cisterna. The virus is released from the cell following rupture. (McNulty, Curran and McFerran, 1976; Stair, Mebus, Twiehaus and Underdahl, 1973).

Biochemical Properties

The two types of particles can be separated in CsCl density gradients in which their buoyant densities are 1.36 gm/ml and 1.38 gm/ml for the smooth or complete and rough or incomplete particles respectively (Bridger, 1978; Bridger and Woode, 1976; Elias, 1977; Holmes, Ruck, Bishop and Davidson, 1975). The genome of all rotaviruses consists of 11 segments of dsRNA as resolved by polyacrylamide gel electrophoresis and the molecular weights of the genome segment range between 2.2×10^6 and 0.2×10^6 (Barnett,

Egbert and Spendlove, 1978; Newman, Brown, Bridger and Woode, 1975; Kalica, Wyatt and Kapikian, 1978; Rodger, Schnagl and Holmes, 1977; Todd and McNulty, 1976; 1977), and size variation has also been reported in the dsRNA of rotaviruses either from the same or different species (Kalica, Sereno, Wyatt, Mebus, Chanock and Kapikian, 1978; Rodger and Holmes, 1979; Verly and Cohen, 1977).

The electrophoretic migration patterns of human rotavirus dsRNA occur in 'long' and 'short' patterns. This division is based on the large differences in the mobilities of the 10th and 11th segments of the genome (Espejo, Calderon, Gonzalez, Salomon, Mortuscilli and Romero, 1979). These electrophoretotypes have been utilised in epidemiological studies of outbreaks of infantile rotavirus gastroenteritis. Thus, Schnagl, Rodger and Holmes (1981), identified three new electrophoretotypes in a series of outbreaks over 4 successive years in children in Australia; while Albert, Soenarto and Bishop (1982) noted that 9 different electrophoretotypes occurred among 85 children in Yogyakarta, Indonesia.

The complete virus particle is composed of 8 - 10 polypeptides, while the single shelled or incomplete particle is made up of 4 - 5 structural polypeptides. The gene coding assignments of virtually all the 11 segments of the dsRNA of rotaviruses have been worked out. Thus segments 1 - 6 encode for the polypeptides VP1 - VP6, with

the VP6 being the major internal protein of the single shelled particle responsible for the common antigen reaction and subgroup specificities; while segments 9 of human and simian (SA-11) rotaviruses and 8 or 9 of calf rotaviruses encode for the VP7, the major outer shell polypeptide. VP7 is glycosylated and responsible for both neutralising antibody specificity and initiation of infection (Bastardo, McKimm-Breschkin, Sonza, Mercer and Holmes, 1981; Kalica, Greenberg, Wyatt, Flores, Sereno, Kapikian and Chanock, 1981; McCrae and McCorquodale, 1982; Mason, Graham and Estes, 1983; Matsuno and Inouye, 1983; Rodger, Schnagl and Holmes, 1977).

Antigenicity

All rotaviruses, regardless of the species from which they were isolated, were thought to share a common antigen until recently when isolates from piglets, calves, lambs, chickens and man were shown to lack the group antigen (Bridger, 1980; McNulty, Allan, Todd, McFerran and McCracken, 1981; Rodger, Bishop and Holmes, 1982; Saif, Bohl, Theil, Cross and House, 1980; Snodgrass, Campbell and Herring, personal communication). The group antigen can be demonstrated by a number of techniques including IF (Woode, Bridger, Jones, Flewett, Bryden, Davies and White, 1976), CF (Kapikian, Cline, Kim, Kalica, Wyatt, VanKirk, Chanock, James and Vaughn, 1976), IEOP (Grauballe, Gemer, Meyling and Hornsleth, 1977), ID (Sharp and Littlejohns, 1981; Woode, Bridger, Jones, Flewett, Bryden, Davies and White, 1976),

IEM (Bridger and Woode, 1975) and ELISA (Ellens and de Leeuw, 1977; Yolken, Barbour, Wyatt, Kalica, Kapikian and Chanock, 1978).

Nevertheless, it is also possible to distinguish between isolates from different species and indeed from the same species by serum neutralisation tests (Bryden, Davies, Hadley and Flewett, 1975; McNulty, Allan, Todd, McFerran, McKillop, Collins and McCracken, 1980; Thouless, Bryden, Flewett, Woode, Bridger, Snodgrass and Herring, 1977); by special ELISAS, (Yolken, Wyatt, Zissis, Brandt, Rodriguez, Kim, Parrott, Urrutia, Mata, Greenberg, Kapikian and Chanock, 1978; Zissis and Lambert, 1980); and, more recently, by IAHA (Kapikian, Cline, Greenberg, Wyatt, Kalica, Banks, James, Flores and Chanock, 1981).

Subgroups

Although 'serotypes' of human rotavirus have been distinguished using the CF and ELISA techniques (Yolken, Wyatt, Zissis, Brandt, Rodriguez, Kim, Parrott, Urrutia, Mata, Greenberg, Kapikian and Chanock, 1978; Zissis and Lambert, 1978 and 1980; Zissis, Lambert, Kapsenberg, Enders and Mutanda, 1981), it was assumed that these 'serotypes' would correspond to those previously defined by conventional neutralisation assays. However, when the serotypes of a wide variety of rotaviruses were investigated by means of IAHA it was found that the results were in close agreement with the serotypes as defined by CF, ELISA, IEM and RIA but differed

markedly from serotypes as defined by serum neutralisation assays. These unexpected findings prompted the idea that these differences were coded for by different genes (Kapikian, Cline, Greenberg, Wyatt, Kalica, Banks, James, Flores and Chanock, 1981). In the light of the above findings, Kapikian and colleagues suggested that the term 'serotype' be reserved exclusively for the antigen that reacts with neutralising antibodies, while the term 'subgroup' be used for the specificity detected by specific CF, ELISA, IEM, RIA and IAHA. There are two subgroups of human rotavirus, although all animal rotavirus strains belong to subgroup I. A correlation exists between the subgroup and RNA mobility patterns with segments 10 and 11 migrating slower in subgroup I strains than in subgroup II strains of human rotavirus (Kalica, Greenberg, Espejo, Flores, Wyatt, Kapikian and Chanock, 1981).

Serotypes

Earlier, the presence of 2 serotypes of human rotaviruses defined by NT had been reported (Thouless, Bryden and Flewett, 1978) Later, other workers identified 3 serotypes and speculated that there could be up to 5 different serotypes of human rotavirus (Flewett, Thouless, Pilford, Bryden and Candeias, 1978; Beards, Pilford, Thouless and Flewett, 1980; Sato, Inaba, Miura, Tokuhisa and Matumoto, 1982; Wyatt, Greenberg, James, Pittman, Kalica, Flores, Chanock and Kapikian, 1982).

On the other hand, the only report so far of the existence of serotypes in rotaviruses from animal hosts is that found in the avian species where 3 serotypes have been distinguished by the serum neutralisation tests. The serotypes were designated Ch 1 from chickens and Ty 1 and Ty 3 from turkeys (McNulty, Allan, Todd, McFerran, McKillop, Collins and McCracken, 1980). The relationship between two haemagglutinating types of calf rotavirus detected by Spence, Fauvel, Petro and Babiuk (1977), and neutralising serotypes is not known.

Cultivation and Propagation

With the exception of SA-11 and the related 'O' agent which were isolated in South Africa but not characterised initially as rotavirus (Malherbe and Strickland-Cholmley, 1967), the first rotaviruses to be isolated, propagated and characterised as such were the calf rotaviruses; as a result four widely used strains, namely Lincoln and Cody (Mebus, Kono, Underdahl and Twiehaus, 1971), UK Compton (Bridger and Woode, 1975) and Northern Ireland (McNulty, Allan and McFerran, 1976) were recognised. However the majority of strains did not multiply in tissue culture (Bridger and Woode, 1975; McNulty, Allan and McFerran, 1976; Mebus, Kono, Underdahl and Twiehaus, 1971). Earlier attempts to isolate human rotavirus in both bovine tissue and human organ cultures were unsuccessful (Albrey and Murphy, 1976; Wyatt, Kapikian, Thornhill, Sereno, Kim and Chanock, 1974). However non-productive infection with human rotavirus and

rotaviruses from other species was achieved when monkey kidney (LLC-MK2) and pig kidney monolayers were infected and centrifuged at 2100-3000 x g for 1-2hr. (Banatvala, Totterdell, Chrystie and Woode, 1975; Bryden, Davies, Thouless and Flewett, 1977; Thouless, Bryden, Flewett, Woode, Bridger, Snodgrass and Herring, 1977). Centrifugation of specimens at such speeds is unlikely to result in the sedimentation of virus but Banatvala et al.(1975), felt that the procedure may in some way enhance virus-cell attachment, especially as all positive specimens did not fluoresce when centrifugation was omitted. It has also been reported that centrifugation of cultures infected with different strains of chlamydiae and other rickettsiae are routinely carried out at similar speeds to enhance infectivity (Evans and Woodland, 1983, Weiss and Dressler, 1960).

The role of proteolytic enzymes which were known to enhance the infectivity of reoviruses (Spendlove, McClain, and Lennette, 1970; Spendlove and Schaffer, 1965), was investigated and the infectivity of non-cultivable rotaviruses was found to be enhanced (Barnett, Spendlove and Clark, 1979; Theil, Bohl and Saif, 1978). Unlike the reoviruses which were affected by a wide range of proteolytic enzymes, only trypsin enhanced the infectivity of rotavirus; but not chymotrypsin or carboxypepsidase A or B (Barnett, Spendlove and Clark, 1979).

By using trypsin, many other workers have been able to isolate and propagate calf, canine and, more recently, human rotaviruses in-vitro by pre-treatment of the faecal virus inoculum with trypsin and by the incorporation of trypsin in the maintenance medium (Almeida, Hall, Banatvala, Totterdell and Chrystie, 1978; Babiuk, Mohammed, Spence, Fauvel and Petro, 1977; Clark, Barnett and Spendlove, 1979; Hoshino, Wyatt, Scott and Appel, 1982; Sato, Inaba, Shinozaki, Fujii and Matumoto, 1981; Urasawa, Urasawa and Taniguchi, 1981; McNulty, Allan, Todd, McFerran, McKillop, Collins and McCracken, 1980). By incorporating trypsin into the culture medium, Graham and Estes (1980) enhanced the infectivity of rotavirus between 10 and 1000 fold. Initially, the mode of action of trypsin was unknown but, recently, it was suggested by two separate groups that the enzyme acts directly on the viral particles in the extracellular environment, converting non-infectious particles to infectious forms, and allowing multiple cycles of replication to occur in normally restrictive cells (Clark, Roth, Clark, Barnett and Spendlove, 1981; Estes, Graham and Mason, 1981).

It would appear that the use of MA104 cells, in conjunction with trypsin and rolling of the cultures enhances infectivity (Hasegawa Matsuno, Inouye, Kono, Tsurukubo, Mukoyama and Saito, 1982; Birch, Rodger, Marshall and Gust, 1983).

Epizootiology

Although calf rotavirus infection is essentially a disease of young animals in their first few weeks of life, nevertheless the virus has been isolated from adult animals showing no clinical disease (Woode, 1978a; Woode and Bridger, 1975). The disease is ubiquitous, having been reported from different parts of the world with temperate, subtropical or tropical climates. High titres of circulating rotavirus antibody are present in cows and this is thought to be as a result of continual subclinical infections (Woode and Bridger, 1975). The virus is known to survive adverse conditions (Woode, 1978a) and may not be inactivated by commonly used disinfectants (Snodgrass and Herring, 1977; Tan and Schnagl, 1981). It has been suggested recently that rotavirus can be spread in aerosols as it is stable at low and high relative humidities (Moe and Harper, 1983).

Infection with rotavirus is transmitted via the faecal-oral route and it has been suggested that contamination of the environment by infected calves or cows is the common source of infection to the calves (Woode and Bridger, 1975). The seasonal epidemics which occur more frequently in the cold or winter months in humans (Brandt, Kim, Yolken, Kapikian, Arrobio, Rodriguez, Wyatt, Chanock and Parrott, 1979; Bryden, Davies, Hadley and Flewett, 1975; Black, Merson, Rahman, Yunus, Alim, Huq, Yolken and Curlin, 1980) have also been observed in calves, with rotavirus

diarrhoea more liable to occur in winter and spring months (Snodgrass, personal communications). It has also been observed that sows tend to shed more rotavirus particles before and immediately after farrowing, thus exposing their piglets to a high risk of infection (Benfield, Stotz, Moore and McAdaragh, 1982). Whether this occurs in cows is not known, but in bovine mammillitis, immunosuppression at parturition leading to recrudescence of bovid herpesvirus 2 infection has been observed (Martin, Wells, Lauder and Martin, 1975).

Clinical Signs, Pathology, Pathogenesis and Mechanism of Inducing Diarrhoea

The clinical signs of both the natural and experimental rotavirus infections in animals and man have been fully described (Mebus, Underdahl, Rhodes and Twiehaus, 1969; Middleton, Szymanski, Abbot, Bortolussi and Hamilton, 1974; Shepherd, Truslow, Walker-Smith, Bird, Cutting, Darnell and Barker, 1975; Snodgrass, Herring and Gray, 1976). The incubation period varies between 15 hours and 4 days and the predominant signs, depending on the severity of the disease, are diarrhoea, depression, anorexia and dehydration, with vomiting in piglets and infants. Fever may or may not be present. The duration in experimentally infected animals is usually 3 - 9 days and the disease can be fatal (Mebus, Underdahl, Rhodes and Twiehaus, 1969; Snodgrass, Herring and Gray, 1976).

Knowledge of the pathology of rotavirus infection has mostly been acquired from studies on experimentally infected animals, which agrees with that observed in naturally infected calves (Pearson, McNulty and Logan, 1978). Mature villous epithelial cells (the villous epithelial cells excluding goblet cells and immature enterocytes of the small intestine) are the main targets for the replication of the virus as demonstrated by immunofluorescent staining. Infected enterocytes degenerate, desquamate and are subsequently replaced by cuboidal cells; consequently, there is stunting of the villi (Mebus and Newman, 1977; Mebus, Stair, Underdahl and Twiehaus, 1971; Snodgrass, Angus and Gray, 1977). Adhesion and fusion of the stunted villi although observed is not a common feature. Crypt epithelia are stimulated to hyperplasia, generating new undifferentiated cells which migrate onto the villi to replace lost cells (Snodgrass, Ferguson, Allan, Angus and Mitchell, 1979). It has been observed that the new immature cells from the crypts are resistant to reinfection as they are thought to lack specific receptors for the infecting virus (Halpin and Caple, 1976; Holmes, Rodger, Schnagl, Ruck, Gust, Bishop and Barnes, 1976; Stair, Mebus, Twiehaus and Underdahl, 1973).

Knowledge of the mechanisms of diarrhoea due to rotavirus infection has been derived largely by analogy from a pathologically similar virus, the transmissible gastroenteritis virus of swine. Rotaviruses selectively infect and destroy mature villous absorptive cells;

resulting in atrophy of the villi and impaired absorptive capacity. Thus materials entering the small intestine by ingestion or secretion are poorly absorbed and tended to pass along to the colon. When the amount of material exceeds the absorptive capacity of the colon, diarrhoea results (Moon, 1978).

Intestinal villous absorptive cells also have digestive as well as absorptive functions (Halpin and Caple, 1976), so in the event of rotavirus infection the digestive capacity of the intestine is also impaired. For example, loss of brush border diasccharidases lead to lactase deficiency; and ingested lactose is poorly digested (Ferguson, Paul, and Snodgrass, 1981; Moon, 1978). It has also been observed that the absorption of D-xylose was severely impaired in calves infected with rotavirus (Woode, Smith and Dennis, 1978). Thus the capacity of infected intestine to handle lactose is impaired in two ways: by malabsorption and maldigestion. Undigested and malabsorbed food in the intestinal lumen tends to retain water in the lumen by osmotic activity; this further contributes to diarrhoea. Although the inability to digest lactose could lead to osmotic diarrhoea, it has been reported that rotavirus infected lambs tolerate normal dietary levels of lactose (Ferguson, Paul and Snodgrass, 1981).

Diagnosis

The fact that rotavirus particles are normally present in large numbers (up to 10^{10} /gm of faeces) has made diagnosis by electron microscopy relatively straightforward; but the sophistication and high cost of the equipment and the need for highly trained personnel preclude its use as a common diagnostic tool. Electron microscopy is also limited in the number of samples that can be handled at any one time. It is therefore not surprising that alternative methods such as IF, CF and IEOP have been used for diagnosis. Another method which is now widely accepted because of its sensitivity, reproducibility, ease of application and suitability for large-scale use and which requires very small amounts of reagents, is the ELISA technique. Radioimmunoassay (Kalica, Purcell, Sereno, Wyatt, Kim, Chanock and Kapikian, 1977) which is based on similar principles to the ELISA and is of equal sensitivity is not used commonly for diagnosis, because of the potential hazard to the operators and the high costs of running the sophisticated equipment. All of these methods which are based on antibody/antigen reactions have one disadvantage in that they are not able to detect those rotaviruses that lack the group antigen. To overcome this problem, the viral nucleic acid can be directly extracted from faeces and identified by polyacrylamide gel analysis (Herring, Inglis, Ojeh, Snodgrass and Menzies, 1982; Theil, McCloskey, Saif, Redman, Bohl, Hancock, Kohler and Moorhead, 1981).

Immunity

About a decade ago a live-attenuated vaccine for oral administration to calves immediately after birth was developed (Mebus, White, Bass and Twiehaus, 1973). This vaccine was marketed in the USA as 'Scourvax-Reo' (Norden Laboratories, Lincoln, Nebraska). It has also been used experimentally to protect colostrum-deprived gnotobiotic calves against virulent challenge using the UK strain of calf, human and foal rotaviruses (Woode, Bew and Dennis, 1978; Woode and Crouch, 1978). In 1976, a modified live rotavirus-coronavirus vaccine called Scourvax II (Norden Laboratories, Lincoln, Nebraska) was also released in the USA for oral administration in calves. This vaccine was later marketed under a new name, 'Calf guard' (Norden Laboratories, Lincoln, Nebraska) and was approved for intramuscular injection of pregnant cows as well as for oral administration in calves. Although there have been reports supporting the efficacy of these products (Thurber, Bass and Beckenhauer, 1977; Twiehaus, Mebus and Bass, 1975) some other workers after conducting independent field trials with the vaccines have questioned their efficacy (Acres and Radostits, 1976; de Leeuw, Ellens, Talmon, Zimmer and Kommerij, 1980; Myers and Snodgrass, 1982). Thus Acres and Radostits (1976) observed no difference in the incidence and severity of diarrhoea between vaccinated and control calves, while Myers and Snodgrass (1982) found that there was no

difference in the colostral and milk antibody titres from vaccinated and non-vaccinated beef heifers, and therefore questioned the reliability of the vaccine on the basis of low antigenic stimulation.

It is known that many young animals which become infected with rotavirus already have high levels of circulating antibodies and Snodgrass and Wells (1978a, 1978b, 1978c) showed that whereas serum antibody does not correlate with protection against rotavirus infection, antibody in the gut lumen is essential for effective protection. For these reasons investigators turned their attention to the role of passive immunity in rotavirus infections. Thus Bridger and Brown (1981) and Lecce, King and Mock (1976), protected piglets against challenge by feeding them immune colostrum from cows. The levels of passive immunity conferred on neonatal calves and lambs by feeding them colostrum and milk from cows and ewes immunised either prior to mating or during the gestation period have also been investigated (Fahey, Snodgrass, Campbell, Dawson and Burrells, 1981; Snodgrass, Wells, Fahey, Herring and Campbell, 1979; Snodgrass, Fahey, Wells, Campbell and Whitelaw, 1980; Wells, Snodgrass, Herring and Dawson, 1978). The results showed that (i) vaccinated cows and ewes produced colostrum and milk with significantly higher titres of antibody to rotavirus than that of non-vaccinated dams. (ii) Lambs fed with colostrum and milk from vaccinated ewes were protected against rotavirus challenge. The virus

neutralising antibody activity was associated with IgG₁ in both colostrum and milk, although IgG concentration in mammary secretions of vaccinates and non-vaccinates did not differ. (iii) High levels of antibody persisted in both colostrum and milk for at least 28 days. In experiments where calves were fed on colostrum and milk with high levels of antibody and challenged with high doses of rotavirus, the calves showed signs of diarrhoea but the incubation and prepatent periods were prolonged significantly, compared with those of calves born to non-vaccinated cows.

Control By Management

Because morbidity in susceptible herds is often very high (90 - 100%), large quantities of viral particles are excreted by infected animals and this results in contamination of the environment (Woode, 1978a & b; Woode and Bridger, 1975). Although control of rotavirus infection in beef and dairy herds by good management alone is impracticable on a large scale, experiments with smaller herds have given encouraging results, whereby diarrhoea was reduced in calves by application of sanitary measures (de Leeuw, Ellens, Talmon, Zimmer and Kommerij, 1980; Van Opdenbosch and Wellemans, 1982). It has also been reported that overcrowding, the use of few calving boxes, delay of first colostrum intake of more than 1hr. and worsening of general hygiene all contribute to the diarrhoea. Improvement of these conditions when applied consistently resulted in reduced diarrhoea (Moerman, de Leeuw, van Zijderveld, Baanvinger and Tiessink, 1982).

Vaccination

Although the efficacy of the live-attenuated vaccine has been questioned by different workers, it is still used in the USA. Its failure in some herds has been attributed to the fact that the vaccine is neutralised by antibody containing colostrum in the gut of the calves (de Leeuw, Ellens, Talmon, Zimmer and Kommerij, 1980) and also the fact that it requires at least 7 days (range 7-21 days) to induce protection; a period when the calves are at high risk (Woode, Bew and Dennis, 1978).

On the other hand, the experimental procedure of passively protecting calves through the feeding of colostrum containing rotavirus antibody from immunised dams has proved successful even in field trials (Van Opdenbosch and Wellemans, 1982; Snodgrass, Stewart, Taylor, Krautil and Smith, 1982).

B. ENTERIC CORONAVIRUSES

Coronaviruses consist of ssRNA with M.W. of 3.8×10^6 and 4 to 6 structural polypeptides. They are pleomorphic particles with sizes ranging between 80 - 150 nm in diameter and surrounded by envelopes which contain radially arranged projections known as peplomers. The peplomers are of various shapes; filamentous, spherical, tear or petal shape. The family Coronaviridae consists of a number of species

including the transmissible gastroenteritis virus of swine (TGE) and infectious bronchitis virus of birds (IB). Antibodies to bovine coronavirus are widespread in sheep (Tzipori, personal communication) and detection of coronavirus-like particles has been reported from sheep (Pass, Penhale, Wilcox and Batey, 1982; Tzipori, Smith, Makin and McCaughan, 1978). Enteric coronaviruses have also been isolated from dogs (Binn, Lazar, Eddy and Kajima, 1970), pigs (Pensaert, Debouck and Reynolds, 1981), turkeys and man (Caul, Paver and Clarke, 1975; Holmes, 1979).

The bovine enteric coronaviruses have petal-shaped peplomers and were first described in the USA by Stair, Rhodes, White and Mebus (1972), who isolated the virus from diarrhoeic calves and successfully reproduced the infection in both colostrum-deprived and colostrum-fed calves. Later the virus was detected in the UK and Denmark (Bridger, Woode and Meyling, 1978), Canada (Acres, Laing, Saunders and Radostits, 1975), and New Zealand (Horner, Hunter and Kirkbride, 1975) and is probably globally distributed. Isolates of bovine enteric coronavirus from different countries were antigenically related (Cilli and Castrucci, 1981; Dea, Roy and Begin, 1980b).

Calves are usually infected at 1 - 3 weeks of age. The infection is characterised by depression, fever, salivation associated with ulcerative lesions in the oral mucosa, and diarrhoea which may or may not contain mucus and blood. In

experimentally infected calves, the incubation period before the onset of diarrhoea varies from 9 - 25 hr. The lesions consist of large amount of yellowish fluid in the intestinal lumen and petechiae of the abomasal mucosa. At the onset of diarrhoea the columnar epithelial cells of the small and large intestine appears normal but as the disease progresses the villi become severely affected (Mebus, Stair, Rhodes and Twiehaus, 1973a). The pathogenesis of diarrhoea in calves resembles that described for rotavirus infection. Initially the infection involves the epithelial cells of the anterior small intestine and then spreads posteriorly. As the infection progresses, cells are lost and become replaced by immature cuboidal or squamous cells (Mebus, Stair, Rhodes and Twiehaus, 1973a).

The virus has been adapted with considerable difficulty to grow in primary cultures of calf kidney (Mebus, Stair, Rhodes and Twiehaus, 1973b), tracheal organ culture (Bridger, Woode, and Meyling, 1978) and a number of bovine continuous cell lines (Dea, Roy and Begin, 1980a; Inaba, Sato, Kurogi, Takahashi, Ito, Omori, Goto and Matumoto, 1976). Generally most field strains are very difficult to cultivate.

Diagnosis is based on electron microscopic examination of faeces (Chasey and Lucas, 1977), IF staining of infected cells or cryostat sections of infected intestine (Jasper-Fayer and Thorsen, 1978), HAI (Bridger, Woode and Meyling, 1978), HEHA (van Blaken, de Leeuw, Ellens and

Straver, 1978/79) or ELISA (Callebaut, Debouck and Pensaert, 1982; Rodak, Babiuk and Acres, 1982).

Prevention of coronavirus infection in calves has been achieved by oral administration of a live attenuated vaccine (Mebus, Stair, Rhodes and Twiehaus, 1973b; Thurber, Bass and Beckenhauer, 1977). 'Calfguard' (Norden Laboratories, Lincoln, Nebraska), comprising a mixture of rotavirus and coronavirus for both oral (calves) and parenteral (pregnant cows) administration has been available in the USA for a number of years but the efficacy of the coronavirus component in the field has yet to be proved. The serological response of vaccinated heifers was not significantly different from non-vaccinated ones (Myers and Snodgrass, 1982).

A transmissible, haemagglutinating coronavirus-like agent called 'Breda Agent' (Woode, Reed, Runnels, Herrig and Hill, 1982) and isolated from 14 out of 47 calves in an outbreak of acute diarrhoea in the USA may be an antigenically distinct virus from the bovine enteric coronavirus. The 'Breda Agent' has not yet been reported by any other workers and attempts to cultivate it in tissue and organ cultures as well as in embryonated eggs have been unsuccessful.

C. THE SMALL ROUND VIRUSES

The small round viruses include astroviruses, caliciviruses and parvoviruses. They are so called because

of their size (22 - 30 nm) and circular or spherical shape. Although each has been associated with neonatal calf diarrhoea, their role, significance and economic importance are not yet clear.

(a). Astroviruses

Astroviruses have an average diameter of 29nm, contain ssRNA and exhibit a 5 or 6 pointed stellate configuration superimposed on circular outline. They were first described in electron micrographs of diarrhoeic faeces of children (Madeley and Cosgrove, 1975). Since then they have been reported in calves (Woode and Bridger, 1978), lambs (Snodgrass and Gray, 1977), deer (Tzipori, Menzies and Gray, 1981), turkeys (McNulty, Curran and McFerran, 1980) and cats (Hoshino, Zimmer, Moise and Scott, 1981). They are generally required in large numbers to produce disease (Flewett, 1978). Experimentally, Woode and Bridger (1978) were able to induce the infection in gnotobiotic calves with their calf isolates whereas Snodgrass and Gray (1977) obtained only a mild diarrhoea in experimentally infected gnotobiotic lambs with the lamb isolate. In the calf experiment, only mature villi were infected with partial villous atrophy. Infected cells sloughed off at the onset of diarrhoea and were replaced by cuboidal cells from the crypt.

Recently, Lee and Kurtz (1981) serially propagated the human astrovirus first in human embryonic kidney cells and then on LLC-MK2 cells by incorporating trypsin in the maintenance medium. Two serotypes of human astroviruses have also been defined (Lee and Kurtz, 1982).

Diagnosis is mainly by EM and IF staining. There appears to be no serological relationship between the human and animal isolates (Woode and Bridger, 1978; Snodgrass and Gray, 1977; Lee and Kurtz, 1982).

(b). Caliciviruses

Members of the new family Caliciviridae have been known as pathogens in cats (Zwillenberg and Burki, 1966), pigs (Madin, 1970) and probably sea lions (Smith, Akers, Madin and Vedros, 1973). They measure about 31nm in diameter, contain a ssRNA genome and have a well defined star or lattice-shaped surface configuration. Caliciviruses differ from astroviruses by having only 6 pointed stars, are bigger, have less well defined edges, with dark hollow centres and surface hollows that appear round or oval, while astroviruses have an unbroken edge and a surface hollow that is triangular (Madaley, 1979; McNulty, Curran and McFerran, 1981). Caliciviruses have been described in electron micrographs of faeces of children with gastroenteritis (Flewett and Davies, 1976; Madaley and Cosgrove, 1976).

The presence of calicivirus-like particles, termed 'Newbury Agent' in acute gastroenteritis of calves has also been reported but only in mixed infections with rotavirus, coronavirus or astrovirus (Woode and Bridger, 1978) So far no field outbreak of calf diarrhoea has been attributed solely to caliciviruses, although in experimentally infected calves the 'Newbury Agent' caused a mild to severe diarrhoea with malabsorption and villous atrophy (Woode and Bridger, 1978).

Diagnosis is mainly by direct electron microscopy.

(c). Parvoviruses

The parvoviruses are small ssDNA viruses measuring between 22 and 25 nm in diameter. Although they have been isolated from calves (Abinanti and Warfield, 1961; Bates, Storz and Reed, 1972; Huck and Wood, 1975; Storz, Leary and Bates, 1978), they tend not to be commonly associated with bovine neonatal diarrhoea. However, parvoviruses are responsible for serious infections of cats (feline panleucopaenia) and, more recently in dogs, in which they cause acute enteritis and myocarditis of puppies, often with high mortality. The virus replicates within cell nuclei, particularly of rapidly dividing cells such as the crypt cells in the intestine. For this reason, parvovirus infections are usually more severe than those caused by rota- or corona-viruses.

Parvovirus can be diagnosed by IF staining, by EM of intestinal content in which very large number of virus particles can be detected and also by HAI of gut contents.

BACTERIAL CAUSES OF NEONATAL DIARRHOEA

A. ESCHERICHIA COLI (E. coli)

E. coli are Gram-negative bacilli which occur as normal inhabitants of the intestinal tracts of animals and man. Some strains however are enteropathogenic, able to multiply rapidly and cause disease (Buxton and Fraser, 1977; Moon, 1974).

These pathogenic strains have been called enterotoxigenic E. coli (ETEC) and possess two main virulence factors which distinguish them from non-pathogenic E. coli. The two factors, namely, the production of adhesive antigen (pili or fimbrae) and enterotoxins are both essential for the pathogenicity of ETEC. Both factors are plasmid controlled (Smith and Halls, 1967; Moon, 1980). Pili enable ETEC to adhere to the small intestine and enterotoxins are produced at the gut surface.

There are several enterotoxins produced by ETEC, but the important one in calves is referred to as heat stable (ST) enterotoxin this has a low M.W., is non-immunogenic and is

not inactivated by heat even at 100°C (Guinee, Jansen, Wordstrom and Sellwood, 1981). There are two types of ST (STa and STb) recognised on the basis of their solubility in methanol (Burgess, Bywater, Cowley, Mullan and Newsome, 1978) and detected by two different bioassay systems. All bovine ETEC seem to produce only STa (Sherwood, Snodgrass and Lawson, 1983) which is detected by the infant mouse assay and ligated loop inoculation method in neonatal calves and piglets (Burgess, Bywater, Cowley, Mullan and Newsome, 1978).

ETEC usually cause diarrhoea in calves less than 3 days old (Smith and Halls, 1967; Morin, Lariviere, Lallier, Begin, Ray and Ethier, 1978; Acres, Laing, Saunders and Radostits, 1975 ; Moon, 1974). Experimental infection in calves less than 24 hr. old has been reported (Smith and Halls 1967; Tzipori, Makin, Smith and Krautil, 1981). Infection is via the oral route and, because the pH of the neonatal stomach is near neutral, the organisms can pass through to the small intestine.

Diagnosis of E. coli diarrhoea is based on:

- i. history of profuse watery diarrhoea with peracute fatal dehydration and death of individuals within a few days of birth.

- ii. slide agglutination or ELISA on E. coli cultures grown on minca-Isovitalex medium to enhance the detection of K99

(Guinee, Veldkamp and Jansen, 1977). ELISA can also be performed directly on faecal samples to detect K99 (Ellens, de Leeuw and Rozemond, 1978 and 1979).

iii. IF or Giemsa staining of histological sections of intestine from moribound animals.

iv. infant mouse assay for ST.

Protection of calves against ETEC by vaccination with K99 pili has been attempted with good results. Calves born to vaccinated pregnant cows were protected against challenge with K99 positive strains of E. coli (Acres, Isaacson, Babiuk and Kapitany 1979; Nagy, 1980; Snodgrass, Nagy, Sherwood and Campbell, 1982). It is hoped that a polyvalent vaccine incorporating K88, K99 and 987P will give a broad spectrum of protection to calves, lambs and pigs (Moon, 1980).

B. SALMONELLAE

These are Gram-negative bacteria which, as a result of their rapid spread, can assume epidemic proportions in calves. They infect calves 3 - 6 weeks old and older animals. In Britain two important species are S. typhimurium and S. dublin as they are regularly associated with enteric salmonellosis in cattle (Sojka and Field, 1970;

Sojka, Wray Hudson and Benson, 1975). Calves are usually at risk in affected herds as infected adults become carriers for life and excrete the organisms, although they are not of common occurrence in home-reared calves (Snodgrass, Sherwood, Terzalo and Synge, 1982).

C. CAMPYLOBACTERS

These organisms are small curved or comma-shaped Gram-negative rods, which infect a variety of animals including man. Their importance in relation to neonatal diarrhoea in calves is not yet clear. The organisms have been isolated from both normal and diarrhoeic calves (Snodgrass, Sherwood, Terzolo and Synge, 1982). Infections with Campylobacter spp have been reproduced in calves using pure cultures of isolates (Al-Mashat and Taylor, 1980a, 1980b and 1981) but further work is needed to establish their aetiological significance.

PROTOZOAN CAUSES OF NEONATAL DIARRHOEA

CRYPTOSPORIDIA

Cryptosporidia are protozoan parasites measuring about 3 - 6 μ m in diameter, which infect the microvillus surface of intestinal epithelial cells (Angus, 1983). The parasites were first observed in mice by Tyzzer in 1907. As with other coccidian parasites, cryptosporidia have a two-stage reproductive cycle - the asexual stage (schizogony) followed

by the sexual stage (gametogony). There has been conflicting reports in the literature as to whether the parasites are extracellular (Panciera, Thomassen and Garner, 1971; Tzipori, Campbell, Sherwood, Snodgrass and Whitelaw, 1980) or intracellular (Inman and Takeuchi, 1979; Pearson and Logan, 1978 and 1983). Also, whether they are host specific and therefore have many species (Vetterling, Jervis, Merrill and Sprinz 1971; Iseki, 1979) or are a single genus parasite in which case they may present a potential zoonosis (Tzipori, Angus, Campbell and Gray, 1980).

Cryptosporidiosis has been reported in many species including calves (Angus, 1983; Pohlenz, Moon, Cheville and Bemrick 1978). It was first reported in cattle in the USA (Panciera, Thomassen and Garner, 1971). Thereafter, outbreaks of calf cryptosporidiosis have been reported in many countries including Canada (Morin, Lariviere and Lallier, 1976), UK (Pearson and Logan, 1978; Snodgrass, Angus, Gray, Keir and Clerihew, 1980; Tzipori, Campbell, Sherwood, Snodgrass and Whitelaw, 1980) and Australia (Jarrett and Snodgrass, 1981).

Calves are readily infected when the oocysts containing 4 sporozites are ingested. The incubation period is between 2 - 5 days, often with no clinical signs or with depression and anorexia as the first clinical signs observed (Tzipori, 1981), followed by diarrhoea, debilitation and a chronic course (Panciera, Thomassen and Garner, 1971) or death in

newborn animals (Tzipori, 1981).

Pathological changes associated with cryptosporidiosis are found in the ileum and large intestine. These include shortening and flattening of the epithelial surfaces and fusion of adjacent villi.

To date no effective treatment has been found against the parasites (Snodgrass, Angus, Gray, Keir and Clerihew, 1980). A drug which was eventually found to be effective against the parasites was not recommended for use as its therapeutic level appeared to be toxic for calves (Moon, Woode and Ahrens, 1982).

Diagnosis of cryptosporidiosis is made by demonstrating oocysts in faecal smears stained with Giemsa (Pohlenz, Moon, Cheville and Bemrick, 1978), modified Ziehl-Neelsen or dilute carbol fuchsin. The parasite can also be identified by flotation methods or by histological examination of gut sections for the presence of the parasites.

AIMS OF THIS STUDY

Interest in rotavirus infections received little attention until electron microscopy revealed that faecal samples from diarrhoeic calves and children with non-bacterial gastroenteritis contained virus particles

morphologically resembling reovirus; which were indistinguishable from each other and are now termed rotaviruses (Flewett, Bryden, Davies, Woode, Bridger and Derrick, 1974). Since then considerable progress has been made in knowledge of the morphogenesis, biochemical and biophysical properties, cultural characteristics, antigenic relationship and pathogenesis of rotaviruses in man and animals, but unfortunately little is known about the epidemiology and immunology of these infections.

More recently there have been an increasing number of reports of subgroups and serotypes among rotaviruses from human sources which have major implications for the epidemiology of human infections and the development of immuno-prophylactic strategies. For these reasons it was decided, in this present work, to examine serological and other variations among rotaviruses from calves by a variety of tests and to investigate the practical significance of the findings by in-vivo cross-protection studies.

Attempts to devise suitable techniques for serotyping of field strains of bovine rotaviruses on a large scale would also be made.

CHAPTER 2GENERAL MATERIALS AND METHODS

The materials and methods used in this thesis are listed as follows:-

MEDIAVirus and Cell Culture Growth Medium

	Cell Culture Growth Medium	Virus Medium
E '59 10x concentration(1) (Gibco, Europe, UK)	10.0% v/v	-
Medium 199 10x conc. (1) (Gibco, Europe, UK)	-	10.0% v/v
BSA	-	0.5% v/v
FBS (2)	10.0% v/v	-
Sodium bicarbonate	0.2% w/v	0.2% w/v
Lactalbumin hydrolysate	1.0% v/v	1.0% v/v
Yeast extract	-	1.0% v/v
HEPES (Sigma Chemicals Co. UK)	-	0.025M
Penicillin (Glaxo, UK)	100 IU/ml	100 IU/ml
Streptomycin(Glaxo, UK)	200 µg/ml	200 µg/ml
Polymixin (Wellcome Fdns.,UK)	50 IU/ml	50 IU/ml
Fungizone (Squibb, UK)	-	0.05mg/ml
Trypsin(Sigma Chemicals Co. UK)	-	5-10 µg/ml

Plaque Assay Medium

BME 10x concentration(1) (Gibco, Europe, UK)	10.0% v/v
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(1) Based on Earles Balanced Salts: 0.12M NaCl, 0.005M KCl, 0.001M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.001M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0018M CaCl_2 with L-glutamine and without sodium bicarbonate.

(2) Mycoplasma free, inactivated at 56°C for 30 min.

Plaque Assay Medium cont'd.

BSA	0.5% v/v
Lactalbumin hydrolysate	1.0% v/v
DEAE Dextran	50 µg/ml
Trypsin	1-2 µg/ml
Noble Agar (Difco, UK)	0.6%
Amino acid 50x concentration (Gibco, Europe, UK)	5.0%
Antibiotics	same as above

Faecal Virus Extraction Medium

Maintenance Medium 199 (Working Strength) containing:

Penicillin	400 IU/ml
Polymixin	200 IU/ml
Streptomycin	800 µg/ml
Genticin (Nicholas Labs. Ltd., UK)	500 IU/ml

BUFFERSPhosphate Buffered Saline (PBS) pH 7.2.

0.14M NaCl, 0.003M KCl, 0.008M Na₂HP0₄, 0.0015M KH₂P0₄, in distilled water and autoclaved at 15lb pressure for 15 min.

Tris-HCl buffer pH 7.5

20mM Trizma in distilled water. pH adjusted to 7.5 using 1M HCl and sterilised by filtration through a 0.45µm millipore membrane. All media and buffers were prepared using sterile distilled water.

ELISA buffers

Coating antibody (carbonate/bicarbonate) buffer, pH 9.6. The coating antibody buffer consisted of: 0.05M Na_2CO_3 , 0.05M NaHCO_3 , 0.004M NaN_3 in distilled water.

Plate washing solution (PBS/T)

This consisted of 0.05% Tween 20 (polyethylene sorbitan monolaureate, Sigma Chemicals Co. UK) in PBS.

Substrate (DEA) buffer pH 9.8

This was made up as follows:

Diethanolamine (DEA) 100ml. Distilled water, 840ml, MgCl_2 0.005M NaN_3 0.004M and the pH adjusted to 9.8 with 1M HCl.

Enzyme substrate (p-nitrophenyl di-sodium phosphate, Sigma Chemicals Co. UK) was prepared when required by dissolving 1 tablet (5mg) in 5ml of substrate buffer.

All ELISA reagents were stored at 4°C and discarded after 14 days.

Cells and Cell Culture Procedures

1. MA104 cells - an embryonic rhesus monkey kidney continuous cell line was obtained at the 56th subculture level from Wellcome Foundations, UK. Other cell lines were available in the Institute, namely:

2. Vero cells - an African green monkey kidney continuous cell line.

3. LLC-MK2 cells - an adult rhesus monkey kidney continuous cell line.

4. MDBK cells - an adult bovine steer kidney continuous cell line.

5. and 6. Primary bovine (EBK) and ovine (EOK) embryonic kidney cells were prepared as described by Paul (1965) and grown in cell culture growth medium 199.

All cells were grown and maintained either in medium 199 or E'59 (MA104 cells) and split at a ratio of 1:3 every third day. For investigative purposes cells were seeded at a concentration of 2×10^5 cells/ml (continuous cell lines) and 3×10^5 cells/ml (primary cell cultures) and dispensed in 0.1ml amounts in microtitre plates (Tissue Culture Grade, Sterilin, UK).

Viruses

1. The UK (Compton) strain of calf rotavirus (CRV) was originally isolated and propagated in calf kidney cells at IRAD Compton, UK (Bridger and Woode, 1975), cloned in MA104 cells by Dr. R.G. Wyatt, National Institute of Allergy and Infectious Diseases, Maryland, USA, from whom the strain was obtained.

2. The Northern Ireland strain of CRV was obtained by courtesy of Dr. M.S. McNulty, Stormont, Belfast. This virus was originally isolated in calf kidney cells but adapted to MDBK cells after the 4th passage. It was further adapted to grow in MA104 cells in this laboratory.

3. The Nebraska calf diarrhoea virus (Lincoln strain) was obtained from Prof. C.A. Mebus, Lincoln, Nebraska, USA. It was isolated originally on EBK cell culture and passaged over 39 times in the same cell system before being adapted to MA104 cells.

4. The simian strain of rotavirus (SA-11) was kindly supplied by Dr. B. Totterdell, St. Thomas Hospital, London. The virus had been propagated in LLC-MK2 cells but was adapted to MA104 cell in this laboratory.

Electron Microscopic (EM) Examination of Faecal Samples

An approximately 10% v/v suspension of faecal sample was made in distilled water and centrifuged at 1200g for 10 min. One drop of the faecal supernatant was then applied to a formvar carbon-coated 400-mesh grid for 2 min. Excess supernatant was removed by blotting paper and the rest allowed to dry on the grid. One drop of 1.0% ammonium molybdate stain, pH6.0, was then added to the grid and left for 45 sec, after which excess stain was removed by means of blotting paper and the grid allowed to dry. The preparation was then examined on a Siemens electron microscope at a magnification of x 40,000.

ELISA for the Screening of Field Samples

The method used was a slight modification of the procedure recommended by Fahey, Snodgrass, Campbell, Dawson and Burrells (1981) and Yolken, Barbour, Wyatt, Kalica, Kapikian and Chanock (1978), designed as a double sandwich technique for the detection of antigen.

Step 1. Flat bottomed polystyrene microelisa plates (Dynatech M129 A or B, UK) were used as the solid phase to attach the antibody. To each well was added 100 μ l of rabbit IgG to CRV diluted at 1/1600 in coating antibody (carbonate/bicarbonate) buffer pH9.6 and the plates left overnight at 4°C.

Step 2. The following morning after washing the plates with PBS/T solution, 100 μ l of the test sample was added to 6 wells per sample and then incubated for 3 hr. at 37°C. Known positive and negative faecal samples were included as controls.

Step 3. Plates were again washed in 3 changes of PBS/T solution. The first two wells received 100 μ l of PBS/T. One hundred μ l of a 1/20 dilution of a known positive calf rotavirus antiserum was added to the next two middle wells, while the last two wells received 100 μ l of a known 1/20 dilution of gnotobiotic coronavirus (rotavirus negative) antiserum. The plates were again incubated at 37°C for 1hr.

Step 4. After washing, 100µl of sheep anti-rabbit IgG conjugated to alkaline phosphatase (Alkaline phosphatase Type VII, Sigma Chemicals Co., UK) at 1/160 dilution in PBS/T was added to all 6 wells. The plates were incubated for 1hr. at 37°C and washed.

Step 5. One hundred µl substrate (p-nitrophenyl phosphate, Sigma, Chemicals Co. UK) in substrate buffer was added to all the wells and left at room temperature for the reaction to occur. After 1 hr. the tests were read by measuring the absorbance of light at a wavelength of 405nm using a Titertek Multiskan spectrophotometer (Flow Laboratory, UK). Only those samples that were specifically blocked partially or completely by the calf rotavirus antiserum and also showed a colour reaction in the last two wells which contained anticoronavirus antiserum were regarded as positive. Samples that showed colour in all 6 wells were regarded as non-specific.

ELISA for the Rapid Screening of Rotavirus Excretion in Experimentally Infected Gnotobiotic Lambs

The above procedure was followed but with modification to some of the steps: Samples were tested in 4 wells as against 6 and the whole of Step 3 was omitted.

Purification of CRV from (a) Faeces and (b) Tissue Culture Adapted Viruses

A 20% v/v or w/v faecal sample was made in 20mM

Tris-HCl buffer pH7.5 and disrupted in a bath sonicator for 1 min. Thereafter an equal volume of fluorocarbon (Arcton 113, ICI, UK) was blended with the suspension in a Silverson homogeniser (Silverson Machines Ltd., UK) and the aqueous phase collected after centrifugation at 2640g for 30 min. at 5°C. (This arcton step was omitted with tissue culture adapted viruses). Ten percent sodium dodecyl sulphate (SDS) was added to the supernatant to a final 1% v/v and held for 5 min. at room temperature and then cleared at 10,000g for 20 min. Virus was pelleted from the supernatant at 70,100g for 45 min. The pellets were homogenised in 1-2ml tris buffer, layered onto a discontinuous gradient consisting of 2ml of a solution containing 1.31M CsCl and 1.58M sucrose, overlaid with 2ml of 1.58M sucrose in tris buffer and centrifuged at 154,400g for 60min. at 5°C. The opalescent band which appeared just below the interphase was harvested, diluted four-fold and pelleted. Pellets were resuspended in 1-2ml of tris buffer and layered onto a 5-step 10-46% CsCl w/v gradient, centrifuged at 50,400g for 18hr. at 5°C. The viral band was harvested by an upward displacement with an ISCO density gradient fractionator (MSE Fisons, UK), fitted with a u.v. monitor, using maxiden (Nyegaurd, Oslo) as pump out solution. The fractions containing viral peaks were pooled, density determined by refractometry, diluted with tris buffer and pelleted at 113,500g for 60 mins at 5°C. Occasionally, viral pellets from the CsCl gradient were layered onto a preformed 10% to 40% sucrose gradient centrifuged and harvested as above.

In later experiments, to obviate the use of the fractionator, 1.0 μ g/ml ethidium bromide was added to a 5-step CsCl/sucrose gradient and centrifuged at 50,400g for 18 hr. This gradient consisted of 1.66M sucrose/1.49M CsCl and 1.56M sucrose/1.49M CsCl at the extremities. An intermediate density was achieved by mixing equal volumes of the two extremes and two further steps were achieved by mixing the intermediate solution with the two extremes. The virus band was located by fluorescence under u.v. light, harvested with a syringe, diluted in tris buffer and pelleted. The pellets were examined by EM as described above and used immediately to immunise rabbits or stored at -70°C until required.

Production of Hyperimmune Antisera in Rabbits

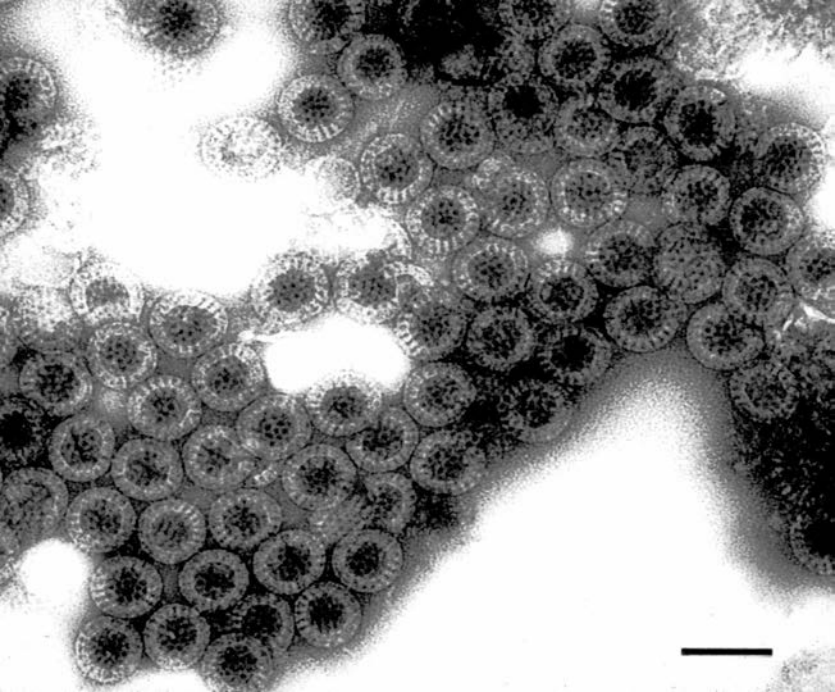
Purified pellets containing greater than 99% complete virions (Fig. 2.1) were diluted to 1 ml in tris buffer with 2% Tween 80 and emulsified by stepwise addition to oil adjuvant, 90% Marcol 52 (Esso) and 10% Arlacel (Sandrin, Chemicals, UK) at a ratio of 1:2, while being emulsified with a Ystral GmbH homogeniser (Funkentsort Dottingen, Western Germany). Rabbits previously shown to be free from antibodies to rotavirus were selected for immunisation. Each rabbit received a deep intramuscular injection of 1.0 ml of the emulsion at two different sites. The injection was repeated 14 days later and the rabbits were bled by cardiac puncture 7 - 10 days after the second injection.

FIGURE 2.1

Electron micrograph of calf rotavirus
showing mainly smooth or complete
particles

Stain: 1% ammonium molybdate

Bar : represents 100nm



Adaptation of Field Strains of CRV to Tissue Culture

A 10-20% faecal filtrate was made in maintenance medium homogenised and centrifuged at 2100g for 30 min. The aqueous phase was filtered through a 0.45µm membrane and treated with 10µg/ml trypsin at 37°C for 1hr. Two hundred µl of the trypsin-treated filtrate was used to infect monolayers of MA104 cells in tubes containing coverslips or in 0.5 ml amounts to infect monolayers in 25 x 3 cm³ Falcon flasks (B-D & Co. California, USA). Virus was allowed to adsorb for 90 min. at 37°C after which the inoculum was removed by washing. The cell cultures were fed with maintenance medium containing 1µg/ml trypsin and incubated at 37°C in roller drums. Monolayers were observed daily for cpe and coverslips were examined by immunofluorescence for evidence of virus replication at each passage level. Virus was passaged blindly every three days up to the 4th passage level when cpe began to appear. Virus replication and cpe were enhanced by treating the virus inoculum with trypsin and incubating the infected cultures in roller drums.

Purification of Tissue Culture Adapted Field Strains of CRV

From the 6th passage level onwards, when virus had started producing a regular and characteristic cpe, the virus was cloned either by the limiting dilution procedure alone or in conjunction with the plaque assay method.

Limiting Dilution

Serial 10-fold dilutions of virus were made in maintenance medium and each dilution was used to infect MA104 cells grown in Falcon flasks (B-D & Co. California, USA). Three Falcon flasks were used per dilution, rolled at 37°C and observed daily for cpe. Three to four days p.i., the flasks with the highest dilution of virus showing cpe were harvested by 3 cycles of rapid freezing and thawing and used to raise virus stock.

Plaque Assay

Two ml of MA104 cell suspension at a concentration of 2×10^5 cells/ml was dispensed into 12 wells of Limbro plates (Flow Laboratories, UK) and allowed to monolayer overnight at 37°C in an incubator containing 5% CO₂ atmosphere. Virus dilution was titrated in triplicate at 0.1 ml/well. Control wells contained maintenance medium in place of virus dilutions. Plates were incubated at 37°C in a 5% CO₂ atmosphere for 90 min. with regular rocking. Thereafter the wells were washed with serum-free maintenance medium and each received 2 ml of plaque assay medium which was allowed to solidify before the plates were returned to the incubator. After 3 - 4 days the plates were stained with 0.001% neutral red in maintenance medium. Excess stain was removed after 1 hr. and the plates were reincubated until plaques developed. The plaques appeared as colourless areas against the red-stained background of living cells. Using a pasteur pipette, single discrete areas of plaques from wells

infected with the highest dilution of virus were selected, resuspended in trypsin containing medium and used to infect monolayers of MA104 cells for virus stock.

Polyacrylamide gel Electrophoresis (PAGE) and Silver Staining Technique

Nucleic Acid Extraction

Faecal samples were diluted 1/4 by weight with 0.1M SDS extraction buffer pH 8.3 (1). An equal volume of 3:2 (v/v) phenol-chloroform mixture was added to the faecal suspension and the sample homogenised using a vortex mixer. The mixture was centrifuged at 1200g for 10 min. and the resultant clear upper layer removed. A sample was then prepared for electrophoresis by the addition of 10 μ l of 25% (w/v) sucrose containing 0.2% bromophenol blue to 30 μ l of the aqueous phase.

Purification of dsRNA from Faeces in CF 11 Column

The principle of the procedure is that in the presence of 15% ethanol, dsRNA will bind to CF 11 cellulose and unbind in its absence. The occasional samples of dsRNA extracted from faeces were further purified in a CF 11 column. A packed column was washed with STE buffer pH 6.98 (2) and equilibrated with STE containing 15% ethanol.

(1) SDS extraction buffer: 0.05M Tris, 0.1M NaCl, 2% SDS

(2) STE buffer: 0.05M Tris, 0.083M NaCl, 0.0011M EDTA

Samples taken up in STE - 15% ethanol buffer were then added to the column, mixed, allowed to stand for 10-15 min. and then washed twice with STE - 15% ethanol buffer. The dsRNA was then removed from the column by the addition of ethanol-free STE buffer and then precipitated with alcohol after 10 μ l of carrier tRNA had been added and the NaCl concentration raised to 0.1M.

Polyacrylamide-gel Electrophoresis (PAGE) for dsRNA

Two slightly different systems were used for the fractionation of genome dsRNA of calf rotavirus in 1.5 mm thick gels:

(1). The 7.5% polyacrylamide slab gel using the continuous buffer system was used to analyse rotavirus from faeces, while, (2)., the 7.5% polyacrylamide gel without a stacking gel using the discontinuous buffer system as recommended by Laemmli (1970) was used for the analysis of dsRNA from purified virions of rotavirus prepared from tissue cultures or infected faeces. For both systems the acrylamide to bis acrylamide ratio was 30:0.8 and polymerisation was achieved by the addition of N,N,N'N' tetramethylethylenediamine (TEMED BDH Chemicals UK) and 10% (w/v) ammonium persulphate. For the continuous system the gel and electrode buffer was 0.036M tris, 0.03M sodium dihydrogen phosphate, 0.0011M EDTA -di-Na (pH7.5) and electrophoresis was performed at room temperature for 18 - 20 hr. at 30mA and 70v. For the discontinuous systems, Laemmli buffers (Laemmli 1970) were used. Electrophoresis was carried out for 11 - 12 hr. at a

constant current of 22mA and 52v. Each track was loaded with either 30-40 μ l (faecal dsRNA) or 10-30 μ l of purified virion dsRNA.

Silver Staining Technique for dsRNA

The gels were stained by using a slight modification of the method recommended by Sammons, Adams and Nishizawa (1981). The gels were fixed in 10% ethanol and 1% acetic acid for 30 min. (for the continuous buffer system) or for 3 - 4 hr. with gentle rocking in 5 or 6 changes of fixative (for the discontinuous buffer system)- This prolonged washing procedure was necessary to rid the gel of SDS and any chloride ions present which would precipitate silver ions during staining if not removed beforehand. The gels were then soaked in degassed 0.011M silver nitrate for 1 hr., rinsed quickly with distilled water and an appropriate amount of degassed 0.75M sodium hydroxide containing 0.1M formaldehyde and 0.0023M sodium borohydride was added to the gel for a maximum of 10 min. or until the desired level of staining was attained. The gel was then soaked in a 0.07M sodium carbonate solution to further enhance the intensity of dsRNA band staining and to stop the development reaction.

SDS-PAGE for Rotavirus Polypeptides

This was carried out in a slab gel of 0.75mm thickness using the discontinuous system of Laemmli (1970). The protein of purified rotavirus samples from infected tissue

culture cells were dissociated to the polypeptide level by boiling at 100°C for 90 sec in a final sample buffer containing 2% SDS, 0.1M 2 - mercaptoethanol, 0.1M tris-HCl buffer pH 6.8 and 0.02% bromophenol blue (tracker dye). Polypeptides along with reference standards (β - galactosidase MW 130,000, phosphorylase A, 92,000, ovotransferrin 76 - 78,000, albumin 66,200, ovoalbumin 45,000, chymotrypsinogen A 25,700, Myoglobin 17,200 and Cytochrome C 12,300) were loaded into the gel tracks and concentrated through a 3% stacking gel prior to resolving in a 10% separating gel in tris-glycine electrode buffer pH 8.6 (1). Electrophoresis was performed at room temperature for approximately 4 hr. with a constant current of 13mA.

Silver Staining Technique for Polypeptides

The silver staining procedure for proteins in polyacrylamide gels as described by Morrissey (1981) was followed. The gel was washed first in a solution consisting of 50% methanol and 10% acetic acid for 30 min. then in a solution of 5% methanol and 7% acetic acid for another 30 min. before being fixed in a 10% glutaraldehyde solution for 30 min. Thereafter the gel was washed in several changes (every 30 min.) of distilled water over a 3 hr. period and then soaked in 5 μ g/ml dithiothreitol for 30 min. after which it was stained with 0.1% silver nitrate for 1 hr. After a quick rinse in distilled water the gel was soaked in a developer consisting of 3% sodium carbonate and 50 μ l of

(1) Tris glycine buffer: 0.25M tris, 0.192M glycine

37% formaldehyde until the desired level of staining was attained, usually within 10 min. The staining reaction was stopped by the addition of 5 ml of 2.3M citric acid directly into the developer and rocked for 10 min. The gel was then washed several times in distilled water over a 30 min. period, dried and photographed.

Immunofluorescence Staining

Infected cells in microtitre plates, flying coverslips or cytopsin microscope slides were washed with warm (37°C) PBS and fixed with chilled acetone for 10-15 min. and then dried with hot air from a hair dryer. Fifty μ l of 1/50 dilution of rabbit hyperimmune antiserum to calf rotavirus were added to each well and incubated at 37°C for 30 min. Plates were washed in three changes of warm PBS and 50 μ l of 1/50 dilution of sheep anti-rabbit immunoglobulin conjugated with fluorescein isothiocyanate (Wellcome Reagents, UK) was added to each well. Further incubation at 37°C for 30 min. was followed by three washings in PBS. The plates were dried and examined for fluorescing cells using a Leitz Ortholux II u.v. light microscope.

Neutralisation Assay

1. Constant-virus Varying Serum Method

Virus was diluted to give 50-100 fluorescent foci per 25 μ l (Beards, Pilford, Thouless and Flewett, 1980; Thouless, Bryden, Flewett, Woode, Bridger, Snodgrass and



Herring, 1977). Sera were inactivated at 56°C for 30 min. and doubling dilutions in serum-free medium were made in transfer plates (Dynatech, UK). Equal volumes of serum dilutions and virus were incubated at 37°C for 2 hr. with appropriate controls. Monolayers of cells in microtitre plates (Tissue Culture Grade, Sterilin, UK) were infected with the serum/virus mixture by aligning the transfer plate over the microtitre plate wells. The plates were centrifuged at 800 - 1000 rpm at 37°C for 1 hr. and incubated at 37°C for 16 - 18 hr., after which they were washed, fixed and stained IF. Titres were expressed as the reciprocal of the highest dilution giving a 50% reduction of fluorescent foci when compared with the control wells.

2. Constant-Serum Varying Virus

Half \log_{10} dilutions of extracted faeces treated with 10 $\mu\text{g}/\text{ml}$ trypsin incubated at 37°C for 1 hr. were made in serum-free medium. Fifty μl of each dilution of virus was titrated against 50 μl of inactivated serum containing 4 antibody units (1 Unit = the amount of antibody which neutralised 100 TCID₅₀ of homologous rotavirus) in a transfer plate and left at 37°C for 2 hr. Monolayers of MA104 cells in microtitre plates were infected as above, allowed to adsorb for 90 min. and then washed carefully with warm maintenance medium. This step was necessary in order to remove the otherwise toxic materials present in the faecal extract. Thereafter medium was added to each well and the plates centrifuged as above, incubated for 16-18 hr. and stained by IF.

CHAPTER 3PROPAGATION OF CALF ROTAVIRUS ISOLATES IN:

i. Gnotobiotic Lambs

ii. Tissue Culture

i. PROPAGATION OF CALF ROTAVIRUS ISOLATES IN GNOTOBIOTIC
LAMBSINTRODUCTION

Initially strains of rotavirus from humans (Albrey and Murphy, 1976; Banatvala, Totterdell, Chrystie and Woode, 1975), pigs (Debouck and Pensaert, 1979), lambs (McNulty, Allan, Pearson, McFerran, Curran and McCracken, 1976; Snodgrass, Herring and Gray, 1976) and some calf strains (McNulty, Allan and McFerran, 1976), would not propagate in tissue culture and as such the only available means of multiplication was by inoculation of gnotobiotic animals. Thus Mebus, Kono, Underdahl and Twiehaus (1971), successfully multiplied calf rotavirus (Cody strain) in several serial passages in gnotobiotic calves, while the porcine rotavirus had been passaged in gnotobiotic piglets (Debouck and Pensaert, 1979). However, experimental cross-species infection for the multiplication of rotaviruses had been in practice for a long time. Thus human rotaviruses had been propagated in several animal species such as piglets (Bridger, Woode, Jones, Flewett,

Bryden and Davies, 1975; Middleton, Petric and Szymanski, 1975; Wyatt, James, Bohl, Theil, Saif, Kalica, Greenberg, Kapikian and Chanock, 1980), calves (Mebus, Wyatt, Sharpee, Sereno, Kalica, Kapikian and Twiehaus, 1976) and monkeys (Wyatt, Sly, London, Palmer, Kalica, VanKirk, Chanock and Kapikian, 1976). Also animal to animal species propagation has been reported, thus calf rotavirus has been propagated in piglets (Woode and Bridger, 1975; Tzipori, Makin and Smith, 1980). Generally, mild or asymptomatic infections have been observed for most of these cross-species experimental infections, but a few workers have reported induction of diarrhoea with heterologous virus strains (Woode and Bridger, 1975; Tzipori, Makin and Smith, 1980).

In the work described in this section, 12 isolates of calf rotavirus which initially failed to infect tissue culture cells were inoculated into gnotobiotic lambs for the multiplication of virus and production of convalescent antisera.

MATERIALS AND METHODS

Lambs

Twelve male hysterectomy-derived, colostrum-deprived gnotobiotic lambs, obtained according to the method of Hart, Mackay, McVittie and Mellor (1971), were reared singly in plastic isolators.

Rotavirus Faecal Specimens

Twelve calf faecal specimens containing rotavirus were used. All were from outbreaks of diarrhoea in calves submitted to the Moredun Institute from Veterinary Investigation Centres (VIC) in Scotland and parts of England during 1980 - 81 for routine diagnosis. The basis of the selection of these 12 isolates was:

- (a) the diverse geographical origin of the samples,
- (b) the presence in electron microscopic examination of faeces (EM) of rotavirus particles to the exclusion of other enteric viruses, together with a positive result for rotavirus by the ELISA test (carried out by Miss I. Campbell, Moredun Institute), and
- (c) only specimens of at least 3 ml in volume were selected, since it was found that smaller amounts were inadequate for the extraction procedure employed.

The age, clinical history and geographical location of calves from which isolates were obtained are summarised in Table 3.1.

Inoculation of Gnotobiotic Lamb

A 10% v/v or w/v faecal suspension, depending on the consistency of the faecal material, was made for each of the 12 samples (care being taken not to cross contaminate the

TABLE 3.1

AGES OF CALVES, CLINICAL HISTORY AND LOCATION OF HERD
FROM WHICH ISOLATES WERE OBTAINED

Isolate No.	Age of calf	Herd	
		Clinical History	Location (County)
637	8days	Scouring	Aberdeenshire
639	1wk	Scouring	Invernessshire
641	2wk	Outbreak of scour in suckled calves aged between 2wk and 3mth. Number affected, approx.30	Dumfriesshire
642	3wk	Scouring	Aberdeenshire
646	1wk	Sudden onset of diarrhoea; Number affected 6. 3 dead. No scour problem for 2 years.	Caithness
649	1wk	Annual problem with scours in calves. Cows tend to be in good health.	Invernessshire
651	<1wk	Bought-in calves, started to scour 2 days later.	East Lothian
657	1-4wk	Scouring calves reared out of doors	Fife
663	1-2wk	Scouring	Ayrshire
666	Calf**	Scouring	Shropshire
669	Calf**	Scouring	Worcestershire
678	Calf**	Scouring	Yorkshire

** Ages of calves not available.

samples), in serum-free maintenance medium 199, hand shaken and then sonicated in a bath for 1 min. An equal volume of fluorocarbon (arcton 113, ICI, UK) was mixed with each suspension using a Silverson homogeniser. The aqueous phase was collected after centrifugation at 2100g for 30 min. at 5°C and filtered through a 0.45µm Millipore membrane. The filtrates were checked for sterility by standard bacteriological methods and also examined for the presence of rotavirus particles by EM.

Three ml of bacteria-free filtrate were administered orally to each gnotobiotic lamb on the second day of life. The lambs were fitted with harnesses (Fig 3.1) for faecal collection and were observed clinically. Faeces were examined daily for rotavirus by ELISA and EM and pooled faeces from each lamb was purified and examined by PAGE.

Convalescent antiserum was obtained from each infected lamb by venepuncture 21 days p.i.. Positive faecal samples from each lamb were pooled, aliquoted and stored at -70°C until required.

RESULTS

Multiplication of rotavirus occurred in all 12 lambs, virus being excreted in the faeces from the first or second day to a maximum of 9 days p.i. (Fig. 3.2). No overt diarrhoea occurred in any of the lambs, although the faeces became loose and yellowish, coincident with virus excretion.

FIGURE 3.1




Gnotobiotic lamb in plastic isolator
showing harness fitted for faecal collection



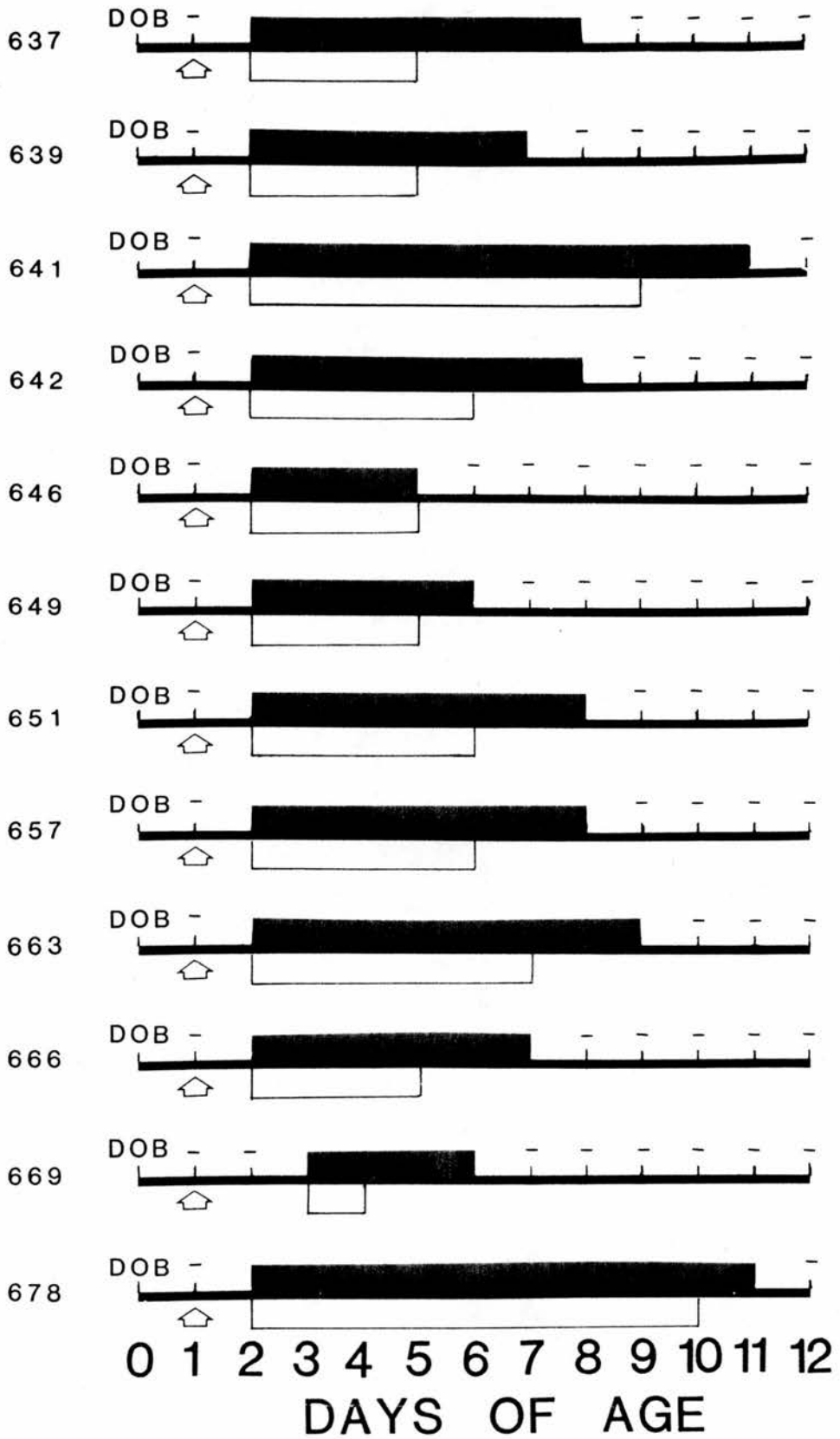
893

FIGURE 3.2.

Excretion of rotavirus in the faeces of lambs inoculated with calf rotavirus as detected by ELISA and EM

- DOB Day of birth
-  Age at inoculation
- No virus detected
-  Duration of virus excretion detected by ELISA
-  Duration of virus excretion detected by EM

LAMB
NO.



One lamb (678) showed a transient anorexia but all others remained clinically normal. In general the ELISA was a more sensitive technique for detecting virus excretion than EM.

Examination of the aliquoted faecal samples by PAGE showed the viral genome present in 8 samples to comprise 11 segments of double stranded (ds)RNA as typical of all rotaviruses (Newman, Brown, Bridger and Woode, 1975; Rodger, Schnagl and Holmes, 1975). No two virus strains showed the same migration pattern (Fig. 3.3). However, 4 of the 12 preparations (Fig. 3.3 tracks 3, 7, 10 and 11) contained more than 11 segments of dsRNA; these were therefore considered to reflect the presence of more than one strain of rotavirus in the original calf faeces and were subsequently excluded from further studies.

All the convalescent antisera contained rotavirus antibodies, and were used in serological studies (Chapter 4).

ii. PROPAGATION OF CALF ROTAVIRUS ISOLATES IN TISSUE CULTURE

INTRODUCTION

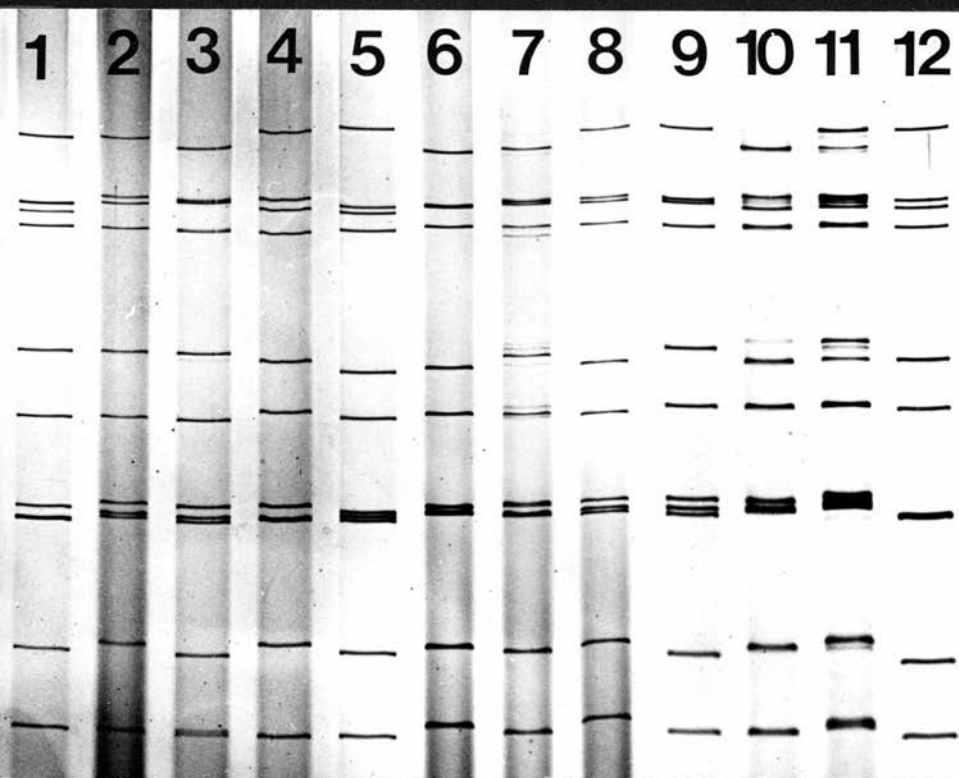
After Mebus, Underdahl, Rhodes and Twiehaus (1969) had shown rotaviruses to be aetiologically important in neonatal calf diarrhoea, numerous attempts were made to isolate and propagate the virus in-vitro since it was more convenient to use tissue culture grown viruses as a source of antigen

FIGURE 3.3

Identification by 7.5% PAGE of dsRNA in 12 calf rotavirus strains in lamb faeces

Tracks 1. 637	Tracks 7. 657
2. 639	8. 669
3. 646	9. 649
4. 641	10. 663
5. 642	11. 666
6. 651	12. 678

Note: Tracks 3, 7, 10 & 11 show more ^{than} 11 segments



(Fernelius, Ritchie, Classick, Norman and Mebus, 1972; Welch and Twiehaus, 1973). Although 4 strains namely Lincoln, Cody (Mebus, Kono, Underdahl and Twiehaus, 1971), UK Compton (Bridger and Woode, 1975) and Northern Ireland (McNulty, Allan, and McFerran, 1976) were first isolated and propagated in tissue culture in the 1970's, detailed virological and serological studies were hampered for a long time because of the difficulties encountered in routinely isolating and propagating other rotaviruses in tissue culture. However, these difficulties have now been largely overcome by incubating the virus inoculum with trypsin before inoculation of cell culture, by incorporating trypsin in the maintenance medium, and by using roller drum cultures of MA104 or BSC-1 cells (Almeida, Hall, Banatvala, Totterdell and Chrystie, 1978; Babiuk, Mohammed, Spence, Fauvel and Petro, 1977; Theil, Bohl, Saif, 1978; Spendlove, McClain and Lennette, 1970). More recently the beneficial effects of trypsin have been reported with human rotaviruses, when serial passages of different strains were achieved in MA104 cells (Sato, Inaba, Shinozaki, Fujii and Matumoto, 1981; Urasawa, Urasawa and Taniguchi, 1981; Hasegawa, Matsuno, Inouye, Kono, Tsurukubo, Mukoyama and Saito, 1982). Routine isolation and cultivation of bovine rotaviruses in cell culture have also been described (Bachmann and Hess, 1981).

Because it proved impossible in the early stages of this investigation to isolate rotavirus from calves using most of the cell lines maintained at the Moredun Institute

at the commencement of this investigation, this section is concerned with attempts to find a suitable cell system for the isolation and propagation of calf rotaviruses to facilitate further characterisation studies of the strains.

Because the isolates were of calf origin, it was assumed that they would grow best in primary bovine kidney cells and this assumption was strengthened by the fact that the three reference strains used in this investigation, namely UK (Compton), Northern Ireland and Lincoln were isolated originally in primary bovine kidney cells and one of them (Northern Ireland strain) was subsequently adapted to a bovine continuous cell line (MDBK).

Apart from investigating primary kidney cell cultures of EBK and EOK, the suitability of cell lines derived from other animal species was also assessed for their ability to support the growth and multiplication of the calf rotavirus isolates. Monkey kidney cell lines were included since BSC - 1 cells and MA104 cells have been used to isolate and propagate calf rotaviruses (Babuik, Mohammed, Spence, Fauvel and Petro, 1977; Smith, Estes, Graham and Gerba, 1979), while LLC-MK2 cells are known to be capable of supporting the replication of a variety of animal and human rotaviruses (Bantvala, Totterdell, Chrystie and Woode, 1975; Bryden, Davies, Thouless and Flewett, 1977; Thouless, Bryden, Flewett, Woode, Bridger, Snodgrass and Herring, 1977).

MATERIALS AND METHODSCells

EBK and EOK cells were seeded in 1 ml amounts at a concentration of 3×10^5 /ml in test tubes containing flying coverslips and incubated overnight in growth medium 199.

2. The continuous cell lines MDBK, Vero, LLC-MK2 and MA104 were seeded at a concentration of 2×10^5 /ml and dispensed in 1 ml amounts into test tubes containing coverslips and allowed to monolayer overnight at 37°C.

Viruses

The viruses used were obtained from the 8 aliquots of lamb faeces described earlier. The UK (Compton) strain of calf rotavirus was used as control. Viruses were treated with 10µg/ml trypsin at 37°C for 1hr. Two hundred µl of trypsin-treated virus was used to infect monolayers of the different cells. After 90 min. of adsorption the infected cultures were incubated in roller drums at 37°C for 24-48hr.

Propagation and Adaptation Studies

On the basis of the results obtained (vide infra), MA104 cells were used routinely for the isolation and propagation of the different isolates. Also on the basis of the cross-neutralisation reactions (see Chapter 4) two of the isolates - 639 and 678 - were selected for adaptation

and propagation on MA104 cells. The cultures were observed daily for cpe. Every 2 - 3 days, the monolayers were disrupted and passaged. This process was repeated until cpe appeared regularly and sufficient virus was available for stock.

Coverslips from each passage level were stained IF. Also, in some occasional tubes in which cells had detached from the glass surface, the supernatant fluids from the cultures were centrifuged on to the surface of microscope slides by means of a cytospin centrifuge (Shandon, Southern, UK). The cells were then fixed with chilled acetone and stained by IF.

Cloning of 639 and 678 Strains

Strains 639 and 678 were cloned first by three passages at terminal dilution and then by three plaque purifications (Chapter 2).

The dsRNA of cloned strains 639 and 678 were compared with the original faecal virus in a 7.5% discontinuous polyacrylamide slab gel. This was to ensure that the viruses had not been contaminated nor undergone mutation during the cloning process.

RESULTS

All 8 isolates of calf rotavirus replicated in MA104 cells, but not in any other cell type. On the other hand the UK (Compton) strain showed evidence of replication in all the cell types.

Immunofluorescence stained coverslips obtained at each passage level showed that strains 639 and 678 were replicating. By the 4th or 5th passage level, cpe began to appear as an initial rounding of cells with indistinct outline, followed by 'heaping' or aggregation of dead cells (Fig. 3.4b). Some of the cells assumed a spindle shape, with one end attached to the glass surface, whilst the others floated in the medium. By the sixth or seventh passage levels, as the cpe progressed the cell sheet became completely destroyed after 2 or 3 days (Fig. 3.4b,c.).

The plaques (Fig. 3.5) of the cloned viruses appeared diffuse and tended to coalesce at lower dilutions of the virus, whereas at higher dilutions (10^{-5}) single plaques could be picked. No plaques were observed in the control wells.

The comparison of the dsRNA of faecal virions and the tissue culture cloned virions of strains 639 and 678 was similar even after the 13th passage (Fig 3.6).

FIGURE 3.4

Propagation of Strains 639 and 678 in MA104 Cells

Fig. 3.4a

Uninfected monolayers of MA104 cells

x 360

Fig. 3.4b

Early cpe, showing focus of cpe. 24hr. p.i.

Note: rounding of cells

x 360

Fig. 3.4c

Advanced cpe, showing destruction of more cells.

48-72 hr. p.i.

Note: many cells have detached, while some cells
are spindle shaped

x 360

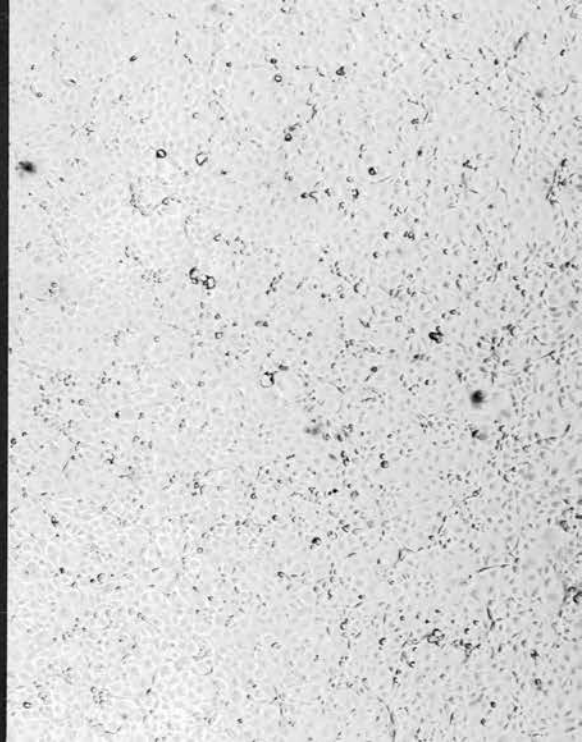
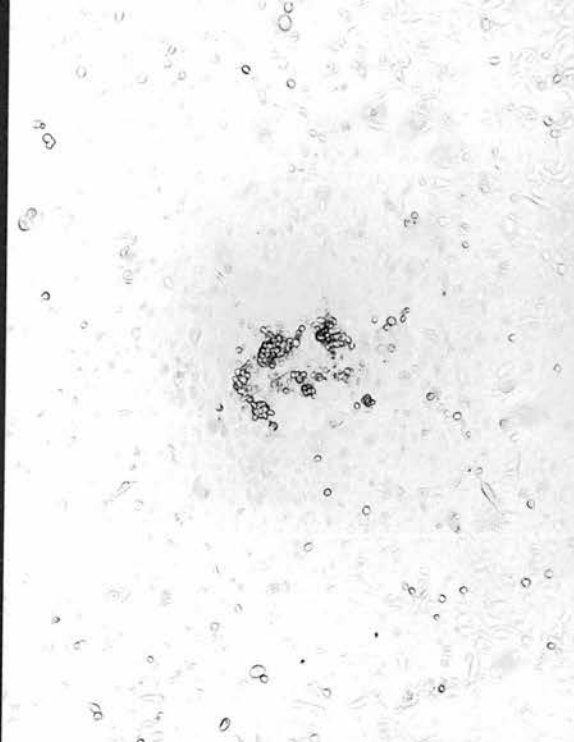
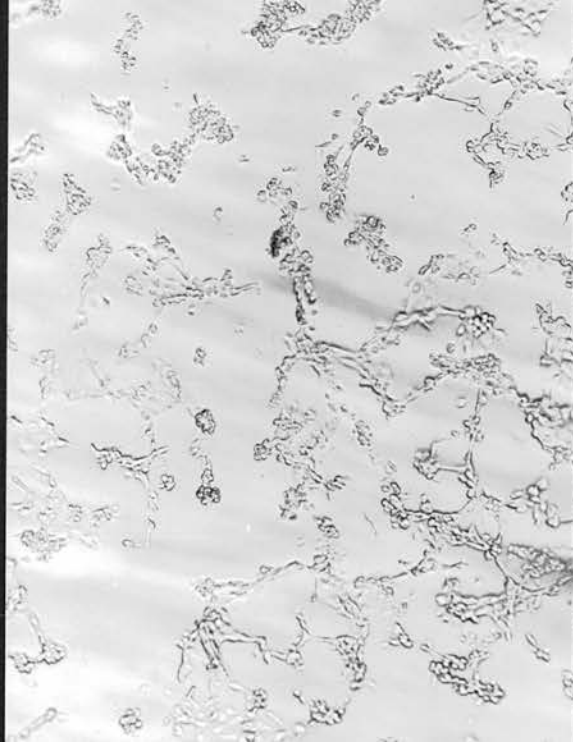


FIGURE 3.5

Plaque Assay of Strain 678 in MA104 Cells

Wells 1-4 are infected with 10^{-6} , 10^{-5} , 10^{-4} and 10^{-3} dilutions of virus respectively

Wells 5 contains uninfected control

Note: Coalescing of plaques at higher dilutions of virus: wells 3 & 4

1

2

3

4

5

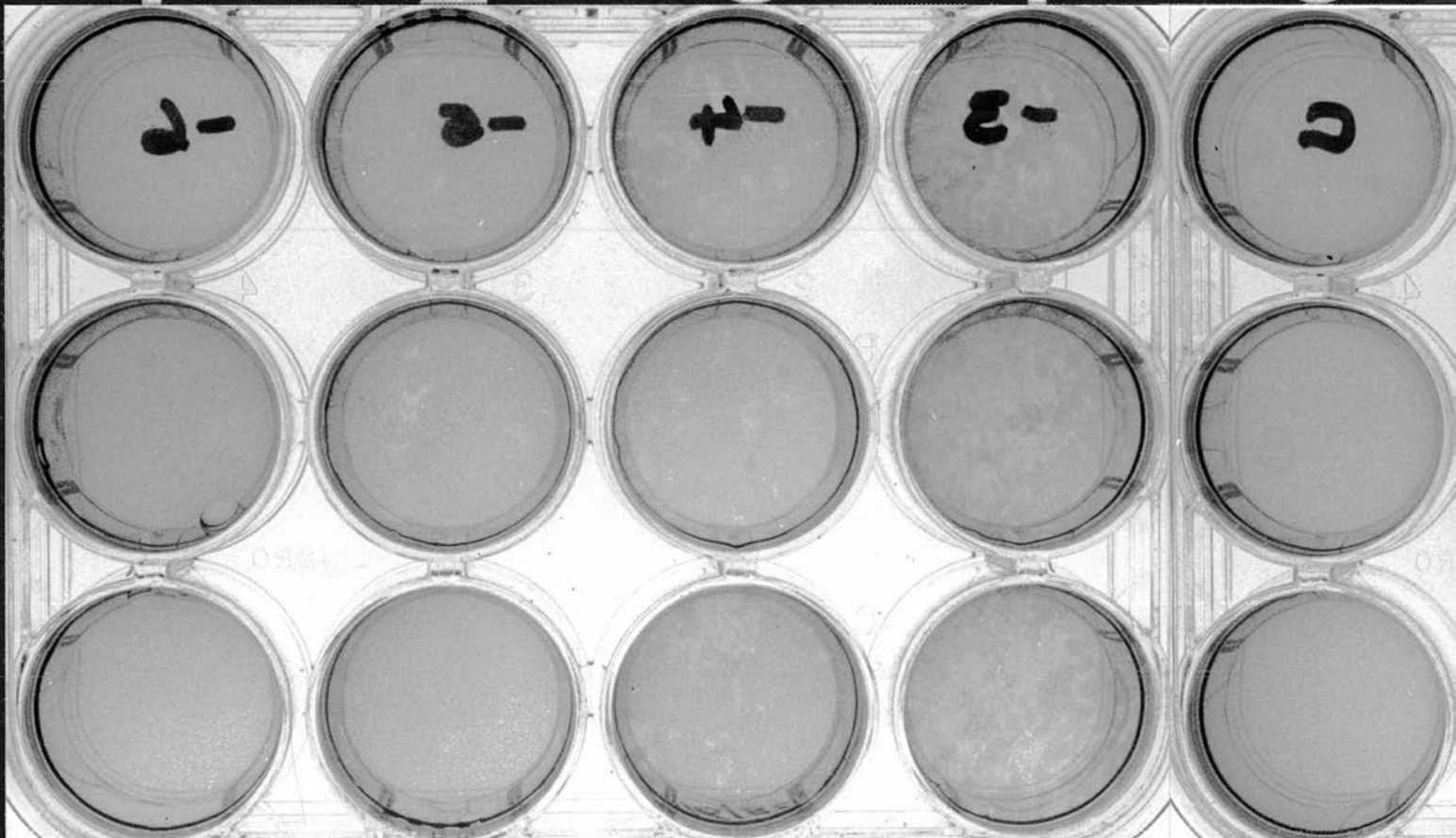


FIGURE 3.6

A comparison of the dsRNA of the faecal virus with the tissue culture adapted form of the same virus in a 7.5% discontinuous gel.

- Tracks 1. Strain 639 faecal virus
2. Faecal and tissue culture virus of strain 639 coelectrophoresed
 3. Strain 639 tissue culture virus
 4. Strain 678 faecal virus
 5. Faecal and tissue culture virus of strain 678 coelectrophoresed
 6. Strain 678 tissue culture virus

1**2****3****4****5****6**

iii. THE EFFECT OF TRYPSIN ON THE SERIAL PASSAGE OF ISOLATES
639 AND 678

After it had been established that strains 639 and 678 had been fully adapted to grow in-vitro, it was decided to investigate the effect of trypsin on the propagation of these strains.

MATERIALS AND METHODS

Three concentrations of trypsin (10 μ g/ml, 5 μ g/ml and 0 μ g/ml) were investigated. Each virus strain at known passage levels was treated with a known concentration of trypsin and used to infect monolayers of MA104 cells. After 3 days of incubation the virus was titrated and the titres at different concentrations of trypsin were compared.

RESULTS

No appreciable change in the virus titre was observed when the concentration of trypsin was varied from 10 μ g/ml to 5 μ g/ml. On the other hand when trypsin was omitted completely, both for the treatment of the virus inoculum and in the maintenance medium, there was a considerable drop in the virus infectivity (Table 3.2).

TABLE 3.2

THE EFFECT OF VARYING CONCENTRATIONS OF TRYPSIN ON ROTAVIRUS
INFECTIVITY

Virus	Passage level	Con. of trypsin µg/ml (*)	Titre in MA104 log ₁₀ TCID ₅₀ /ml
639	8	10	6.2
678	7	10	5.9
639	10	5	5.9
678	9	5	5.9
639	9	0	4.5
678	8	0	4.8

(*) The concentration of trypsin in maintenance medium remained at 1µg/ml through the test, while the concentration of trypsin on the virus inoculum was varied as shown.

iv. INFECTION OF REFRACTORY CELLS USING TWO TISSUE CULTURE
ADAPTED ISOLATES

This experiment was designed to ascertain whether the two calf rotavirus isolates 639 and 678 which had been adapted to growth in MA104 cells would infect and replicate in those cell types which were initially refractory.

MATERIALS AND METHODS

Cells

With the exception of LLC-MK2, all the cells listed in Section ii of this chapter were used.

Viruses

Isolates 639 and 678 at the 12th and 13th passage level respectively in MA104 cells were employed. They were later adapted and passaged 3 times in the various cell types.

RESULTS

Both isolates 639 and 678 infected and replicated in all the cell culture types tested by IF staining (Table 3.3). However, infectivity in the various cell types was lower than that obtained in MA104 cells. Adaptation of the viruses by passaging them 3 times in the various cell types did not seem to enhance their infectivity for these cells as the titres before and after adaptation did not seem to vary significantly. In some of the cell types e.g. EBK and EOK

TABLE 3.3

INFECTION OF PREVIOUSLY INSUSCEPTIBLE CELLS WITH TISSUE
CULTURE STRAINS 639 AND 678

Virus	Pass. level in MA104 (Cell Type)	Cell	IF	Titre log ₁₀ TCID ₅₀ /ml in MA104 (CellType)
639	12	MA104	+	6.3
678	13	MA104	+	5.8
639	0 (3)a	EBK	+	4.9 (4.6)b
678	0 (3)a	EBK	+	3.8 (4.9)b
639	0 (3)a	EOK	+	4.8 (4.8)b
678	0 (3)a	EOK	+	3.8 (2.8)b
639	0 (3)a	MDBK	+	3.1 (3.8)b
678	0 (3)a	MDBK	+	3.8 (3.8)b
639	0 (3)a	Vero	+	3.8 (4.3)b
678	0 (3)a	Vero	+	2.8 (2.8)b
639(*)	0	MA104	+	4.9
678(*)	0	MA104	+	5.0

a Passage levels of virus in the cell type concerned.

b Virus titre in the cell concerned after adaptation
and three passages.

+ Presence of fluorescing cells

(*) Faecal virus.

the virus titre of 639 approached that of the faecal virus in MA104 cells. By contrast, the titres obtained with isolate 678 in these various cell types were considerably lower than that of the faecal virus in MA104 cells.

DISCUSSION

One of the major difficulties in this investigation was the fact that none of the cell types initially available was capable of supporting the growth and propagation of field strains of calf rotaviruses. For this reason the experiments described in this chapter were designed to find cell types capable of producing high titres of rotaviruses so that further characterisation of the viral isolates could be carried out. Until this was achieved it was decided to study the growth of calf rotavirus in gnotobiotic lambs in the hope that they would readily support viral replication and shed large amounts of virus in their faeces. The use of these gnotobiotic lambs would also prove useful as a means of preparing specific antiserum against each of the viruses isolated.

A review of the literature showed that the method of infecting gnotobiotic neonates of one species with rotavirus from another species has been exploited extensively by a number of workers who induced diarrhoea in gnotobiotic calves, lambs piglets and monkeys infected with rotavirus from infants suffering from gastroenteritis, (Bridger, Woode, Jones, Flewett, Bryden and Davies, 1975; Snodgrass,

Madeley, Wells and Angus, 1977; Torres-Medina, Wyatt, Mebus, Underdahl and Kapikian, 1976; Wyatt, Sly, London, Palmer, Kalica, VanKirk, Chanock and Kapikian, 1976). Virus shedding patterns in infected gnotobiotic piglets were consistent with those observed in infants with diarrhoea, and the maximum amount of virus in the faeces occurred shortly before or at the onset of diarrhoea (Middleton, Petric and Szymanski, 1975). More recently, Wyatt, James, Bohl, Theil, Saif, Kalica, Greenberg, Kapikian and Channock (1980), successfully passaged the Wa strain of human rotavirus 11 times in gnotobiotic piglets and observed 'moderate' diarrhoea during some of the passages and only inapparent infection with others. In this respect, their results were similar to those of Mebus, Wyatt, Sharpee, Sereno, Kalica, Kapikian and Twiehaus (1976) who induced an inconsistent diarrhoea of less than 2 days duration in gnotobiotic calves infected with human rotavirus. Tzipori, Makin and Smith (1980), infected gnotobiotic lambs with isolates from different species including cattle but did not observe diarrhoea, although the virus could be demonstrated from 3 - 5 days after inoculation by EM.

In view of these observations it was interesting to note that the results obtained in this present investigation were in agreement with those of other workers but differed only at some points with those of Tzipori, Makin and Smith (1980), in that all but one lamb (669, Fig 3.2) of the experimentally infected gnotobiotic lambs began to excrete virus 24hr. post infection, at which time there was a

marked change in both the colour and consistency of the faeces.

This investigation afforded an opportunity to compare the sensitivity of the ELISA and EM techniques. The ELISA in these tests not only proved to be more sensitive but was more practicable and confirmed that the virus was excreted for a maximum of 9 days in one of the lambs. Other workers who have compared the detection rate of rotavirus by the EM and ELISA methods also found ELISA to be more sensitive (Brandt, Kim, Rodriguez, Thomas, Yolken, Arrobio, Kapikian, Parrott and Chanock, 1981; Ellens and de Leeuw, 1977). These results obtained with ELISA may well explain the inability of Tzipori, Makin and Smith (1980), to detect virus excretion until the third day post infection and for not more than two days thereafter by their use of the comparatively insensitive EM method. On the other hand, it is possible that the differences between the observations of Tzipori et al. (1980) and those described in this section could be attributed to the different virus strains.

The isolation of calf rotavirus on primary embryonic bovine kidney cells reported earlier (Bridger and Woode, 1975; McNulty, Allan and McFerran, 1976; Mebus, Kono, Underdahl and Twiehaus, 1971), and the fact that rotaviruses from different species have been used to infect LLC-MK2 (Bryden, Davies, Thouless and Flewett, 1977; Thouless, Bryden, Flewett, Woode, Bridger, Snodgrass and Herring, 1977) were shown not to be sufficient basis for attempting

to isolate field calf rotaviruses on primary embryonic bovine and ovine kidney cells as well as on continuous cell cultures of monkey kidneys, Vero, LLC-MK2 and bovine kidney, MDBK. Some other workers have also found that BHK-21 and Vero cells were relatively resistant to infection with calf, lamb and pig rotavirus isolates (McNulty, Allan, Pearson, McFerran, Curran and McCracken, 1976). However, in this investigation all the strains under study readily infected MA104 cells only, in contrast to the UK (Compton) strain which infected all the cell types examined.

The beneficial effects of trypsin for the isolation and propagation of rotaviruses have been exploited by many workers (Babiuk, Mohammed, Spence, Fauvel and Petro, 1977; McNulty, Allan, Todd and McFerran, 1979; Sato, Inaba, Shinozaki, Fujii and Matumoto, 1981; Urasawa, Urasawa and Taniguchi, 1981), all of whom employed trypsin routinely for the isolation and propagation of different strains of rotaviruses from calves chickens and humans respectively. There are however a number of reports of the successful isolation and growth of rotaviruses from calves, dogs, infants and chicken without the use of trypsin (Bachman and Hess, 1981; Hoshino, Wyatt, Scott and Appel, 1982; Hasegawa, Matsuno, Inouye, Kono, Tsurukubo, Mukoyama and Saito, 1982; McNulty, Allan and McFerran, 1976; McNulty, Allan, Todd, McFerran, McKillop, Collins and McCracken, 1980). There are also reports that some strains of rotaviruses are less dependent on trypsin for subsequent passages following the

initial trypsin-associated isolation. The reasons for these are not known but it would appear that the need for trypsin as an aid in the isolation and replication of rotaviruses from field specimens depends to some extent on the strain of virus in question. In this present study, results indicated that pre-treatment of virus inocula with trypsin and incubation of cultures in roller drums were essential for continuous propagation of field isolates of rotavirus.

The mechanism of the action of trypsin (Clark, Roth, Clark, Barnett and Spendlove, 1981; Graham and Estes, 1980) on rotavirus infectivity, whereby non-infectious virus was converted to infectious forms, might explain why there was over 10-fold drop in virus infectivity when trypsin was withdrawn. (Table 3.2). Graham and Estes (1980) noted that when trypsin was present in the culture medium, during multiple cycles of virus replication the infectivity of the virus was enhanced between 10- and 1000-fold.

Because the UK (Compton) strain of rotavirus was capable of replicating in all cell types examined, attempts were made to grow two strains, 639 and 678, on those cell cultures that were previously found to be insusceptible to infection with freshly isolated strains of rotaviruses. Surprisingly, it was found that all the culture systems were now able to support the growth of both strains. Although the reasons for these unexpected results were not clearly established it would appear that tissue culture adapted viruses (both of which had undergone at least 12 serial

passages in MA104 cells and a further 3 serial transfers in the different cell systems under study), have acquired a much wider range of susceptible host cells. Since both 639 and 678 adapted strains had also been purified by terminal dilution and cloning procedures, it was possible that the methods had selected a population of viruses that was specially adapted to in-vitro cultivation.

Examination by PAGE of the dsRNA of 12 rotavirus isolates showed that 4 samples had mixed segment profiles. This situation may have arisen when two samples were inadvertently mixed in the laboratory or it may signify a dual or sequential infection by two different strains of rotavirus (Spencer, Avendano and Garcia, 1983). Examination of the dsRNA of the original calf faeces with the dsRNA of the lamb faeces of these 4 samples showed that their genome segments were similar which means that the original calf inoculum had been mixed from the field. The occurrence of a mixed segment profile was first reported by Lourenco, Nicholas, Cohen, Scherrer and Bricout (1981) in children suffering from rotavirus diarrhoea.

A situation that highlighted the good quality control aspects of the PAGE in monitoring tissue culture adaptation of rotavirus arose during the plaquing process of the two strains 639 and 678. One of the strains became contaminated between the 2nd and 3rd stages of purification by an entirely different strain. This contamination was detected when the dsRNA of the original virus was compared by PAGE with the

product of the second stage of plaque assay. The plaque process had to be continued with an earlier stock of virus. This same advantage had also been exploited by Taniguchi, Urasawa and Urasawa, (1982) when adapting human rotavirus strains to tissue culture.

In conclusion, the experiments described in this chapter fulfilled their purpose in that calf rotavirus strains multiplied in gnotobiotic lambs with the production of large quantities of virus in faeces which were, in turn, adapted and propagated in tissue culture. However, only MA104 cells were found suitable for the isolation and propagation of these viruses.

CHAPTER 4STUDIES ON THE SEROLOGICAL RELATIONSHIPS OF CALF
ROTAVIRUS ISOLATESi. IMMUNOFLUORESCENCE AND CROSS-NEUTRALISATION STUDIESINTRODUCTION

The serological relationships of bovine rotavirus have not been studied as extensively as those of human rotavirus in which at least 4 serotypes have been distinguished (Beards, Pilford, Thouless and Flewett, 1980; Flewett, Thouless, Pilford, and Bryden, 1978; Thouless, Bryden and Flewett, 1978; Sato, Inaba, Miura, Tokuhisa and Matumoto, 1982; Wyatt, Greenberg, James, Pittman, Kalica, Flores, Chanock and Kapikian, 1982; Urasawa, Urasawa and Taniguchi, 1982), and those of the avian species in which at least 3 serotypes have been reported (McNulty, Allan, Todd, McFerran, McKillop, Collins and McCracken, 1980) by serum neutralisation assays.

Because other workers have used complement-fixation, immune-electron microscopy and the ELISA tests to distinguish human rotavirus serologically (Yolken, Wyatt, Zissis, Brandt, Rodriguez, Kim, Parrott, Urrutia, Mata, Greenberg, Kapikian and Chanock, 1978; Zissis and Lambert, 1978; 1980), it has been suggested that the term subgroup be used to describe antigens detected by these broad serological reactions which involve the major internal core

protein, whereas the term serotype be used exclusively for antigens involved in serum neutralisation reactions at least one of which is associated with the surface glycoprotein of the outer shell (Kapikian, Cline, Greenberg, Wyatt, Kalica, Banks, James, Flores and Chanock, 1981)

Two strains of bovine rotavirus have been distinguished by haemagglutination-inhibition (Spence, Fauvel, Petro and Babiuk, 1977), and while Murakami, Nishioka, Hashiguchi and Kaniyasu (1981) were also of the opinion that more than one serotype of bovine rotavirus might exist they have recently confirmed this by differentiating at least 2 serotypes by serum neutralisation tests (Murakami, Nishioka, Hashiguchi and Kuniyasu (1983).

In view of these observations it was decided to investigate the relationships between strains of bovine rotavirus by serum neutralisation tests and also to attempt to define the prevalence of the more important serotypes in faeces and serum samples from various parts of the United Kingdom.

MATERIALS AND METHODS

Viruses

The 8 calf strains described in chapter 3 and three tissue culture adapted reference strains, UK (Compton), Northern Ireland and Lincoln were used.

Cells

MA104 cells were used throughout the experiments.

Sera

i. Convalescent antisera were prepared in gnotobiotic lambs to the 8 faecal viruses (Chapter 3).

ii. Hyperimmune antisera to the 8 purified faecal virions were prepared in rabbits previously shown to be free of antibodies to tissue culture (UK Compton) calf rotavirus.

iii. Hyperimmune antisera to purified tissue culture virions of the tissue culture adapted strains were also prepared in rabbits.

All sera were inactivated at 56°C for 30 min. before use.

Immunofluorescent Tests

Indirect immunofluorescent staining was carried out on monolayers of MA104 cells infected with the 8 calf strains of rotavirus. Plates were stained with gnotobiotic lamb convalescent antisera to the 8 strains of calf rotavirus, followed by a fluorescein-conjugated rabbit anti-sheep globulin (Wellcome reagents).

Neutralisation Assay

The neutralisation focus reduction test with both faecal and tissue culture adapted viruses was carried out on MA104 cells in microtitre plates (Sterilin UK) using a constant amount of virus (100 TCID₅₀). Neutralisation titres (NT) were expressed as the reciprocal of the highest serum dilution which reduced fluorescent foci by 50%.

Strains were assumed to be serologically different on the basis of a 20-fold or greater dilution difference between homologous and heterologous titres.

The antigenic relationship (R) between two strains of rotaviruses was calculated by a slight modification of the method of Archetti and Horsfall (1950), using the formula:

$$R = 100 \sqrt{r_1 \cdot r_2} \quad \%$$

$$\text{where } r_1 = \frac{\text{heterologous titre (Strain 2)}}{\text{homologous titre (Strain 1)}}$$

and

$$r_2 = \frac{\text{heterologous titre (Strain 1)}}{\text{homologous titre (Strain 2)}}$$

As the value of R increases, the more closely related are the two strains antigenically. A value of $R \leq 5\%$ is assumed to show antigenic distinction between two strains, as this R value corresponds to a 20-fold reciprocal difference between

homologous and heterologous titres. This value (5%) is severe in the light of 10-32% used in differentiating between types and sub-types of foot-and-mouth disease virus (Brooksby, 1968, cited by Forman, 1975).

RESULTS

Immunofluorescence

The 8 faecal strains of calf rotavirus showed cross fluorescence with convalescent antisera (Table 4.1a) The difference between the homologous and heterologous titres was never greater than 4-fold variation with the exception of the antiserum to 678 which showed 8-fold variation.

The IF antigenic relatedness between any two strains is given in Table 4.1b. By this method, all the strains seemed to be closely related.

TABLE 4.1a

INDIRECT IMMUNOFLUORESCENT TITRES OF CONVALESCENT
GNOTOBIOTIC LAMB SERA TO 8 STRAINS OF CALF ROTAVIRUS.

Rotavirus	Convalescent antisera							
	637	639	641	642	649	651	669	678
637	<u>256</u>	128	64	128	256	256	128	64
639	64	<u>64</u>	64	128	64	64	64	64
641	128	128	<u>128</u>	128	64	64	256	64
642	512	128	64	<u>128</u>	128	128	64	32
649	256	256	256	256	<u>128</u>	256	128	128
651	256	256	256	256	256	<u>128</u>	256	256
669	64	64	64	64	64	64	<u>128</u>	128
678	64	64	64	128	128	64	128	<u>256</u>

Homologous titres are underlined.

TABLE 4.1b

(R VALUES %)

Rotavirus	Convalescent antisera							
	637	639	641	642	649	651	669	678
637	100							
639	71	100						
641	50	100	100					
642	100	100	50	100				
649	100	100	100	100	100			
651	100	100	100	100	100	100		
669	50	71	100	50	71	71	100	
678	25	50	35	35	71	71	71	100

a Values greater than 100

Neutralisation Test

1. Convalescent Lamb Antisera Reacted with Faecal Virus

All the lamb antisera possessed NT antibody to the homologous rotavirus strains, and also showed varying cross-neutralisation with other strains (Table 4.2a).

Within 6 of the strains (637, 639, 641, 649, 651 and 669), there was a high degree of cross-reactivity, with not more than 8-fold variation between homologous and heterologous titres.

Antiserum to strain 678 showed 4- to 32-fold lower titres against all heterologous antigens, and 678 antigen was neutralised to lower than homologous titre by all other antisera. Strain 642 showed one-way cross-reaction with the group of 6 strains mentioned above with 642 antigen adequately neutralised by the other 6 antisera, while the 642 antiserum neutralised heterologous strains less efficiently.

Similarly, 7 of the 8 strains (Table 4.2b) show a close antigenic relatedness, with their R values ranging between 25 and 100%; while one strain (678), has R values ranging between 4 and 25% in relation to the other 7 strains.

TABLE 4.2a

NT TITRES OF ANTISERA FROM CONVALESCENT GNOTOBIOTIC
LAMBS TO 8 STRAINS OF CALF ROTAVIRUS

Rotavirus	Antiserum to Rotavirus							
	637	639	641	642	649	651	669	678
637	<u>128</u>	128	32	128	128	128	64	16
639	64	<u>128</u>	512	128	64	32	64	8
641	64	64	<u>64</u>	64	64	64	64	32
642	128	128	512	<u>1024</u>	128	128	128	32
649	128	128	32	ND	<u>128</u>	128	64	16
651	128	64	256	256	128	<u>64</u>	64	16
669	128	64	64	64	64	64	<u>128</u>	16
678	32	16	32	16	32	32	16	<u>256</u>

Homologous titres are underlined.
ND Not done.

TABLE 4.2b

(R VALUES %)

Rotavirus	Antiserum to Rotavirus							
	637	639	641	642	649	651	669	678
637	100							
639	71	100						
641	50	50	100					
642	36	100 ^a	71	100				
649	100	71	50	ND	100			
651	100 ^a	100	100 ^a	71	100 ^a	100		
669	71	100	71	25	50	71	100	
678	13	6	25	4	13	18	9	100

a Values greater than 100
ND Not done

2. Hyperimmune Antisera to Faecal Virus, Reacted with Faecal Virus

The 6 strains considered to be similar in their reactions with convalescent antisera were also similar in their reactions with hyperimmune antisera, with not more than a 4-fold variation in titre between homologous and heterologous strains (Table 4.3a). Strain 642 appeared to be more closely related to the 6 strains in these tests, with efficient neutralisation of 642 virus by other antisera, and up to a 16-fold variation in titre of 642 antiserum with heterologous virus strains. Once again, strain 678 showed significant distinction from all other 7 strains, with greater than 20-fold difference between homologous and heterologous titres.

The antigenic relatedness of the 7 strains was even closer, with all R values >35%. Strain 642 (R >50%) was clearly shown to relate to the 6 strains (Table 4.3b). On the other hand, strain 678 continued to show clear distinction from the 7 strains with R values ranging from 2-9%.

This evidence suggested that 7 strains, including 642, were of one serotype, with less than 20-fold difference in titre and R values >35%; and that strain 678 was a distinct serotype.

TABLE 4.3a

NT TITRES ($\times 10^{-2}$) OF HYPERIMMUNE RABBIT ANTISERUM TO FAECAL ROTAVIRUS STRAINS, TO 8 STRAINS OF CALF ROTAVIRUS IN FAECES

Rotavirus	Antiserum to Faecal Rotavirus							
	637	639	641	642	649	651	669	678
637	<u>2048</u>	1024	512	1024	4096	512	256	64
639	2048	<u>1024</u>	1024	2048	8192	1024	256	32
641	1024	512	<u>512</u>	512	2048	1024	256	64
642	4096	1024	2048	<u>8192</u>	128	2048	512	64
649	2048	512	2048	8192	<u>2048</u>	512	512	16
651	512	512	2048	4096	8192	<u>1024</u>	512	32
669	1024	512	512	2048	2048	ND	<u>512</u>	32
678	32	64	64	32	64	32	32	<u>1024</u>

Homologous titres are underlined.
ND Not done.

TABLE 4.3b

(R VALUES %)

Rotavirus	Antiserum to Rotavirus							
	637	639	641	642	649	651	669	678
637	100							
639	100	100						
641	71	100	100					
642	50	50	50	100				
649	100	100	100	100	100			
651	35	71	100	100	100	100		
669	71	50	71	50	100	ND	100	
678	3	4	9	2	2	3	4	100

a Values greater than 100
ND Not done.

3. Hyperimmune Antisera to Tissue Culture Virus Reacted with Tissue Culture Virus.

Cell culture adapted UK (Compton), Northern Ireland, and Lincoln strains were compared with 639 and 678 viruses isolated in cell cultures. The UK (Compton), Northern Ireland and 639 strains appeared identical by cross neutralisation tests (Table 4.4a). The Lincoln strain was less efficiently neutralised by antisera to these 3 strains and thus showed slight one-way variation. However, the differences were never greater than 16-fold, so the Lincoln strain should still be considered as the same serotype.

Antiserum to 678 virus had heterologous titres in the range of 64 to 512-fold less than homologous titre although 678 virus was clearly distinguished by a greater than 20-fold titre difference by only 2 of the other antisera.

Antigenic relationships studied by R values (Table 4.2b) confirmed that UK (Compton), Northern Ireland, Lincoln and 639 strains all belonged to the same serotype ($R > 25\%$), while 678 strain was clearly different ($R < 1-6\%$).

The above evidence suggests the existence of 2 serotypes: serotype 1, typified by isolate 639, which includes 7 of the 8 field strains and UK (Compton), Northern Ireland and Lincoln reference strains; and serotype 2, consisting so far only of isolate 678.

TABLE 4.4a

NT TITRES ($\times 10^{-2}$) OF HYPERIMMUNE RABBIT ANTISERM TO TISSUE CULTURE ADAPTED ROTAVIRUS STRAINS, TO 5 STRAINS OF CALF ROTAVIRUS IN TISSUE CULTURE

Rotavirus	Antiserum to Rotavirus				
	UK(Compton)	Northern Ireland	Lincoln	639	678
UK(Compton)	<u>64</u>	1024	4096	1024	32
Northern Ireland	64	<u>1024</u>	4096	512	32
Lincoln	4	512	<u>4096</u>	64	4
639	64	1024	4096	<u>1024</u>	32
678	2	32	512	256	<u>2048</u>

Homologous titres are underlined

TABLE 4.4b.

(R VALUES %)

Rotavirus	Antiserum to Rotavirus				
	UK(Compton)	Northern Ireland	Lincoln	639	678
UK(Compton)	100				
Northern Ireland	100	100			
Lincoln	25	71	100		
639	100	71	25	100	
678	<1	2	1	6	100

ii. SURVEY FOR SEROTYPES 1 and 2 IN INFECTED CALF FAECES
AND IN ADULT COW SERA

MATERIALS AND METHODS

Faecal Samples

A total of 100 calf faecal samples submitted by the Scottish Veterinary Investigation Centres for diagnostic purposes or collected by Dr. Snodgrass of this Institute as part of his studies on the aetiology of calf scours were investigated. Samples were treated with extraction medium and arcton (Chapter 2) and then centrifuged. The resultant supernatant was incubated with 10µg/ml trypsin for 1 hr. at 37°C before infecting monolayers of MA104 cells.

Adult Cow Sera

Sixty-nine serum samples from adult cows in 6 different herds were studied. These were collected by Dr. Snodgrass from control cows in a vaccine experiment. All sera were inactivated at 56°C for 30 min. before use.

Serotyping Antisera

Heat inactivated hyperimmune rabbit antisera prepared against purified tissue culture grown viruses 639 and 678 were used.

Neutralisation Test (NT) for Serotyping

A constant serum varying-virus assay was used. Half \log_{10} dilutions of faecal virus were incubated for 1hr. with 4 antibody units of both serotype 1 and 2 antisera (Chapter 2); and then inoculated MA104 cells which were stained for fluorescent foci after 16-18hr. incubation at 37°C.

NT for the Prevalence of Antibodies to Serotypes 1 and 2 in Adult Cow Sera

A varying-serum constant virus (100 TCID₅₀) assay with both strains 639 and 678 was used.

RESULTS

Survey for serotypes 1 and 2

Each faecal sample was tested at least three times, and only those that gave consistent results were allocated to serotypes. Allocation was made on the basis of at least a 20-fold reduction in virus titre compared with both the virus control titre and the titre with the other antiserum.

Seventy-three of 100 samples (73%) were neutralised by serotype 1 antiserum, but none was neutralised by serotype 2 antiserum. Nine samples (9%) were not neutralised by either antiserum. Typing of 18 strains (18%) was not possible as they failed to produce fluorescing foci in MA104 cells.

Prevalence of Antibodies to Serotypes 1 and 2 in Cow Sera

All the cows sampled possessed serum neutralising antibodies to calf rotavirus, with titres ranging between 320 and 5120. However, titres to both serotypes showed not more than a 4-fold difference.

iii. COMPARISON OF THE UNNEUTRALISED STRAINS (POTENTIAL SEROTYPES) IN CROSS NEUTRALISATION TESTS

MATERIALS AND METHODS

Four of the 9 strains (411, 683, 1548 and 2484) that were not effectively neutralised by either serotype 1 or 2 antisera were successfully isolated and propagated in MA104 cells, cloned by terminal dilution and used to raise hyperimmune antisera in rabbits.

The UK (Compton) and 639 strains (serotype 1) and the 678 strain (serotype 2) were included with their respective antisera in this comparison.

RESULTS

The distinction of serotypes 1 and 2 was confirmed in this comparison (Tables 4.5a and 4.5b).

Antisera to 3 of the new viruses (411, 683 and 2484) distinguished serotypes 1 and 2 from homologous strains by 32 to 64 fold variation. Likewise these 3 viruses were

neutralised less efficiently by serotype 1 and 2 antisera by at least a 32-fold dilution difference. Conversely, there were close cross neutralisation reactions within these 3 viruses, with not more than 8- to 16-fold variation between their homologous and heterologous titres. This distinction of these 3 viruses from serotypes 1 and 2 on the one hand and the cross neutralisation within this group of 3 viruses on the other hand, clearly establishes them as belonging to a third serotype.

Similarly, these difference were clearly brought out when their antigenic relatedness (R) was compared (Table 4.5b). Serotypes 1 and 2 viruses were less related ($R < 8\%$) to viruses 411, 683 and 2484, which in turn were more related to each other ($R > 25\%$)

Virus 1548 was distinguished by serotype 1 and 2 antisera (32- to 64-fold difference) but less clearly (4- to 8-fold difference) by antisera to 411, 683 and 2484 . The R values between 1548 and the group of three viruses were in the range of 9-18%.

This evidence suggests that 411, 683 and 2484 rotaviruses form a closely related group of viruses distinct from serotypes 1 and 2 , and should be considered as serotype 3. Virus 1548 appears to be of intermediate status with some relatedness to serotype 3 viruses. Whether it represents a separate serotype is a matter of definition.

TABLE 4.5a.

COMPARISON OF CROSS NEUTRALISATION TITRES ($\times 10^{-2}$) BETWEEN SEROTYPES 1 and 2 AND THE POTENTIALLY DIFFERENT SEROTYPES USING HYPERIMMUNE ANTISERA AND TISSUE CULTURE CALF ROTAVIRUS

Rotavirus	Antiserum to Rotavirus						
	Serotype 1		Serotype 2	Potential. Diff. Serotypes			
	UK	639	678	411	683	2484	1548
UK(Com.)	<u>128</u>	128	4	4	4	8	8
639	64	<u>1024</u>	16	8	2	4	8
678	4	32	<u>2048</u>	8	4	32	4
411	4	32	32	<u>256</u>	256	32	16
683	4	32	64	256	<u>128</u>	64	16
2484	4	16	64	64	16	<u>128</u>	32
1548	4	32	32	32	32	32	<u>128</u>

Homologous titres are underlined

TABLE 4.5b.

(R VALUES %)

Rotavirus	Antiserum to Rotavirus						
	Serotype 1		Serotype 2	Potential. Diff. Serotypes			
	UK	639	678	411	683	2484	1548
UK(Com.)	100						
639	25	100					
678	<1	2	100				
411	2	3	2	100			
683	3	2	3	100 ^a	100		
2484	4	2	9	25	25	100	
1548	3	3	2	9	13	18	100

a Values greater than 100

iv. DISTINCTION OF SEROTYPES 1 and 2 BY ELISA

INTRODUCTION

The ELISA technique devised by Engvall and Perlmann (1972), and renowned for its sensitivity, large-scale applicability and economic usage of reagents has been extensively used to detect specific antigens and antibodies. However, the extreme sensitivity of the system requires the reagents to be highly specific so as to avoid the occurrence of non-specific cross reactions. Thus special ELISA procedures have been used in a large scale study to define subgroups of human rotavirus (Yolken, Wyatt, Zissis, Brandt, Rodriguez, Kim, Parrott, Urrutia, Mata, Greenberg, Kapikian and Chanock, 1978; Zissis and Lambert, 1980; Thouless, Beards and Flewett, 1982).

In this section, attempts to devise an ELISA system that would differentiate calf rotavirus isolates and might subsequently be used in large scale surveys for the prevalence of different serotypes of rotavirus are described.

MATERIALS AND METHODS

Preparation of Specific Serotyping Antisera for use as ELISA Coating Antibody

Rabbit hyperimmune antisera to strains 639 and 678 were prepared. The fractionation of these sera and the enzyme

(alkaline phosphatase)-IgG conjugation procedures were performed by Mr. J. Redmond of this Institute. The IgG fraction was isolated on a protein A column by the method of Goding (1976). This fraction which contained 5 μ g/ml of protein was conjugated to alkaline phosphatase (Sigma Type VII) by the method of Engvall and Perlmann (1972). The conjugate was then aliquoted and stored at -20°C until required.

Checkerboard Titration to Determine Optimal Dilution of Coating Antibody, Enzyme-conjugated Antibody and Rotavirus

Four microtitre plates were coated with doubling dilutions of 639 (serotype 1) and 678 (serotype 2) IgG (coating antibody) from 1/50 to 1/1600 in 100 μ l volumes. Each of the four plates was incubated with dilutions (1/10, 1/40, 1/160, and 1/640) of faecal viruses 639 and 678. Alkaline phosphatase-conjugated IgG to 639 and 678 (dilutions 1/25 to 1/1600) were added to each plate (100 μ l/well) in a checkerboard fashion. The optimal dilutions of the reagents were regarded as the maximum dilution which could be read unequivocally as positive by unaided eye. After one hour of incubation with substrate at room temperature, the absorbance value at a wavelength of 405nm (OD 405) for these dilutions was found to be 0.2. The optimal dilutions were 1/400 for both coating antibodies and 1/25 and 1/50 for 639 and 678 conjugates respectively. A 1/10 antigen dilution was used throughout the test since all the dilutions tested were adequate.

Absorption of Hyperimmune Serotype 1 and 2 Sera with SA-11 Virus

Equal volumes of each antiserum were incubated with 100 TCID₅₀ of SA-11 virus at 37°C for 2 hr. The sera were centrifuged at 154,400g for 60 min. at 5°C to remove the antibody-coated SA-11 virus particles. The resultant supernatant sera were used in a blocking ELISA test (ie in Step 3 of the normal ELISA as described in Chapter 2), the absorbed sera being titrated with both homologous and heterologous viruses.

Specificity of the ELISA Test

The UK (Compton), Northern Ireland, 639 and 678 tissue culture viruses together with the faecal 639 and 678 viruses were used to investigate specificity. All the tissue culture strains were used undiluted, while the faecal viruses were used at 1/10 dilution.

For detection of antigen, doubling dilutions of 639 and 678 faecal viruses were titrated in both homologous and heterologous systems with appropriate controls included in each plate. A positive/negative ratio (P/N) for each dilution was calculated from the following equation:

$$P/N = \frac{E(\text{test}) - E(\text{blank})}{E(\text{neg.}) - E(\text{blank})}$$

where

E(test) = OD405 of samples tested
 E(blank) = OD405 of PBS/T control
 E(neg) = OD405 of mean of 6
 negative samples.

P/N values ≤ 2 were regarded as negative.

For the ELISA blocking assay, 100 μ l of 4 antibody units (1 unit was the amount of antibody which neutralised 100 TCID₅₀ of homologous rotavirus) of each of the SA-11 absorbed sera was used to block two of the four wells containing any of the virus strains.

The percentage blocking was calculated from the following equation:

$$\text{Percentage blocking} = \frac{\text{OD (Test)} - \text{OD (Serum)}}{\text{OD (Test)}} \times 100$$

A 50% or greater blocking was considered specific.

RESULTS

Antigen Titration

The results are illustrated in Figs 4.1 and 4.2. In both systems the two serotypes could not be differentiated.

FIGURE 4.1

Titration of strains 639 and 678 (faecal preparations) in 639 coating antiserum and conjugate

- ☆→☆ Strain 639 faeces P/N values versus reciprocal dilution
- *→* Strain 678 faeces P/N values versus reciprocal dilution
- Cut off point P/N values ≤ 2 regarded as negative

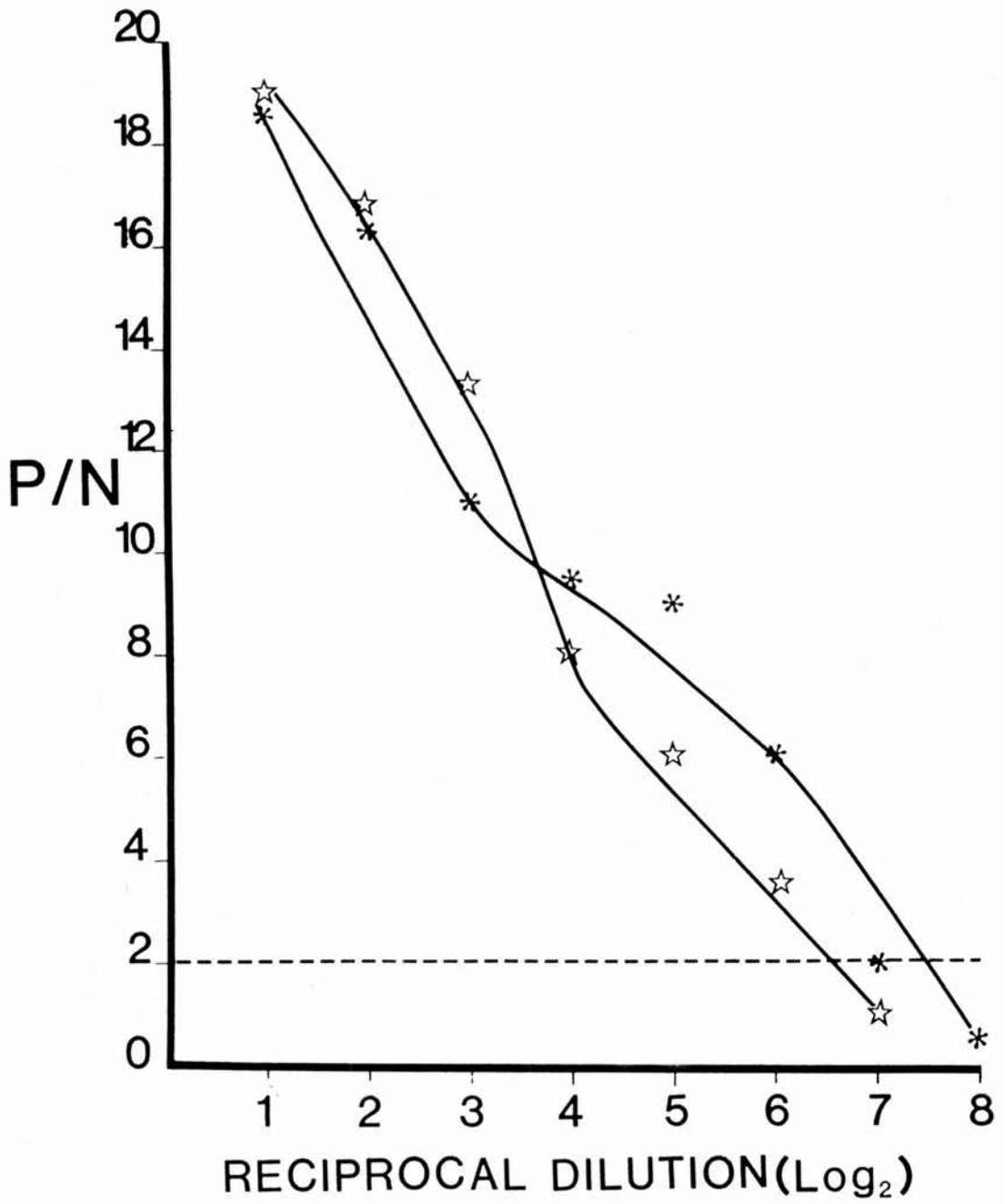
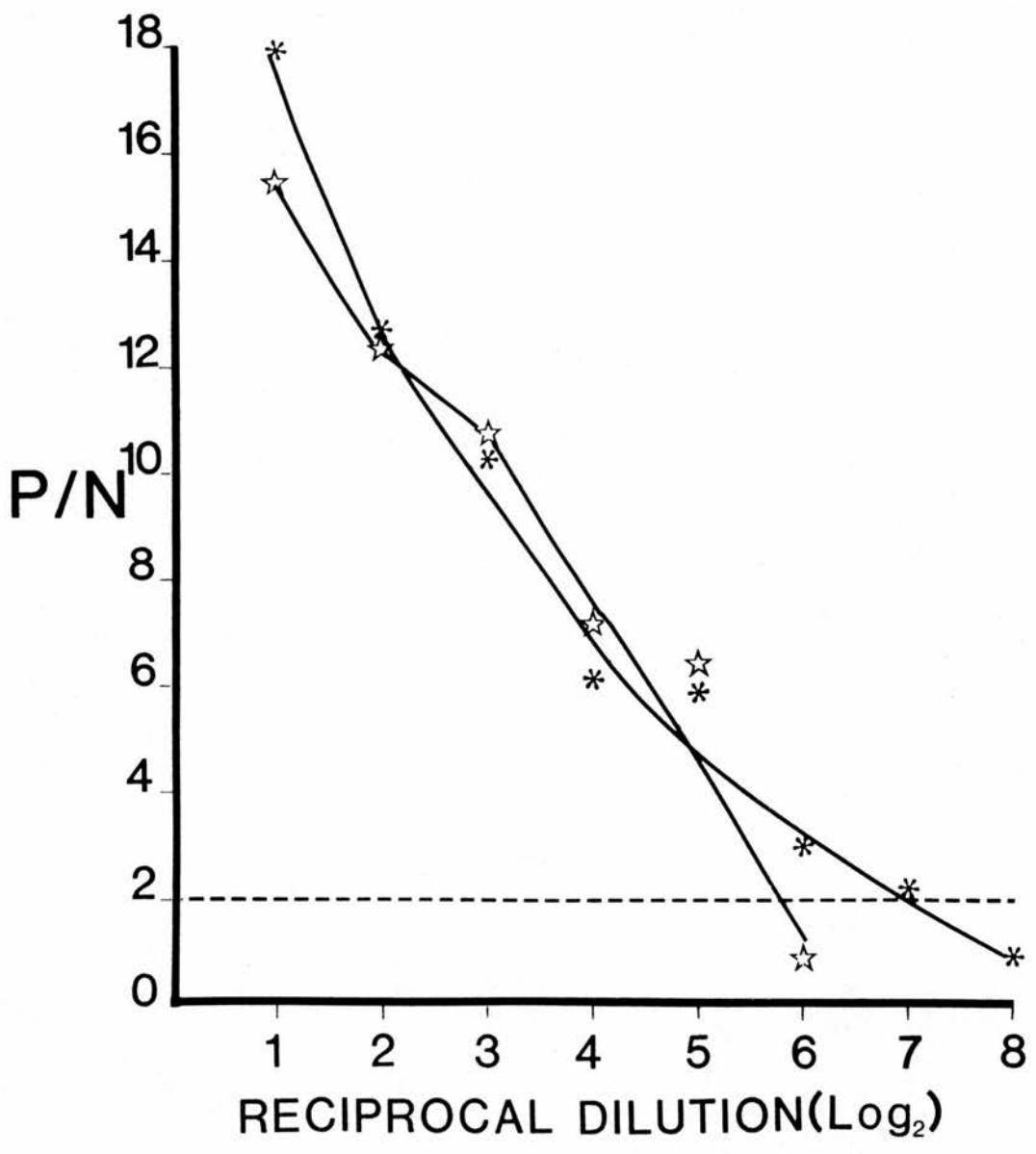


FIGURE 4.2

Titration of strains 639 and 678 (faecal preparations) in 678 coating antiserum and conjugate

- ☆→☆ Strain 639 faeces P/N values versus reciprocal dilution
- *→* Strain 678 faeces P/N values versus reciprocal dilution
- Cut off point P/N values ≤ 2 regarded as negative



Blocking ELISA

Serotype 2 antiserum reduced homologous antigen titre by 70%, but the serotype 1 strains were all reduced by less than 50% (Table 4.6).

With the faecal viruses, no definite pattern could be established, as both the homologous and heterologous blocks were less than 50% (Table 4.6).

v. DISTINCTION OF SEROTYPES 1 and 2 BY HAEMAGGLUTINATION
INHIBITION (HAI) TECHNIQUES

INTRODUCTION

The extensive application of HAI technique for large scale screening of samples has been in vogue for many years, particularly in the field of arbovirus infections (Clarke and Casals, 1958). To date, except for one unconfirmed report involving a human rotavirus (Shinozaki, Fujii, Sato, Takahashi, Ito and Inaba, 1978), haemagglutination by rotavirus has been demonstrated only with animal strains, although not all of these haemagglutinate (Fauvel, Spence, Babiuk, Petro and Bloch, 1978). Amongst those which are known to produce haemagglutinins are the Nebraska calf diarrhoea virus (Inaba, Sato, Takahashi, Kurogi, Satoda, Omori and Matumoto, 1977), SA-11 (Kalica, James and Kapikian, 1978) and avian rotaviruses (Hancock, Gray and Palmer 1983).

TABLE 4.6

RESULTS OF ELISA BLOCKING TESTS USING ABSORBED SEROTYPE
1 and 2 ANTISERA

Virus	Test (OD405)	OD405 (% Reduction) After Blocking with Sera	
		Serotype 1	Serotype 2
UK	0.54	0.24 (56)	0.28 (48)
North. Ireland	0.38	0.14 (63)	0.20 (47)
Lincoln	0.43	0.24 (44)	0.23 (47)
^a 639	0.51	<u>0.25 (51)</u>	0.34 (23)
^a 678	0.64	0.32 (50)	<u>0.19 (70)</u>
^b 639	0.87	<u>0.71 (18)</u>	0.72 (17)
^b 678	0.64	0.52 (19)	<u>0.56 (14)</u>

a Tissue Culture Virus

b Faecal Virus

Homologous Blockings are underlined

The experiments described in this section were attempts to distinguish serotypes 1 and 2 by the HAI method, in the hope of applying it to large scale surveys for the prevalence of different serotypes of rotavirus, if successful.

MATERIAL AND METHODS

Antigen Preparation

The HA antigen was prepared in 3 ways:-

1. From virus (strains 639 and 678) grown in tissue culture using the method of Fauvel, Spence, Babiuk Petro and Bloch (1978).
2. A 10 - 20% faecal suspension of strains 639 and 678 was made in maintenance medium, homogenised and centrifuged. The resultant supernatant was filtered through a 0.45 μ m membrane and used as antigen.
3. Faecal suspensions of strains 639 and 678 were arcton-extracted and purified through a CsCl/sucrose gradient. Pellets were taken up in 0.5 - 1.0 ml of 20mM Tris HCl buffer and used as HA antigen.

Diluent

PBS containing 0.5% BSA was employed as diluent.

Cells

Erythrocytes from human 'O', sheep, rabbit, guinea pig, rat, mouse and goose were washed and prepared as a 0.5% suspension in PBS

Haemagglutination Assay (HA)

HA was carried out in microtitre plates (V-bottom, Sterilin, UK). A serial two-fold dilution of antigen in diluent (50 μ l) with appropriate controls was incubated with equal volumes of each species of erythrocytes at 37°C and at room temperature as described by Clarke and Casals (1958). Tests were read after 1 hr. of incubation.

RESULTS

None of the virus strains, irrespective of the way in which they were prepared, gave satisfactory haemagglutination with any species of erythrocytes tested. Consequently the attempt to devise an HAI was abandoned.

DISCUSSION

This study demonstrated the presence of at least three distinct serotypes of rotaviruses in calves and the possible existence of a fourth. They were defined by a neutralisation assay using fluorescent focus reduction with a 20-fold or greater dilution difference between homologous and heterologous titres (Gaul, Simpson, Woode and Fulton, 1982; Wyatt, Greenberg, James, Pittman, Kalica, Flores

Chanock and Kapikian, 1982) and by an antigenic strain relatedness (R) value of <5%. The fact that the majority of the field strains (73%) surveyed, plus 7 of the 8 strains in the initial studies, as well as the widely used reference strains namely UK (Compton), Northern Ireland and Lincoln, all shared the same serotype suggests that this is the most common serotype in the United Kingdom at present. For the purposes of this thesis this serotype will be referred to as serotype 1 with its representative strains; UK (Compton) and 639. The other distinct strain will be designated serotype 2, with its representative strain 678; because strains 411, 683 and 2484 are antigenically related (Table 4.5b), they will be referred to as serotype 3 and strain 1548 as potential serotype 4.

As yet only one strain of serotype 2 has been established. The failure thus far to isolate another serotype 2 strain raises the question if this strain (678), was non-calf in origin i.e. was this calf infected by rotavirus from another species? Attempts to trace this sample through the Central Veterinary Laboratory, Weybridge, has failed to reveal whether the farm from which the sample originated had any other species of livestock, but have confirmed that it was of calf origin.

The fact that 9 samples were not neutralised by either antiserum to serotypes 1 or 2 could have been due to both serotypes being present in the samples. However, 4 out of 9 were successfully isolated, which indicated that the

unneutralised samples were serotypically distinct. The serotype 3 strains were the second most commonly encountered followed probably by serotype 4.

It was clear from the results obtained in the cross-immunofluorescence tests (Tables 4.1a and b) that serological differentiation by this method was not possible although 678 virus did show a slightly greater degree of variation. This was as expected since it is known that immunofluorescence detects only the common group antigen of rotaviruses (Woode, Bridger, Jones, Flewett, Bryden, Davies and White, 1976; McNulty, Allan, Todd and McFerran, 1979).

In the neutralisation tests the convalescent antisera (Table 4.2a) prepared against the serotype 1 viruses consistently distinguished serotype 2 (678) virus, but at less than 20-fold dilution difference. Similarly, the 678 convalescent antiserum (Table 4.2a) differentiated only one (639) of the serotype 1 viruses by greater than 20-fold difference and reduced the rest by 16-fold variation. There was agreement between the NT serological differentiation of the strains and their antigenic relatedness (R). With the exception of strain 641, all the 6 strains of serotype 1 viruses exhibited closer antigenic relatedness (Table 4.2b). The inability to type correctly strain 641 at this stage is due to the fact that convalescent antisera were used.

Compared to these results, the hyperimmune antisera (Table (4.3a) of the serotype 1 viruses differentiated

serotype 2 (678) virus by greater than 20-fold difference in titre, and by clear distinction of R values. With the use of hyperimmune antisera, strain 641 (Table 4.3b) was clearly shown to be antigenically similar to the serotype 1 viruses.

Thus these and similar results (Tables 4.4a and b) suggest that hyperimmune antisera might be better than convalescent antisera for the typing of rotaviruses. This is in agreement with the findings of Estes and Graham (1980), who reported that convalescent antiserum raised in a gnotobiotic calf did not permit differentiation between porcine, bovine and simian rotaviruses, whereas a hyperimmune antiserum prepared in guinea pigs differentiated these viruses in a plaque neutralisation reduction test; and also with the results of Gaul, Simpson, Woode and Fulton (1982), who observed that a difference of 20-fold or greater between homologous and heterologous neutralisation titres was not always obtained with sera from convalescent non-hyperimmunised animals. On the other hand, it has been observed that convalescent antisera obtained solely from infection in gnotobiotic calves gave a more type-specific reaction rather than hyperimmune antisera which had a broader reaction directed against common viral antigenic determinants (Yolken, Barbour, White, Kalica, Kapikian and Chanock, 1978). This seeming contradiction can be explained by the recently published report of Greenberg, McAuliffe, Valdesuso, Wyatt, Flores, Kalica, Hoshino and Singh (1983), who found that when a gnotobiotic calf was infected by rotavirus the convalescent antiserum response to the sixth

gene product was primarily to the subgroup region of the 42K protein. It is now known that the ELISA technique employed by Yolken, Barbour, Wyatt et al. (1978) detected subgroups rather than serotypes (Kapikian, Cline, Greenberg, Wyatt, Kalica, Banks, James, Flores and Chanock, 1981). Whereas convalescent antisera are better for the detection of subgroups, hyperimmune antisera are preferred for serotyping of rotaviruses.

The serotyping of field strains using the fluorescent focus assay was found to be rapid and reasonably accurate although its general usefulness was limited by the fact that it requires large amounts of infectious virus in the original faecal samples (Beards, Pilford, Thouless and Flewett, 1980; Thouless, Bryden, Flewett, Woode, Bridger, Snodgrass and Herring, 1977). This may explain why no fewer than 18% of the field samples which produced few (<50) or no fluorescent foci could not be investigated adequately. It is also possible that as a number of samples had been stored for over a year at 4°C this may have resulted in degradation of the virus particles although it was noted that some samples kept for 2 years under similar conditions produced adequate number of fluorescent foci.

The presence of high titres (320 - 5120) of serum neutralising antibodies in all samples from the adult cows might indicate that cattle are constantly being infected, presumably by different strains (serotypes), as it was impossible to type any of the sera to one particular strain.

The implication of this is that antibodies in the colostrum of such cows are very likely to provide a broad spectrum of cover to calves against most of the serotypes of rotavirus.

Although the neutralisation of focus reduction assay was rapid, it was, nevertheless, laborious. This prompted the search for a large scale method of serotyping calf rotavirus. The ELISA test has been successfully adapted for subgrouping human rotaviruses by means of specific antisera raised in two different species (Zissis and Lambert, 1980; Thouless, Beards and Flewett, 1982). These workers also found that it was necessary to absorb the sera with rotavirus from another species in order to eliminate or reduce cross-reactivity between the serotype specific antisera. In this present work, the results obtained with the ELISA technique failed to differentiate the two strains 639 and 678 but this could have been due to the fact that antisera used both in the coating of the plates and for the enzyme conjugate were raised in a single animal species or to the fact that the ELISA is not suitable for serotypic differentiation. On the other hand it could be that absorption of the sera with the heterologous SA-11 virus to make them more serotype specific had been achieved at the expense of the sensitivity of the test. Zissis and Lambert (1980) also observed in one of their ELISA systems in which the antisera had been absorbed at 4°C overnight that the procedure resulted in the removal of the type specific antibodies and, consequently, lowering of neutralising antibody titres by 4 to 16 times.

The blocking effect of the absorbed sera showed slight but consistent distinction between serotypes when cell culture virus was used, but not faecal virus. However, if all the samples to be screened must first be adapted to growth in tissue cultures then its usefulness as a rapid diagnostic test is defeated. Nevertheless, serotyping of human rotavirus using special ELISAS using faecal samples has been reported (Thouless, Beards and Flewett, 1982).

Haemagglutination was attempted because of reports that two strains of calf rotavirus had been differentiated by this method (Spence, Fauvel, Petro and Babiuk, 1978). However, in this present study the HAI was not contemplated beyond the HA stage since it was apparent that the two strains 639 and 678 did not possess haemagglutinins and because Fauvel, Spence, Babiuk, Petro and Bloch (1977), also failed to demonstrate haemagglutination by a UK strain in contrast to two Canadian strains.

In conclusion, the results in this chapter show that there are serotypic differences in bovine rotaviruses with the establishment of three or more serotypes and that one serotype is predominant in the UK. It would also appear that the serum neutralisation assays conducted either by the cpe methods of Murakami, Nishioka, Hashiguchi and Kuniyasu (1983) or by the fluorescent focus reduction tests of Beards, Pilford, Thouless and Flewett (1980), Thouless, Bryden, Flewett, Woode, Bridger, Snodgrass and Herring, (1977) will remain the methods of choice for serotyping bovine rotaviruses since the ELISA and HAI systems proved to be unsuitable.

CHAPTER 5i. VARIATION IN CALF ROTAVIRUS STRAINS:
STUDY BY GENOME dsRNA ANALYSISINTRODUCTION

The genome of rotavirus consists of 11 segments of dsRNA with molecular weights ranging approximately from 2.0×10^6 to 0.2×10^6 . The segments can be readily separated by polyacrylamide gel electrophoresis (PAGE). Comparison of RNA from different animal and human rotavirus isolates has revealed differences in the mobilities of these segments (Espejo, Calderon and Gonzalez, 1977; Kalica, Wyatt and Kapikian, 1978; Rodger and Holmes, 1979; Sabara, Deregt, Babiuk and Misra, 1982). Human strains can be subdivided into 'long' and 'short' RNA patterns (electrophoretotypes), based on the large and consistent differences in the mobilities of segments 10 and 11. Recently a direct correlation has been established between these electrophoretotypes and the subgroup classification. Thus it has been shown that human rotaviruses with the 'short' pattern belonged to subgroup I while those with the 'long' pattern belonged to subgroup II (Kalica, Greenberg, Espejo, Flores, Wyatt, Kapikian and Chanock, 1981). To date, no distinct 'short' and 'long' patterns have been observed within the genome of animal rotaviruses, although some reports have shown that calf and most other animal rotaviruses studied belonged to subgroup I (Greenberg, McAuliffe, Valdesuso, Wyatt, Flores, Kalica, Hoshino and

Singh, 1983; Kapikian, Cline, Greenberg, Wyatt, Kalica, Banks, James, Flores and Chanock, 1981; Thouless, Beards and Flewett, 1982).

Rotavirus strain differentiation by RNA electrophoresis has been used by several workers to study the epidemiology of human rotavirus (Albert, Bishop and Shann, 1983; Albert, Soenarto and Bishop, 1982; Espejo, Calderon, Gonzalez, Salomon, Mortuscilli and Romero, 1979; Espejo, Munoz, Serafin and Romero, 1980; Lourenco, Nicholas, Cohen, Scherrer and Bricout, 1981; Pereira, Azeredo, Leite, Candeias, Racz, Linhares, Gabbay and Trabulsi, 1983) but these are of limited value due to the enormous number of different patterns both between and within species (Sabara, Deregt, Babiuk and Misra, 1982).

The aims of this investigation were to study outbreaks of calf rotavirus diarrhoea with a view to identifying the number of strains involved in each outbreak and to look for any correlation between serotype and electrophoretype.

MATERIALS AND METHODS

A total of 129 rotavirus positive faecal samples from calves not more than 4 weeks of age from 30 different farms in Scotland and northern England were studied. They were collected by Dr. Snodgrass or arrived by post in 1982, during a survey into the aetiology of calf scours. At least four samples were examined from each farm. One particular

farm was sampled over a three month period (August-October) as it had an intractable rotavirus problem.

Eight separate samples representing the four different serotypes of bovine rotavirus were also investigated.

The dsRNA of both the faecal and the tissue culture adapted rotaviruses was extracted with a phenol-chloroform mixture. Some faecal samples were further purified by CF11 column chromatography. Two different PAGE systems were employed in analysing the dsRNA:

1. The faecal samples were analysed in a 7.5% PAGE using the continuous buffer system; while
2. the dsRNA of the 8 strains, representing the 4 different serotypes, were analysed in a 7.5% SDS-PAGE utilising the discontinuous buffer system.

RESULTS

The majority of the faecal samples consisted of the characteristic 11 segments of dsRNA of rotaviruses. Three samples from 2 farms showed profiles consisting of more than 11 segments. These 3 samples were considered to denote mixed or sequential infections in the calves from which they were obtained. One hundred and twenty-eight of the 129 samples exhibited the typical pattern of segments 1-4, 5-6, 7-9 and 10-11 migrating in four groups, with segments 7-9 in particular often very close or comigrating (Fig. 5.1a). In

one outbreak, however, one strain (discovered earlier by Dr. Herring, Moredun Institute) showed a complete departure from this general pattern of the rotaviruses and exhibited a different pattern which is illustrated in Fig. 5.1b. This unusual sample was later shown to be uncultivable and lacked the rotavirus common antigen. It is referred to as calf rotavirus group B in Chapter 6.

In 25 of the 30 farms (Table 5.1) only one strain of rotavirus was involved in the outbreak. This represented 98 samples of the 129 investigated. The dsRNA of the farm sampled over the three month period is illustrated in Fig. 5.2. Three representative samples have been taken from each month; the genome segment of the rotavirus showed that one single strain was responsible for the outbreak. In 5 of the 30 farms more than one strain was involved in the outbreak; this represented 31 of the 129 samples investigated. On one farm outbreak, 3 different strains were involved (Fig. 5.3, tracks 1-4, 5 and 7), while in another outbreak 4 different strains were identified (Fig. 5.3, tracks 9-10, 11, 12 and 13. page 121).

The comparison of the dsRNA from the different serotypes using the SDS-PAGE discontinuous buffer system revealed that all the preparations (Fig. 5.4, page 122) exhibited the characteristic migration pattern of rotaviruses. The only slight difference being that serotype 2, strain 678, had very closely migrating segments 7, 8 and 9.

FIGURE 5.1

dsRNA of rotavirus showing characteristic 11
segments

FIGURE 5.1a

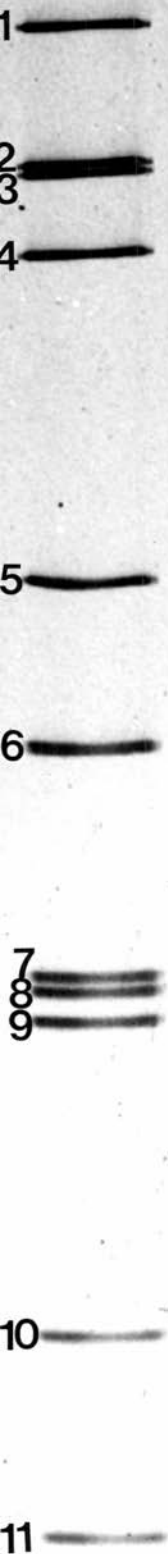
Pattern from normal calf rotavirus

FIGURE 5.1b

Pattern from calf rotavirus group B

Note: Segments 5&6 migrate together and
segment 9 migrates in group with 10
and 11

a



b

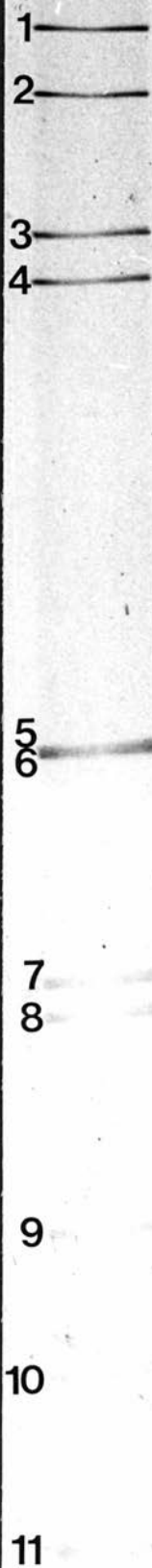


TABLE 5.1

STRAINS OF ROTAVIRUS INVOLVED IN OUTBREAKS OF CALF SCOUR

No. of Strains Involved in Each Outbreak	No. of Farms
25	1
3*	2
1	3
1	4
30	TOTAL

* Three samples from 2 of these farms had mixed dsRNA segments.

FIGURE 5.2

dsRNA of strains of calf rotavirus involved
in an outbreak over a three month period
(August to October)

Tracks 1 - 3. Samples from August
4 - 6 Samples from September
7 - 9 Samples from October

Note: The identical nature of all the
strains over the three month
sampling period.

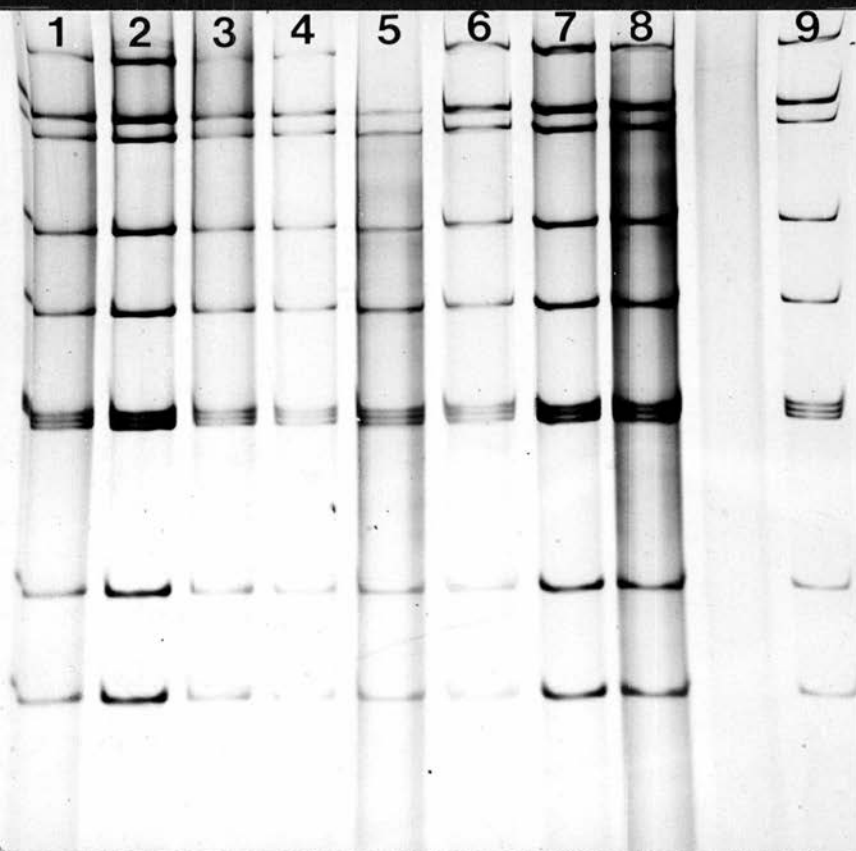


FIGURE 5.3

dsRNA of strains of calf rotavirus involved in outbreaks, showing the presence of more than one strain in each outbreak.

Tracks 1 - 7 A single farm outbreak showing the involvement of 3 different strains
(See text, page 117)

9 - 13 Outbreak from a single farm showing the involvement of 4 different strains (See text, page 117)

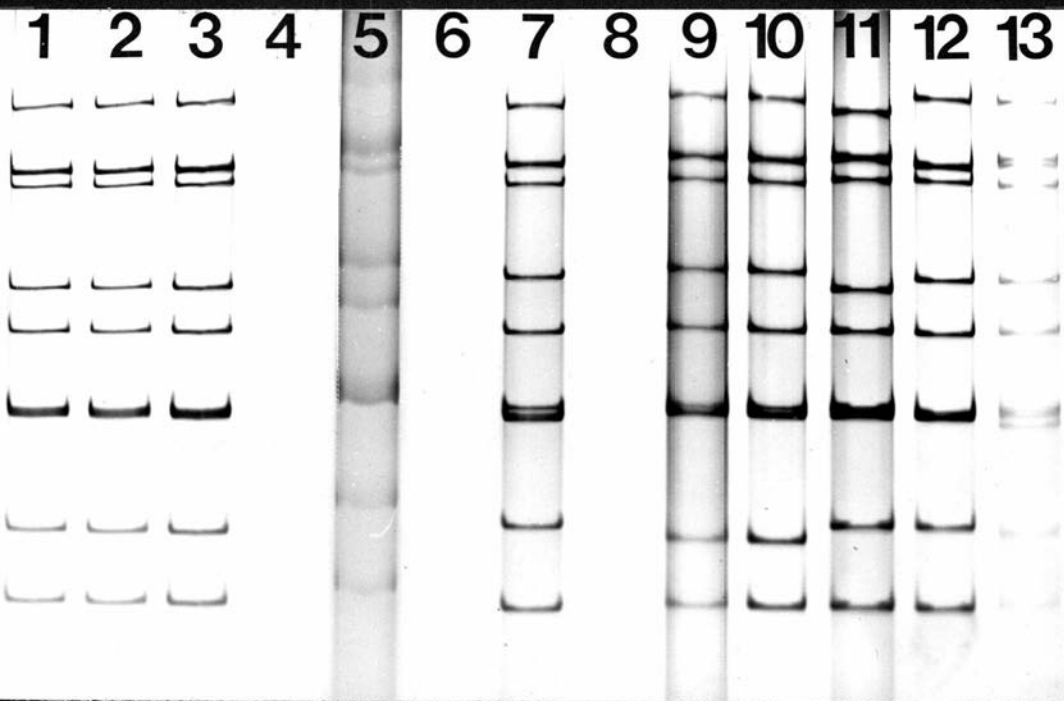


FIGURE 5.4

Comparison of dsRNA of 4 different serotypes
of calf rotavirus

- Tracks 1 - 3. Serotype 1
 4. Serotype 2
 5 - 7. Serotype 3
 8. Provisional serotype 4

1

2

3

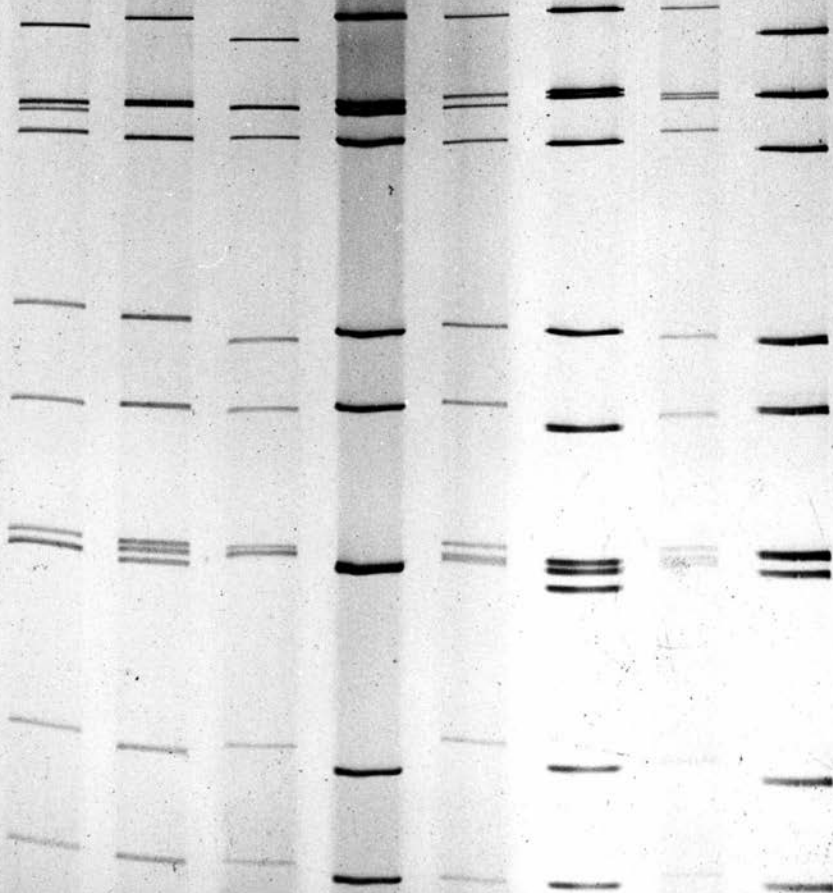
4

5

6

7

8



ii. COMPARISON OF THE POLYPEPTIDES OF BOVINE ROTAVIRUS
SEROTYPES

INTRODUCTION

The structure of rotavirus has been the subject of several investigations, resulting initially in a number of conflicting interpretations as regards the total number of polypeptides (Bridger and Woode, 1976; McCrae and Faulkner-Valle, 1981; McNulty, 1979; Newman, Brown, Bridger and Woode, 1975; Rodger, Schnagl and Holmes, 1975; Rodger Schnagl and Holmes, 1977; Thouless, 1979; Todd and McNulty, 1976). However, the consensus view is in the range of 8 - 10 polypeptides, (Matthews, 1982).

A major glycosylated outer polypeptide (VP7) has been shown to elicit neutralising antibodies (Bastardo, McKimm-Breschkin, Sonza, Mercer and Holmes, 1981; Kalica, Flores and Greenberg, 1983; Killen and Dimmock, 1982; Matsuno and Mukoyama, 1979, Rodger, Schnagl and Holmes, 1977). However, it has been suggested that because of the complex nature of rotaviruses, more than one polypeptide may well be involved in neutralisation reactions (Beards, Pilford, Thouless and Flewett, 1980; Bastardo, McKimm-Breschkin, Sonza, Mercer and Holmes, 1981). The outer polypeptides are important not only for neutralisation, but are also concerned with infectivity in the complete double shelled rotavirus (Bridger and Woode, 1976; Elias, 1977). Unfortunately, these outer shell polypeptides tend to disintegrate during CsCl/sucrose

gradient purification (Novo and Esparza, 1981); to enhance their stability therefore divalent cations (Ca^{++} or Sr^{++}) are added to buffers throughout the purification process (Cohen, Larporte, Charpillienne and Sherrer, 1979; Shirley, Beards, Thouless and Flewett, 1981).

In this section the VP7 of the different serotypes of bovine rotavirus is compared.

MATERIALS AND METHODS

Viruses

The UK (Compton), Northern Ireland, Lincoln and strain 639, all belonging to serotype 1, isolate 678 (serotype 2), strain 411, 683, 2484 (serotype 3) and 1548 provisionally classified as serotype 4 were used in this experiment. All had undergone at least 11 passages in MA104 cells and with the exception of the Lincoln and Northern Ireland isolates, had been cloned either by plaque purification or by passage at terminal dilutions. All the viruses were purified through CsCl /sucrose gradients.

EM

Purified virions were examined by EM and the proportion of smooth to rough particles estimated.

PAGE

The samples were analysed by SDS-PAGE a and silver staining technique for polypeptides.

RESULTS

VP7 was detected in preparations of UK (Compton), Northern Ireland, Lincoln, 639, 678 and 683, but not in 411, 1548 and 2484 (Fig 5.5). In the preparations in which it was detected, the VP7 from each strain migrated differently. Nevertheless they migrated between ovoalbumin (45,000 MW) and chymotrypsinogen A (25,000 MW).

There was good correlation between the presence of VP7 in the gels and the proportion of smooth particles. The proportion of complete virions estimated by EM for the different viruses was greater than 50% for the 6 viruses in which VP7 was detected and <15% for the 3 viruses in which VP7 was not detected (Table 5.2, page 127).

DISCUSSION

The results of these investigations demonstrate that in the majority of outbreaks of calf rotavirus one strain was usually responsible. In the 5 farms in which more than one strain was detected, one strain appeared to predominate, but larger samples would be required to assess this quantitatively. These results are in agreement with those of other workers in the human rotavirus field who have found

FIGURE 5.5

Comparison of the polypeptides (VP7) of bovine rotavirus serotypes

Track 1 contains standard proteins made up of:

β	β-galactosidase	M.W. 130,000
p	phosphorylase A	92,000
ot	ovotransferin	76-78,000
a	albumin	66,200
oa	ovoalbumin	45,000
c	chymotrypsinogen A	25,700
m	myoglobin	17,200
cy	cytochrome C	12,300
2.	UK (Compton)	(Serotype 1)
3.	Northern Ireland	"
4.	Lincoln	"
5.	639	"
6.	accidental loading	
7.	678	(Serotype 2)
8.	411	(Serotype 3)
9.	1548	(Potential serotype 4)
10.	2484	(Serotype 3)
11.	683	(Serotype 3)
12.	2484	(Serotype 3) repeat

Note: Arrows show VP7 of different strains

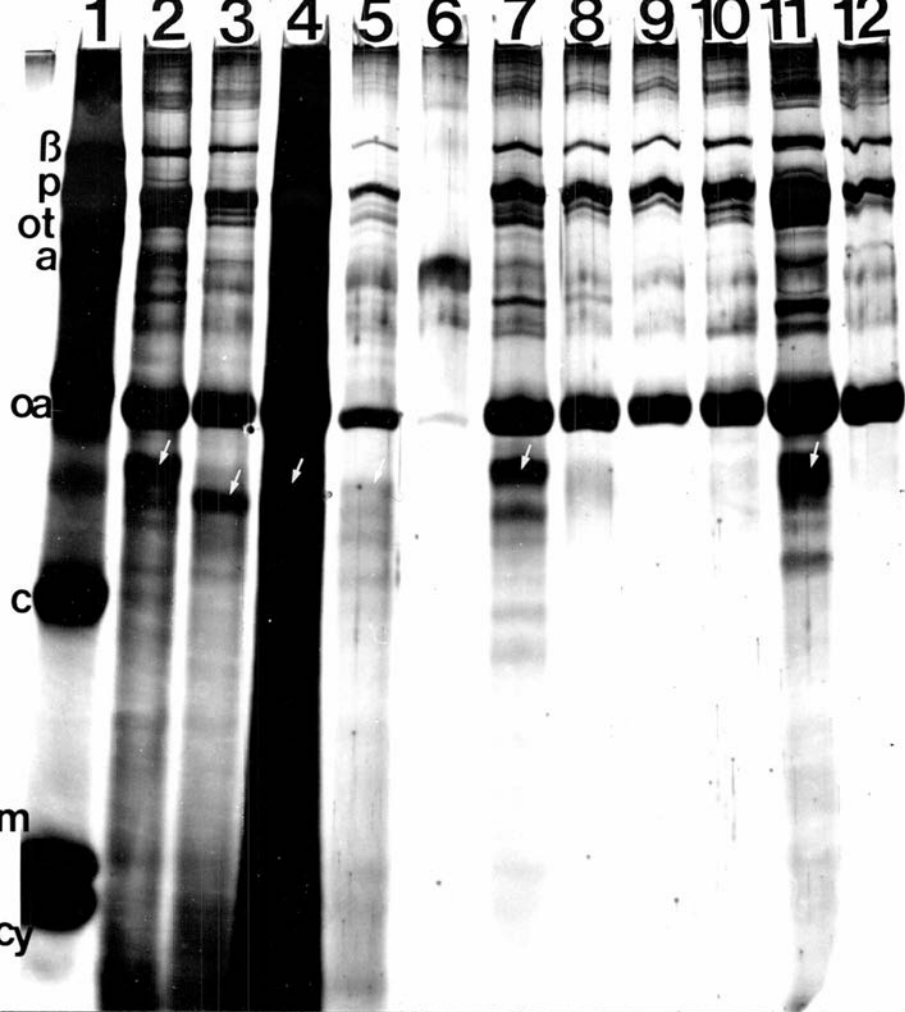


TABLE 5.2

PROPORTION OF SMOOTH PARTICLES IN VIRUS PREPARATIONS

Virus Strain	Percentage Smooth Particles
UK (Compton)	68
Northern Ireland	60
Lincoln	70
639	54
678	90
683	63
411	10
1548	11
2484	15

that in outbreaks of rotavirus gastroenteritis in children, one strain among the many that might be involved was usually predominant (Espejo, Munoz, Serafin and Romero, 1980; Rodger, Bishop, Birch, McLean and Holmes, 1981; Follett and Desselberger, 1983). The PAGE analysis has the additional advantage of detecting the unusual types of rotavirus that may be co-circulating with normal rotavirus, as was the case with the calf rotavirus group B.

The comparison of dsRNA of the serotypes did not reveal any significant departure from the characteristic mobility pattern of rotaviruses. As yet, no direct correlation has been established between electrophoretotypes and serotypes of calf rotavirus strains and, by analogy with human rotavirus strains, such correlation probably does not exist. In the absence of further isolates of serotype 2, the significance of the co-migration of segments 7,8 and 9 in strain 678 is not clear. The serotype 3 and potential serotype 4 rotaviruses could not be distinguished (electrophoretically) from the serotype 1 strains, as they all exhibited the characteristic rotavirus pattern.

In the initial investigation involving 12 strains of calf rotavirus (Chapter 3), four of 12 samples were mixed; but in this present study, 3 of 129 sampled were mixed. It has been suggested that mixed segment profiles might be common from field cases of calf rotavirus (Sabara, Deregt, Babiuk and Misra, 1982). If that were the case, then the small proportion of 3 out of 129 samples might be due to the

fact that 7.5% PAGE using the continuous buffer system employed in analysing these samples was not as sensitive as the high resolving gels of Sabara et al. (1982). Also in the case of 4 of the 12 samples, inoculation of gnotobiotic samples might have allowed strains hitherto in very low undetectable amounts by in-vitro methods to multiply.

Genetic analysis of SA-11 and reassortants of the human (Wa) strain and the UK bovine rotaviruses have identified RNA segments 9 of the human (Wa) and SA 11 as coding for the protein that elicits neutralising antibodies (Flores, Greenberg, Myshinski, Kalica, Wyatt, Kapikian and Chanock, 1982; Greenberg, Wyatt, Kapikian, Kalica, Flores and Jones, 1982; Kalica, Greenberg, Wyatt, Flores, Sereno, Kapikian and Chanock, 1981; Kalica, Flores and Greenberg, 1983; Mason, Graham and Estes, 1983). This protein has been identified as the outer polypeptide VP7 (Bastardo, McKimm-Breschkin, Sonza, Mercer and Holmes, 1981; McCrae and McCorquodale, 1982). The presence of this polypeptide in some strains and its absence in others (Fig. 5.5) after CsCl/sucrose gradient purification, despite the use of divalent cations (Ca⁺⁺) in the buffers, was not clear. The most likely explanation was that some strains of calf rotavirus lose their outer capsids more easily than others. Novo and Esparza (1981) reported the same observation with isolates of bovine rotaviruses; while the outer shelled capsid of the mouse rotavirus was equally easily lost with handling (Woode, Bridger, Jones, Flewett, Bryden, Davies and White, 1976). It was interesting to observe that those strains

lacking VP7 were difficult to adapt to tissue culture propagation, requiring higher concentrations of trypsin and longer incubation (data not shown). Although these viruses belonged to serotype 3 and potential serotype 4, it would require comparison of more isolates of the same serotypes to ascertain whether this lack of VP7 in their tissue culture preparations is a constant feature of these serotypes. Nevertheless, this underscores a potential problem if vaccines with those serotypes are contemplated.

Comparison of virions prepared from faeces and tissue culture showed that VP7 from tissue culture preparations tended to stain less intensely than in preparations from infected faeces (data not shown), although no difference was observed in the VP7 when faecal and tissue culture virions of the UK (Compton) strain was compared (Thouless, 1979). Unfortunately, the comparison of the faecal and tissue culture virions of these strains without the VP7 was not possible as faecal samples had been depleted.

In summary, the investigations described in this chapter showed that in each individual herd outbreak, only one strain of calf rotavirus was involved, although multiple strain infections did occur on a minority of farms. The different electrophoretotypes as seen by PAGE could not be correlated with serotypes. It also reveals that the important outer shell polypeptide VP7 showed variation in migration pattern; and was only present in the tissue culture preparations of some strains but not in others.

CHAPTER 6COMPARISON OF VIRULENCE OF DIFFERENT STRAINS AND
SEROTYPES OF BOVINE ROTAVIRUS IN ISOLATED CALF GUT LOOPSINTRODUCTION

Apart from studying the pathogenesis of individual isolates of rotaviruses in gnotobiotic animals (Halpin and Caple, 1976; Lecce, King and Mock, 1976; Mebus, Stair, Underdahl and Twiehaus, 1971; Pearson and McNulty, 1977) few workers have actually compared the virulence of different strains (Carpio, Bellamy and Babiuk, 1981; Logan, Pearson and McNulty, 1979).

The technique of inoculating ligated calf gut loops for differentiating between enteropathogenic and non-enteropathogenic strains of E. coli is well established (Myers and Guinee, 1976; Smith and Halls, 1967). Recently, Carpio, Bellamy and Babiuk (1981) used this technique to compare the virulence of 4 different Canadian isolates of bovine rotaviruses. While variation in virulence was observed, no information on serotypes was available.

In this chapter, studies on the virulence of different strains and serotypes of bovine rotavirus in ligated calf gut loops are presented.

MATERIALS AND METHODSAnimal

One 2, day, old gnotobiotic calf was used. Examination of faecal samples prior to inoculation showed the gut to be free of bacteria.

Viruses

A total of 19 strains of rotavirus was studied, of which 9 were faecal filtrate viruses, 5 were tissue culture adapted viruses, and, in 5, both faecal and tissue culture sources were used. All the strains were of calf origin, with the exception of 3 - one piglet, one lamb and one simian. All cell culture adapted viruses were grown in MA104 cells. The nature and treatment of each virus strain is summarised in Table 6.1

Faecal samples were prepared as 20 - 25% w/v or v/v suspensions in either serum-free maintenance medium or PBS pH7.2 and passed through a Millipore membrane filter of 0.45µm average pore diameter. The inoculum of all the faecal viruses except calf group B and piglet group C was standardised to $3.0 \log_{10}$ TCID₅₀/ml in MA104 cells, while the inoculum for the cell culture viruses was standardised to $6.0 \log_{10}$ TCID₅₀/ml. Most of the strains were inoculated into two separate loops in proximal and middle small intestine.

TABLE 6.1

STRAINS OF ROTAVIRUS INOCULATED INTO CALF GUT LOOPS

Virus Strains	Passage Level in MA104 Cells	Virus Inoculum	
		Tissue Culture	Faeces
UK (Compton)	6(a)	+	-
Northern Ireland	4(a)	+	-
Lincoln	6(a)	+	-
639 (Serotype 1)	13	+	+
678 (" 2)	12	+	+
411 (" 3)	12	+	+
683 (" 3)	11	+	+
2484 (" 3)	11	+	+
1548 (" 4)	11	+	-
SA-11 Simian rotavirus	6(a)	+	-
G753 (Serotype 1)	-	-	+
D28 (" 1)	-	-	+
D128/34 (" 1)	-	-	+
D194/1 (" 1)	-	-	+
D531/42 (" 1)	-	-	+
D2038 (" 1)	-	-	+
Lamb Rotavirus Group A	-	-	+
Calf Rotavirus Group B	-	-	+(b)
Piglet Rotavirus Group C	-	-	+(b)

(a) Pasaged for unknown number of times in other laboratories.

(b) Used as 25% faecal filtrate.

Surgical Procedure

General anaesthesia was induced with a 4% halothane (Fluothane, ICI, UK), oxygen and nitrous oxide gaseous mixture and maintained throughout the operation on a 1.5% mixture of the same by means of a closed circuit system (Fluotec 3, Cyrane Ltd, Keighley, UK).

A 15 - 20 cm ventro-abdominal incision was made along the linea alba and the ileo-caecal junction of the small intestine was located. An initial ligature using a broad cotton tape was applied 2 - 3 metres proximal to the ileo-caecal junction and to the Peyers patches. Fifty-four isolated loops approximately 7 - 10 cm in length were then made using braided nylon suture at both ends of each loop, with a 5 cm gap between each loop. Loops were divided into 5 sets of 10 or 11 with broad cotton tape in order to demarcate each set. Each loop in a set was inoculated with 2 ml of the appropriate strain of virus using a plastic syringe and 26 gauge needle. Midway along each set of loops positive and negative control samples were inoculated, consisting of G753 (positive control), maintenance medium and rotavirus negative faecal filtrates (negative controls).

Approximately 3 litres of warm (37°C) sterile Ringer's solution prepared according to the method of Gingerich (1981) was administered to the calf intravenously during surgery and throughout the 24 hr period of the experiment. Analgesia was maintained by an initial injection of Rompun

(0.1ml/50kg, Bayer, UK) and thereafter maintained by regular 4-hourly deep intramuscular injections of pethidine (1mg/kg, Evans Medical, UK). Following surgery, the calf was placed in a pen by itself. Post operative recovery was uneventful and the animal was soon up on all four feet, alert and showing no obvious signs of discomfort.

Necropsy Procedure

Approximately 24 hr. post-inoculation, the calf was deeply anaesthetised by an intravenous injection of pentobarbitone sodium (May & Baker, UK). Portions of all loops were removed and fixed. The calf was then killed by an overdose of pentobarbitone sodium.

Histological and Cryostat Processing

Tissues for histology were opened along the length of the intestine and fixed flat, mucosal surface uppermost, in 10% buffered formol saline. Twenty-four hours later the specimens were trimmed and divided into small pieces for routine processing in paraffin-wax blocks. Sections were cut at 5 μ m and stained with haematoxylin and eosin (H & E). These procedures were carried out by Mr. K.W. Angus and Miss G. Hutchison of the Moredun Institute.

Portions of intestine for cryostat sectioning were filled with Tissue-Tek II (Miles Laboratories, Illinois, USA) and frozen immediately in a CO₂-isopentane freezing

mixture and stored at -70°C for subsequent IF examination.

Immunofluorescent Staining

Frozen transverse sections were cut ($6\mu\text{m}$ thick) on a cryostat (Frigocut, Mod.27,00, Reichert-jung, W. Germany), mounted on glass slides and fixed in chilled acetone. The sections were then stained first with a gnotobiotic calf antiserum to calf rotavirus, followed by a fluorescein-conjugated rabbit anti-sheep globulin (Wellcome Reagents) and then examined using a u.v. light microscope.

Histological Examination

H & E stained sections were examined, particular attention being paid to the size and shape of the villi, epithelial cell morphology and the extent of cellular infiltrations within the lamina propria. In each section ten well orientated villi and crypts were measured by eyepiece graticule calibrated on a reference measurement slide (Leitz, W. Germany).

Statistical Analysis

The negative controls in each set of loops were combined and the results compared with the test measurements in that set, by one-way analysis of variance.

RESULTSMacroscopic Observations

Most of the loops appeared normal, while others were distended with fluid which was either frothy, haemorrhagic or serous.

Histological Examination

Five sets of loops (A to E from distal to proximal) were infected. The examination of the negative control loops in each set showed that the mucosa contained long and slender finger-like villi (508-651 μ m in length), with columnar epithelial cells (Fig 6.1a). The villi in the proximal loops were taller (Figs 6.2 and 6.3). No cellular infiltrations were observed in the control loops apart from mononuclear cells.

Viruses inoculated in the proximal loops (Fig 6.3 sets D and E) produced only mild pathological changes of cellular infiltrations but caused no significant damage to either the crypts or the villi. On the other hand, when the same viruses were inoculated into the middle and distal loops (Fig 6.2 sets A B and C), significant pathological changes were observed in the villi, resulting in abnormal shapes and sizes (Fig 6.1b), replacement of columnar epithelial cells by cuboidal cells (Fig 6.1c) and marked neutrophilic cellular infiltrations. For example, the serotype 1 viruses (G753, UK(Compton), Northern Ireland and Lincoln) and the

FIGURE 6.1

Inoculation of rotavirus strains into calf
gut loops

FIGURE 6.1a

Control loop showing normal villi with
columnar epithelial cells

Set A Distal small intestine (ileum)

H & E x 360

FIGURE 6.1b

Infected loop with G753

Note: Stunting of villi, abnormal shapes and
increased cellularity of the lamina
propria

Set A Distal small intestine (ileum)

H & E x 360

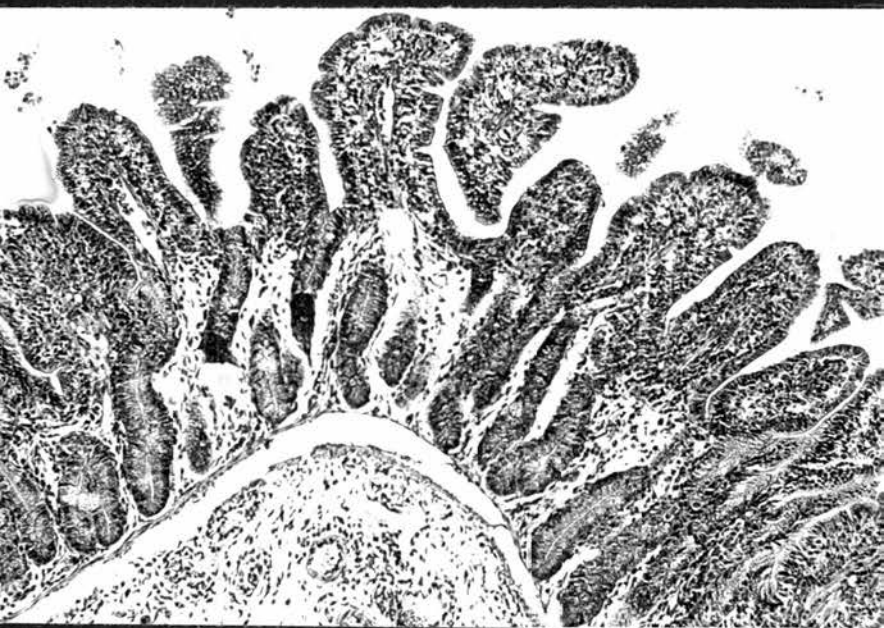


FIGURE 6.1c

Infected villi

Note: Replacement of columnar epithelial cells by cuboidal cells and massive cellular infiltration in the lamina propria

H & E x 1440

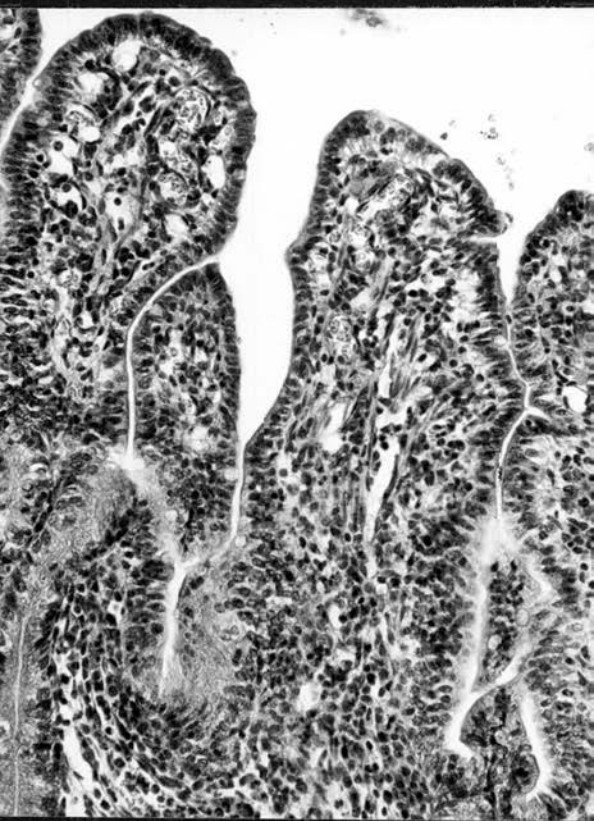


FIGURE 6.2

Tissue Changes Induced in Calf Gut Loop
Inoculated with Strains of Rotavirus.

+

Measurements (Mean - se) of villous height and crypt depth in sites A, B and C (distal portion) of the small intestine.

A	B	C
1. UK(Compton)	1. 411 t/c	1. D128/34 f/f
2. Northern Ireland	2. 411 f/f	2. D531/42 f/f
3. Lincoln	3. 683 t/c	3. Calf group B f/f
4. G753 f/f	4. 683 f/f	4. G753 f/f
5. 639 t/c	5. G753 f/f	5. Lamb group A f/f
6. 639 f/f	6. 1548 t/c	6. Piglet group C f/f
7. 678 t/c	7. 2484 t/c	7. D194 f/f
8. 678 f/f	8. 2484 f/f	8. D128 f/f
	9. SA-11 t/c	9. D2038 f/f

C = Controls: Combined values for maintenance medium and negative faecal filtrate inocula

t/c Tissue culture virus

f/f Faecal virus

The levels of significance of the deviations from the controls are:

x p <0.050

xx p <0.010

xxx p <0.001

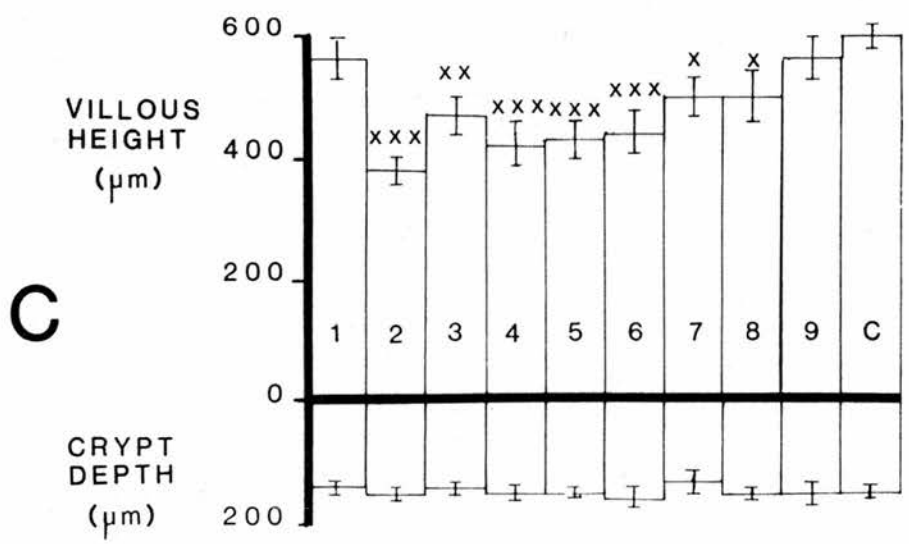
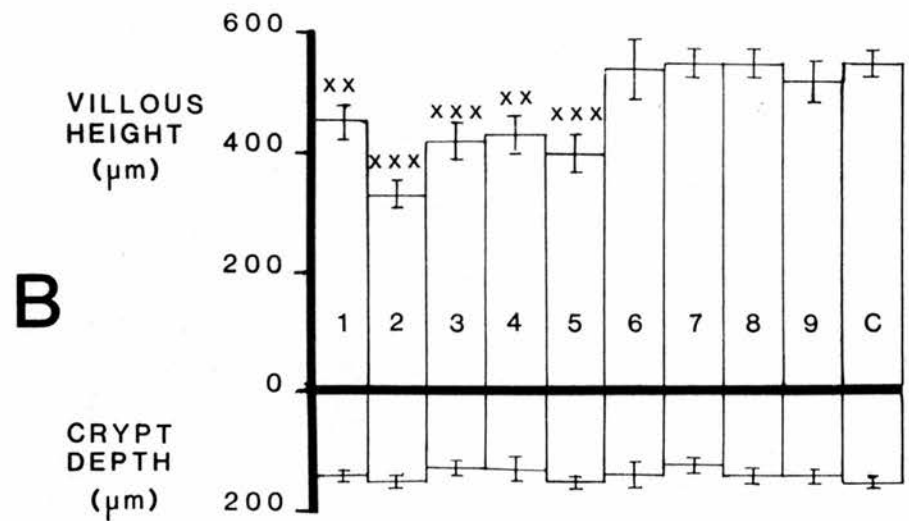
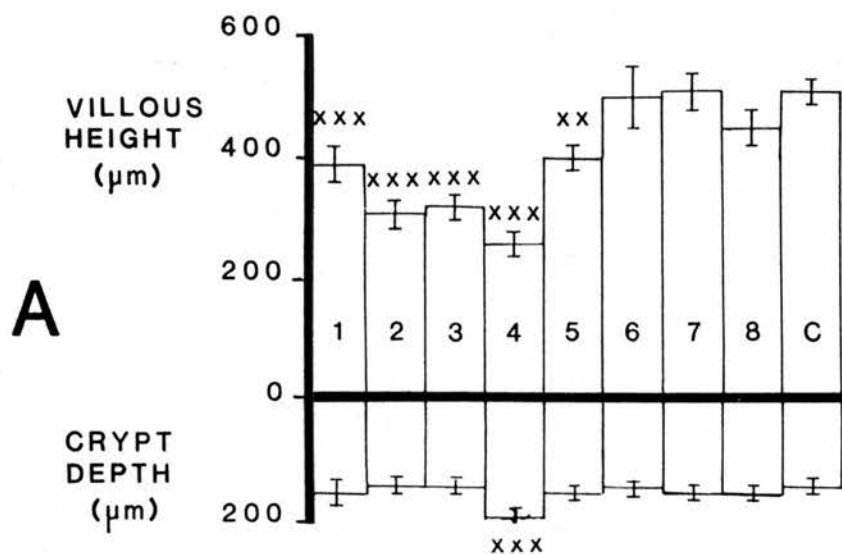


FIGURE 6.3

Tissue Changes Induced in Calf Gut Loops
Inoculated with Different Strains of Rotavirus

+
 Measurements (Mean - se) of villous height and crypt depth
 in sites D and E (proximal portion) of the small intestine

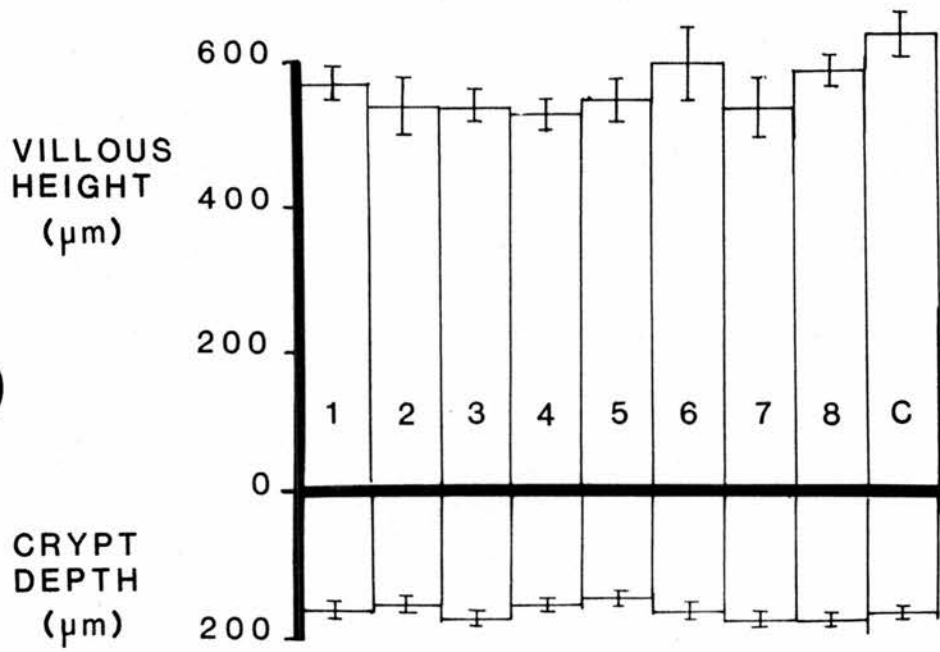
D	E
1. UK(Compton)	1. 411 t/c
2. Northern Ireland	2. 411 f/f
3. Lincoln	3. 683 t/c
4. G753 f/f	4. 683 f/f
5. 639 t/c	5. G753 f/f
6. 639 f/f	6. 1548 t/c
7. 678 t/c	7. 2484 t/c
8. 678 f/f	8. 2484 f/f
	9. SA-11 t/c

C = Controls: Combined values for maintenance medium
 and negative faecal filtrate inocula

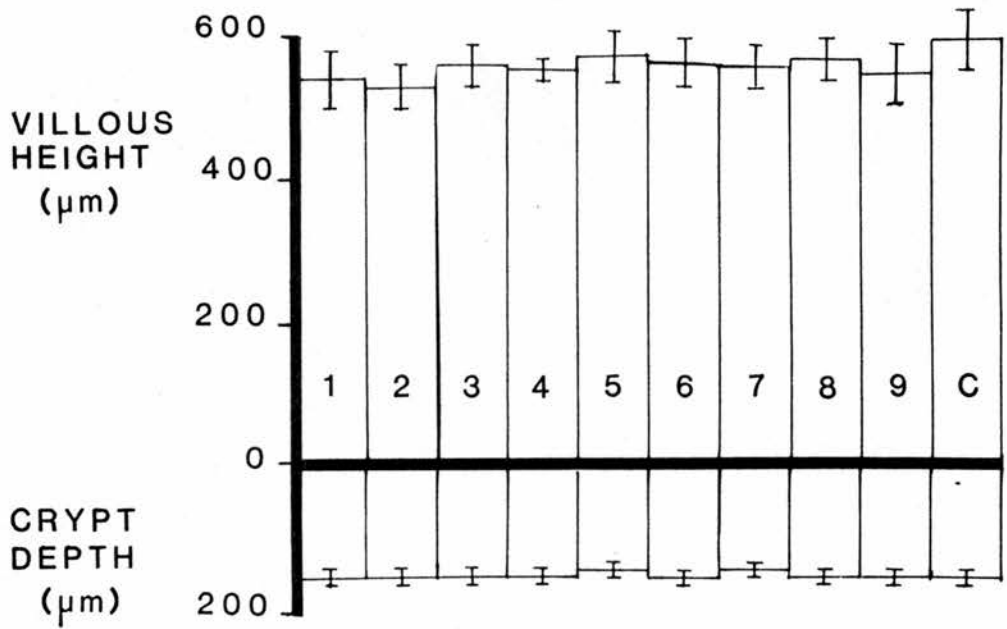
t/c Tissue culture virus

f/f Faecal virus

D



E



serotype 3 viruses (411 and 683) significantly reduced the villous height ($p < 0.001$ and $p < 0.01$ respectively) in middle and distal small intestine, but failed to evoke similar responses in proximal loops. Neither the faecal nor the tissue culture forms of serotype 2 virus significantly reduced the villi in any sections of the intestine.

The effect of all these viruses on the crypt was not significant, other than one strain G753 (Fig. 6.2A4) which significantly increased the depth of the crypt while at the same time it significantly reduced the length of the villi. But as the effect was limited to only this section of the gut and not produced by the same virus strain in the other parts of the gut it was regarded as of little importance.

The pathology of the rest of the middle and distal portions of the gut showed that some strains produced a mixture of shortened and normal villi, whereas others showed very little pathological changes apart from slight to moderate infiltration of neutrophils (Table 6.2 a, b and c) Moderate mononuclear cell infiltrations occurred in the lamina propria, but this was a common feature in specimens prepared from both the control and infected loops and therefore of no significance.

The most significant and quantifiable lesion produced was the reduction in villous height. For this reason, this was chosen as a marker for virulence, and viruses were compared in their ability to cause villous atrophy.

TABLE 6.2a

TISSUE CHANGES INDUCED BY ROTAVIRUS STRAINS IN THE MIDDLE AND
DISTAL SMALL INTESTINE OF GNOTOBIOTIC CALF.

Virus	Nature of villus			Epithelium at prox. 1/3	Cells infil. lamina propria
	Finger-like	Stunted	Swollen		
Control	+	-	-	columnar	mononu- clear
UK(Com.)	i. cone shaped ii. leaf shaped	+	+	cuboidal	neutro- phil
North. Ireland.	mixed	more stunted	moderate	mostly cuboidal	capil- laries plugged with neu- trophil
Lincoln	club shaped	+	moderate	cuboidal	neutro- phil, eosino- phil
G753	broad shaped	+	+	cuboidal	neutro- phils in lumen of crypt and capi- llaries
639*	mixed	+	+	cuboidal	neutro- phil
639**	mixed	+ -	+ -	columnar cuboidal	neutro- phil
678*	mixed	+ -	+ -	mainly columnar	neutro- phil
678**	mixed	+ -	+ -	mainly columnar	neutro- phil, eosino- phil

* Tissue Culture Virus

** Faecal Virus

+ Evidence of stunted and swollen villi

- Absence of stunted and swollen villi

TABLE 6.2b

TISSUE CHANGES INDUCED BY ROTAVIRUS STRAINS IN THE MIDDLE AND
DISTAL SMALL INTESTINE OF GNOTOBIOTIC CALF

Virus	Nature of Villus			Epithelium on prox.1/3	Cell infil. lamina propria
	Finger-like	Stunted	Swollen		
Control	+	-	-	columnar	mononu- clear
411*	Leaf- like	+	+	cuboidal	neutrophil
411**	Broad shape	+	+	cuboidal	neutrophil
683*	Broad- shape	+	+	cuboidal	neutrophil
683**	Cone shape	+	+	cuboidal	neutrophil
G753	Broad- shape	+	+	cuboidal	neutrophil
1548	Finger- like	-	moderate	columnar	neutrophil
2484*	mixed	+ -	moderate	columnar	neutrophil
2484**	Finger- like	-	-	columnar	neutrophil
SAll	Finger- like	-	-	columnar	neutrophil

* Tissue Culture Virus

** Faecal Virus

+ Evidence of stunted and swollen villi

- Absence of stunted and swollen villi

TABLE 6.2c

TISSUE CHANGES INDUCED BY ROTAVIRUS STRAINS IN THE MIDDLE AND
DISTAL SMALL INTESTINE OF GNOTOBIOTIC CALF

Virus	Nature of villus			Epithelium on prox.1/3	Cell infil. lamina propria
	Finger-like	Stunted	Swollen		
Control	+	-	-	columnar	mononu- clear
D128/34	mixed	+	+	columnar	neutrophil
D531/42	leaf- like	+	+	cuboidal	neuteophil
Calf Group B	mixed	+	+	cuboidal	neutrophil
G753	mixed	+	+	cuboidal	neutrophil
Lamb Group A	mixed	+	+	cuboidal	neutrophil
Piglet Group C	mixed	+	+	cuboidal	neutrophil
D194	mixed	+	+	cuboidal	neutrophil
D28	mixed	+	+	cuboidal	neutrophil
D2038	mixed	+	+	columnar	neutrophil

+ Evidence of stunted and swollen villi

- Absence of stunted and swollen villi

Comparison of Tissue Culture and Faecal Viruses

The comparative virulence of tissue culture grown and faecal viruses was compared by estimating the reduction in villous height. The overall virulence of the tissue culture viruses was not significantly different from those of the faecal viruses (Table 6.3). However, in the individual comparisons of viruses used in both tissue culture and faecal preparations, confusing results were obtained. Virus 639 was not pathogenic in faecal preparations but was significantly so in tissue culture form, while virus 411 was pathogenic in both forms but significantly more so in faecal preparations. Virus 678 was not pathogenic in either preparation, while virus 683 was equally pathogenic in both.

Comparison of Serotypic Virulence

The effects of the 4 serotypes on the villi were compared, using reduction in villous height as an index of virulence (Table 6.4). There was considerable variation in individual virus strain virulence within serotypes. Serotype 1 viruses overall were significantly pathogenic ($p < 0.001$), but some (eg D128 and D2038) were non-pathogenic. Similarly, serotype 3 viruses were significantly pathogenic ($p < 0.05$), but virus 2484 caused no lesions. The individual representatives of serotypes 2 and 4 caused no lesions.

TABLE 6.3

COMPARISON OF TISSUE CULTURE AND FAECAL VIRUSES

Virus	Mean Villous Height (μm - se)		P
	Tissue Culture	Faecal Virus	
Overall	458 - 29 (10)	450 - 34 (11)	N.S
639	394 - 21 (22)	503 - 54 (1.0)	<0.05
678	505 - 26 (0.6)	445 - 31 (12)	N.S
411	425 - 25 (23)	326 - 19 (41)	<0.05
683	418 - 32 (24)	432 - 32 (22)	N.S
2484	550 - 20 (0.1)	548 - 18 (0.5)	N.S

() Percentage Reduction from Control Villous Height

N.S Not Significant.

P Level of significance

TABLE 6.4

COMPARISON OF SEROTYPIC VIRULENCE

Serotype	Virus	Mean Villous Height		Villous Height Group Mean	
		μm	\pm se	μm	\pm se
Control	Control site A	508	\pm 18	546	\pm 20
	Control site B	551	\pm 22		
	Control site C	580	\pm 26		
1	UK (Compton)	393	\pm 18	441	\pm 29
	North. Ireland	309	\pm 16		
	Lincoln	318	\pm 16		
	639 *	394	\pm 21		
	639 **	503	\pm 54		
	D128/34	560	\pm 30		
	D531/42	379	\pm 21		
	D194	503	\pm 29		
	D28	449	\pm 21		
D2038	558	\pm 33			
2	678 *	505	\pm 26	475	\pm 30
	678 **	445	\pm 31		
3	411 *	425	\pm 25	450	\pm 30
	411 **	326	\pm 19		
	683 *	418	\pm 32		
	683 **	418	\pm 32		
	2484 *	550	\pm 20		
2484 **	548	\pm 18			
4	1548 *	540	\pm 41	540	\pm 41
Serotype 1 versus	Group Mean	p < 0.001		* Tissue Culture	
Serotype 2 versus	Group Mean	p = N.S		** Faecal Virus	
Serotype 3 versus	Group Mean	p < 0.05			
Serotype 4 versus	Group Mean	p = N.S			

Immunofluorescence

No fluorescence was detected in negative control loops; but specific IF staining was demonstrated in the villi of all infected loops apart from those infected with calf group B and piglet group C rotaviruses. Fluorescing epithelial cells of the distal and proximal portions were generally at the tip of the villi (Fig 6.4), with the number of fluorescing cells being only marginally different between different serotypes and viruses.

DISCUSSION

The use of ligated gut loops was validated in this experiment in the sense that the tissue changes observed in the calf gut following inoculation of different strains of rotavirus were confined to infected loops as judged by the presence and distribution of specific immunofluorescent staining of cryostat sections and histopathological changes.

Control loops were normal, apart from the presence of mononuclear cellular infiltrations which were also seen in infected loops. The only preparations that failed to show fluorescing cells were either the control loops or those infected with calf group B and piglet group C rotaviruses. The inability of these group B and C rotaviruses to induce fluorescence was not unexpected since it had been established that these rotaviruses lacked the group antigen and could not therefore be detected with the reagents used in this experiment. Despite this, however, the fact that they were capable of inducing pathological changes on the

FIGURE 6.4

Immunofluorescent Staining of Cryostat
Sections of Infected Calf Gut Loops

Note: Fluorescing cells at the tip of villus
FITC x 900

calf villi was confirmed by the presence of appreciable shortening of the villi and partial replacement of the columnar epithelial cells by cuboidal cells (Table 6.2 c).

It was observed that all the viruses inoculated at the proximal portions and repeated at the middle and distal sections of the gut did not induce any significant pathological changes at the proximal end as they did distally. This observation agrees very closely with the findings of Snodgrass, Ferguson, Allan, Angus and Mitchell (1979) and Snodgrass, Angus and Gray (1977), who noted that the pathology of rotavirus infections in gnotobiotic lambs was less consistent and less severe in the jejunum than in the midgut (ileum) and those of Mebus, Stair, Underdahl and Twiehaus (1971), who observed that the upper small intestine of the calves infected with the Nebraska Calf Diarrhoea Virus (rotavirus) either did not fluoresce with antigen or had only few fluorescing cells at the tip of the villi. The 'upper small intestine' and the 'jejunum' of these workers, correspond by analogy to the proximal loops (Fig 6.3 sets D and E) in this present experiment. Carpio, Bellamy and Babiuk, (1981) also noted that histopathological changes varied considerably with the isolates tested, but tended to be more consistent in the ileum than in the jejunum. However the absolute decrease in villous length that resulted from infection was greater in the jejunum than in the ileum.

It was difficult to assess the dose-effect of these different rotaviruses. In the end, $3.0 \log_{10}$ TCID₅₀/ml for faecal viruses and $6.0 \log_{10}$ TCID₅₀/ml for tissue culture viruses were used as practical levels, but there is no evidence as to how these relate to epithelial cell infectivity. Comparison of the tissue culture and faecal forms of the same virus showed that they affected the villous height differently; only the tissue culture form of strain 639 significantly reduced the villi, while both the faecal and tissue culture forms of strain 411 significantly reduced the villi (Fig. 6.2A5 and 6.2B1 and 2). On the whole, there was no difference between the effect of tissue culture and faecal viruses (Table 6.3). These individual variations were perhaps due to difficulties in standardizing the dose used. However, there was no evidence generally that in-vitro passages attenuated these rotaviruses.

It is noteworthy that significant changes were observed with a number of other tissue culture strains including UK (Compton), Northern Ireland, Lincoln and 639, all of which belonged to the same serotype, and by strains 411 and 683 which belonged to serotype 3. Thus the fact that all these strains produced significant villous changes in the calf intestine and had undergone at least 12 serial passages in tissue culture, support the suggestion made by Carpio, Bellamy and Babiuk (1981), that in-vitro passages of rotaviruses do not seem to produce a rapid decline in virulence. A similar observation was made by Mebus, Stair, Underdahl and Twiehaus (1971), who after the 14th passage

level with the Nebraska Calf Diarrhoea (rotavirus), noticed stunting of the villi and replacement of the epithelial cells with cuboidal cells.

Comparison of the virulence of the four serotypes showed within the limits of the strains investigated that serotype 1 rotaviruses significantly ($p < 0.001$) reduced the villi, followed by serotype 3 ($p < 0.05$), while serotypes 2 and 4 did not produce any significant effects. Generally most rotaviruses were pathogenic. This led to difficulties in predicting virulence in the strains belonging to serotypes 1 and 3, since highly pathogenic and non-pathogenic strains were observed in both serotypes. Serotypes 2 and 4 were non-pathogenic but as, there was only one representative from each serotype, no firm conclusions could be drawn as to their virulence.

None of the strains caused crypt hypertrophy within the duration of the experiment, apart from one strain G753 (Fig. 6.2A4). These results agree with those of Snodgrass, Ferguson, Allan, Angus and Mitchell (1979), who did not observe crypt hypertrophy until 27 to 42hr. post infection and those of Carpio, Bellamy and Babiuk (1981), who failed to detect crypt hypertrophy in any tissue during the 48hr period of their experiment. However, crypts could be expected to hypertrophy later as a response to villous atrophy.

Unfortunately, IF with different viruses and serotypes did not follow any particular pattern; generally cells at the tip of the villi fluoresced with antigen (Fig 6.4), but the number was only marginally different between strains and serotypes. The fact that the duration of the experiment was approximately 24hr. might have resulted in most of the antigen-laden cells being sloughed off. It has been shown with lamb rotavirus that a 12hr. duration of infection resulted in more and quantifiable fluorescing cells than in infections that lasted between 18 and 27hr. (Snodgrass, Angus and Gray, 1977).

The non-calf viruses used (simian, lamb and piglet) showed the same variations from pathogenic to non-pathogenic as did the calf viruses. Thus even viruses from non-calf origin were as virulent as the average calf virus.

In conclusion, although the technique has some advantages, it has however some drawbacks, with many variables that were difficult to control (eg. dose of virus inocula). Whether the changes observed in-vitro relate to virulence in-vivo is not known. However it was observed that G753 and H799 (Chapter 7) produced watery diarrhoea and depression in gnotobiotic calf passage. If the in-vitro changes can be shown to relate in-vivo, then the technique might be a useful tool. So, apart from concluding that most rotaviruses strains used in this experiment appeared pathogenic in an in-vivo system, it was not possible to conclude that a definite relationship existed between serotypes and virulence.

CHAPTER 7CROSS PROTECTION STUDIES IN GNOTOBIOTIC LAMBSINTRODUCTION

Sequential infections of rotavirus in infants or young children have been reported. In most cases such infections were due to different subgroups, type I followed or preceded by type II (Fonteyne, Zissis and Lambert, 1976; Rodriguez, Kim, Brandt, Yolken, Arrobio, Kapikian, Chanock and Parrott, 1978; Yolken, Wyatt, Zissis, Brandt, Rodriguez, Kim, Parrott, Urrutia, Mata, Greenberg, Kapikian and Chanock, 1978). Furthermore, observations made when rotavirus was given orally to adult volunteers showed that there was no cross protection in subjects that were fed with subgroup II rotavirus and challenged with subgroup I virus (Kapikian, Wyatt, Greenberg, Kalica, Kim, Brandt, Rodriguez, Parrott and Chanock, 1980). Studies on active immunisation of young calves with live-attenuated tissue culture rotavirus vaccine have shown it to be effective experimentally (Woode, Bew and Dennis, 1978) and also in some limited field trials (Mebus, White, Stair, Rhodes and Twiehaus, 1972; Twiehaus, Mebus, and Bass, 1975). However the efficacy of this vaccine has been questioned in large scale blind field trials (Acres and Radostits, 1976; de Leeuw, Ellens, Talmon, Zimmer and Kommerij, 1980).

On the other hand, calves, lambs and piglets have been protected against rotavirus diarrhoea by the continuous

feeding of immune colostrum, thus ensuring that the intestinal mucosa is 'bathed' in antibody (Bridger and Brown, 1981; Lecce, King and Mock, 1976; Snodgrass and Wells, 1976). This concept has led to pregnant cows and ewes being vaccinated with rotavirus in order to increase and prolong the duration of excretion of colostrum and milk antibody (Snodgrass, Fahey, Wells, Campbell and Whitelaw, 1980; Fahey, Snodgrass, Campbell, Dawson and Burrells, 1981). It has also been shown that feeding of immune serum in place of immune colostrum protected lambs from rotavirus diarrhoea, (Snodgrass, Madeley, Wells and Angus, 1977; Snodgrass and Wells, 1978b).

The experiments described in this chapter were designed to evaluate the practical significance of the existence of serotypes of calf rotavirus to a vaccine based on passive immunisation, by comparing the passive protection abilities between these serotypes. To this end hyperimmune rabbit antisera to two serotypes of calf rotavirus were fed to lambs and the effect of challenge with homologous and heterologous serotypes observed.

MATERIALS AND METHODS

Animals

Twelve gnotobiotic lambs kept in pairs in plastic isolators were used.

Viruses

The challenge rotavirus strains used were:

1. G753, first gnotobiotic calf passage of a field calf rotavirus strain. This virus was shown to be serologically identical to serotype I (Chapter 4), and by PAGE to contain only a single rotavirus electrophoretype.
2. H799, the first gnotobiotic calf passage of calf rotavirus strain 678, serotype 2, which had previously been passaged once in a gnotobiotic lamb.

Approximately 25% w/v faecal suspensions were prepared in serum-free maintenance medium, homogenised with equal volumes of arcton, centrifuged and then filtered through a 0.45µm membrane. Filtrates were aliquoted in 5 ml amounts in vials, tested for bacterial sterility and stored at -70°C.

Five ml of filtrate was given orally to each lamb (Table 7.3). The titres of the inocula in MA104 cells are given in Table 7.1.

Preparation of Rabbit Hyperimmune Antiserum

The viruses of serotype 1 (639) and serotype 2 (678) at the 12th and 13th tissue culture passage levels respectively

TABLE 7.1

TITRES OF VIRUS INOCULUM IN MA104 CELLS

Inoculum	Virus Type	Log ₁₀ TCID ₅₀ /ml
G753	Serotype 1	4.6
H799	Serotype 2	5.3

were purified in CsCl/sucrose gradients and inoculated intramuscularly into rabbits which were previously shown to be free of antibodies to calf rotavirus. The serum pool from 6 rabbits for each virus was sterilised by filtration through a 0.45 μ m membrane, using a 90 mm Filter Holder (Millipore Corporation) and tested for neutralising antibodies. The homologous and heterologous antibody titres are given Table in 7.2.

Experimental Design

The twelve gnotobiotic lambs were allocated in pairs to 6 groups. All lambs were fed a normal diet, consisting of sterile evaporated milk diluted in water. Lambs in groups 1 and 2 were challenged on the 2nd day of life with G753 and H799 viruses respectively. Lambs in groups 3 to 6 were fed three times per day at 8-hourly intervals from the 2nd to the 4th day of life with 14ml of antiserum in the normal milk diet per feed. Midway between the 1st and 2nd feeds on the 2nd day of life, these lambs were challenged with 5ml of either G753 (groups 3 and 4) or H799 (groups 5 and 6). The experimental design is summarised in Table 7.3.

Clinical Observations

Lambs were observed daily, particular attention being paid to the consistency of the faeces and the voluntary milk intake.

TABLE 7.2

NEUTRALISING TITRES OF SERUM POOLS

Virus	Serum	
	Serotype 1	Serotype 2
636	<u>51200</u>	1600
678	3200	<u>51200</u>

Homologous titres are underlined

TABLE 7.3

DESIGN OF EXPERIMENT

Group	Lambs	Serum Type	Virus Inoculum
1	H800 and H801	-	G753 (control)
2	H802 and H803	-	H799 (control)
3	H813 and H814	Serotype 1	G753
4	H819 and H820	Serotype 2	G753
5	H817 and H818	Serotype 2	H799
6	H815 and H816	Serotype 1	H799

Faecal Sample Examination

Faecal samples were collected daily from each lamb and examined for the presence of rotavirus by ELISA and a 7.5% PAGE using the continuous buffer system. The positive samples were titrated by ELISA (Ellens and de Leeuw, 1977; Yolken, Babour, Wyatt, Kalica, Kapikian and Chanock, 1978) and the reciprocal of the highest dilution that gave a positive/negative (P/N) ratio of greater than 2 (see Chapter 4) was regarded as the titre of the sample.

Faecal samples were also collected at intervals for bacteriological examination. Nine lambs remained bacteria-free throughout the duration of the experiment, but from three lambs non-enterotoxigenic E. coli (ie K99-) were isolated.

Serum Samples

The serum-fed lambs (groups 3 to 6) were bled at 5 and 23 days of age. The sera were titrated for IF antibody against UK (Compton) calf rotavirus in MA104 cells.

RESULTS

Clinical Observations

The clinical signs following infection of gnotobiotic lambs with serotypes 1 and 2 were similar to those already described in Chapter 3, the only exception being that the

lambs infected with H799 did not show the transient anorexia observed in lamb 678 of Chapter 3.

Virus Excretion and Titration by ELISA

No virus was detected in the faeces of any lamb prior to experimental infection. Control lambs infected with G753 (Group 1), excreted rotavirus for 5 and 6 days respectively (Fig. 7.1). Lambs infected with G753 and fed homologous type 1 antiserum (Group 3) did not excrete rotavirus, while lambs fed heterologous type 2 antiserum (Group 4) excreted rotavirus at similar ELISA titres (Fig. 7.1) and for similar duration as control lambs (Group 1).

(On the other hand), control lambs infected with H799 (Group 2) excreted rotavirus for 5 and 7 days respectively (Fig. 7.1), while lambs infected with the same virus and fed heterologous type antiserum (Group 6) excreted rotavirus from 24hr. post infection and for similar duration as the control lambs (Group 2). Lambs fed serotype 2 antiserum and challenged with homologous virus (Group 5) had a significantly delayed onset of virus excretion of 5 days and thereafter excreted virus for 4 days.

Detection of rotavirus by PAGE gave identical results to detection by ELISA (Fig. 7.1).

FIGURE 7.1

Excretion of Rotavirus as Detected by:

ELISA



and

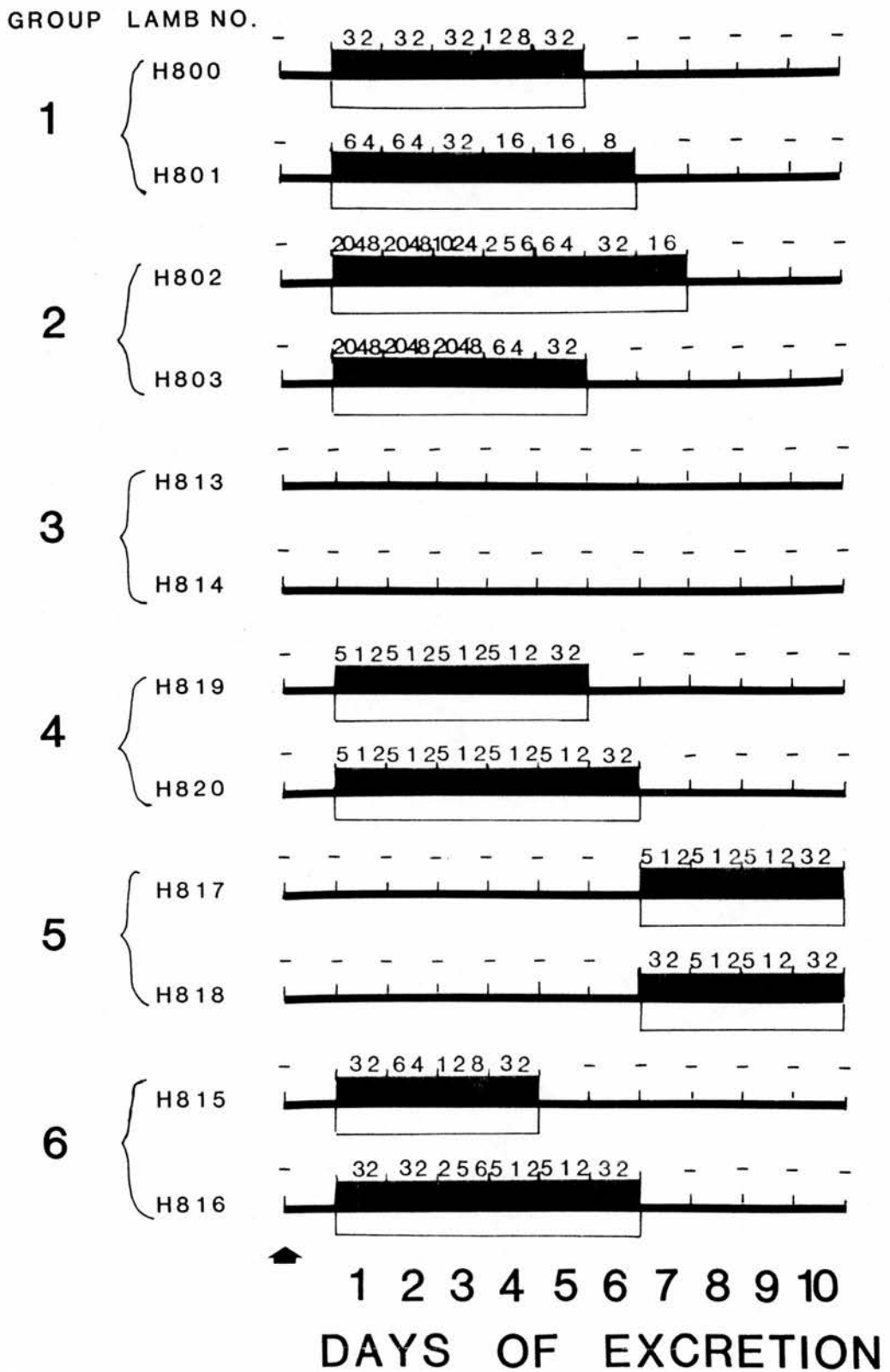
PAGE



- No virus detected

▲ Age at infection

Note: Figures inserted above the bars are the
ELISA reciprocal titres of faecal
dilutions



Serum Antibody Levels

No rotavirus antibody was detected in the serum-fed lambs on day 5 of life. However, antibody to rotavirus was present in the sera of these lambs by day 23 (Table 7.4).

DISCUSSION

This experiment was successful in showing clear distinction between the serotypes in passive immunisation. In one homologous system (Group 3) virus excretion was completely prevented throughout the duration of the experiment, while onset of excretion was substantially delayed in the other (Group 5). On the contrary, in both heterologous systems virus was excreted for similar duration and at similar ELISA titre to the controls. These observations are similar to those reported by Snodgrass and Wells (1976; 1978a and b), who investigated the levels of passive protection conferred on lambs when fed colostrum and serum containing antibodies to lamb rotavirus.

These clear cut results were fortunate to some extent, as it was difficult to estimate before hand the dose of virus and serum required to elicit adequate response. In the event, the report of Snodgrass and Wells (1976), gave guidance as to the amount of serum to feed. By feeding as little as 20 ml of serum containing antibody to rotavirus for three days, Snodgrass and Wells (1976) obtained complete protection in one lamb and delayed response in another. In this experiment 42ml of serum was fed per day for three days to obtain similar results.

TABLE 7.4

ANTIBODY TITRES IN LAMB SERA

Lamb Number	5 Days	23 Days
H813	<10	40
H814	<10	40
H815	<10	80
H816	<10	80
H817	<10	10
H818	<10	10
H819	<10	40
H820	<10	40

Titration of faecal virus was employed to quantitate the amount of virus excretion, lest this was the only way to differentiate the serotypes. In the event this was found to be unnecessary as serotypes were clearly distinguished by virus excretion patterns.

The fact that lambs in group 3 failed to excrete virus in their faeces, unlike lambs in group 5 which shed the virus only after a delayed onset, seemed to suggest that the hyperimmune antiserum had completely neutralised the virus inoculum G753 and prevented infection in group 3 lambs whereas the virus inoculum H799 given to lambs in group 5 was only partially neutralised and was capable of initiating a delayed infection with subsequent shedding of the virus. However, this was not the case because all 4 lambs in both groups developed specific rotavirus antibody thereby confirming that replication of the virus inoculum had occurred.

Judged on the fact that lambs were not protected against heterologous challenge, this experiment highlights a potential problem in passive immunisation. However experiments by Bridger and Brown (1981) and Lecce, King and Mock (1976) have shown that passive immunity has a broad spectrum of activity between species, by the protection of piglets against porcine rotavirus infection using bovine colostrum. The explanation of this phenomenon might lie in the fact that cows are constantly becoming infected by different rotaviruses (Moerman, de Leeuw, van Zijderveld,

Baanvinger and Tiessink, 1982) and, as a result, mount antibody responses to all the infecting serotypes. These antibodies which circulate in the cows are secreted in colostrum and milk. In fact, Bridger and Brown (1981) showed that there were two different strains of rotavirus co-circulating in the cow herd from which their colostrum pool was obtained. One of these strains was shown to have a strong cross relationship with the porcine rotavirus used in their study. Also Snodgrass, Ojeh and Campbell (to be published) have shown that vaccination of pregnant cows given monovalent vaccine induced a good heterologous response when the sera from such cows were assayed in serum neutralisation tests against the different serotypes of bovine rotavirus. Thus, so far as passive immunisation is concerned, this observation suggests that although serotypes are not cross-protective, they may not be of such fundamental importance under practical field conditions.

The question of active immunity with these serotypes remains to be answered. Sequential infection experiments were designed in piglets and then in lambs, but had to be abandoned because of the problem of age resistance to the serotypes in these species. Age resistance to rotavirus infection has also been observed in calves and mice (Tzipori, Makin, Smith and Krautil, 1981; Sheridan, Eydeloth, Vonderfecht and Aurelian, 1983).

In summary, the results of the experiments described above lend support to the conclusion reached in earlier chapters that the two serotypes (639 and 678) of calf rotaviruses exhibited antigenic differences both in in-vitro studies as well as in experimentally infected lambs. These differences are important in understanding the epidemiology of rotavirus infections in animals and merit further study.

CHAPTER 8GENERAL DISCUSSION

Rotaviruses have been shown to be the single most important infectious agent of acute gastroenteritis requiring hospitalisation of infants and young children in both developed and developing countries. It is estimated that in developing countries alone, 5-10 million children under five years of age die every year as a result of diarrhoea (Kapikian, Wyatt, Greenberg, Kalica, Brandt, Rodriguez, Parrott and Chancok, 1980), with rotavirus being the most important pathogen (Black, Merson, Rahman, Yunus, Alim, Huq, Yolken and Curlin, 1980). Similarly, in acute diarrhoea of calves and piglets, rotaviruses have been shown to be of major importance (Lecce, King and Mock, 1976; Snodgrass, Sherwood, Terzolo and Synge, 1982). The main objective of the work presented in this thesis was to investigate the serological variation among bovine rotavirus, as the infection in calves continues to cause serious concern to farmers.

Studies on the 12 strains of bovine rotavirus originally selected for investigation were initially hampered because of the inability of the virus to grow in-vitro. For this reason, gnotobiotic lambs were used to propagate the virus. This simple procedure proved to be useful in the end, in that it provided specific convalescent antisera to each of the strains, yielded large quantities of

virus and proved to be sensitive in detecting the mixed infections in 4 of the 12 samples. At the time these lamb experiments were being performed, major progress was made with techniques applicable to routine cultivation of rotavirus which, when applied to bovine rotavirus, were successful in 12 out of 17 cases. The 5 samples that were uncultivable, were found to have very low titres of virus in the original faecal samples.

These techniques, which involved rolling of infected cultures, treatment of inocula with trypsin and the use of MA104 cells afforded a major breakthrough in this investigation on rotaviruses, by enabling adaptation of many rotavirus strains to cell culture. In this study (Chapter 3), primary cell culture of EBK, EOK and established cell lines (MDBK, LLC-MK2 and Vero) could not be used to isolate bovine rotavirus. In a recent report, similar findings were obtained when 9 trypsin-treated isolates of calf rotavirus, which were cytopathic for MA104 cells failed to replicate in primary bovine embryonic kidney cells (Castrucci, Ferrari, Frigeri, Cilli, Donelli, Angelillo and Bruggi, 1983). It, seems likely, therefore, that trypsin and MA104 cells are two essentials for the successful routine isolation and propagation of rotaviruses (Birch, Rodger, Marshall and Gust, 1983; Urasawa, Urasawa and Taniguchi, 1981).

When this study first commenced, no published information was available on the existence of serotypes of bovine rotavirus, although 2 strains in Canada had been

differentiated by haemagglutination-inhibition tests (Spence, Fauvel, Petro and Babiuk, 1977). Further differentiation of bovine rotavirus by this technique was not possible because not all strains possessed haemagglutinins (Spence, Fauvel, Petro and Babiuk, 1977; Castrucci, Ferrari, Frigeri, Cilli, Donelli, Angelillo and Bruggi, 1983). As research findings on human rotavirus continued to emerge, a new definition of rotavirus serotypes became inevitable. It was found that 2 or 3 human rotavirus 'serotypes' identified using CF and ELISA (Yolken Wyatt, Zissis, Brandt, Rodriguez, Kim, Parrott, Urrutia, Mata, Greenberg, Kapikian, and Chanock, 1978; Zissis and Lambert, 1978; 1980; Zissis, Lambert, Kapsenberg, Enders and Mutanda, 1981), reacted with the inner structural 42K protein of rotaviruses and were different from the serotypes defined by the conventional serum neutralisation tests which reacted with the 7th, 8th or 9th gene product of the outer shell polypeptides. On the basis of these differences the term 'subgroup' has been adopted to define differences between rotaviruses detected by such serological tests as CF, ELISA, IEM, ID and IAHA, whereas the term 'serotype' is used to distinguish between rotaviruses differentiated by the serum neutralisation tests (Kapikian, Cline, Greenberg, Wyatt, Kalica, Banks, James, Flores and Chanock, 1981). To date, 4 serotypes of human rotavirus have been defined (Wyatt, James, Pittman, Hoshino, Greenberg, Kalica, Flores and Kapikian, 1983), either by the conventional neutralisation assay (Sato, Inaba, Miura, Tokuhisa and Matumoto, 1982), fluorescent focus reduction tests (Beards, Pilford, Thouless

and Flewett, 1980) or plaque reduction neutralisation assay (Wyatt, Greenberg, James, Pittman, Kalica, Flores, Chanock and Kapikian, 1982; Wyatt, James, Pittman, Hoshino, Greenberg, Kalica, Flores and Kapikian, 1983; Urasawa, Urasawa and Taniguchi, 1982). Comparison of these three methods yielded similar results.

In this thesis, the fluorescent focus reduction assay was used; serotypes were defined by a 20-fold dilution difference between homologous and heterologous viruses, using hyperimmune antisera and the antigenic relatedness (R) between homologous and heterologous strains. Although R values of <5% between two strains was used to denote distinction, a value of 10-32% as used for differentiating between types and subtypes of foot-and-mouth disease virus (Brooksby, 1968 cited by Forman, 1975) and porcine enteroviruses (Forman, Pass and Connaughton, 1982) would unequivocally separate the strains of bovine rotavirus studied into 3 distinct serotypes, with serotype 1 and 3 showing a >25% relatedness within serotypes whereas between serotypes 1, 2 and 3, there is a <5% relationship.

From these results, therefore, it does seem that these serotypes fall into three biologically different groupings that are either related (>25%) or not (<5%). The exception, virus 1548 has R values (9-18%) which are intermediate between 5 and 25%. This virus may belong to serotype 3 but it is definitely not as closely related. For this reason it is referred to in the text as potentially serotype 4.

The distinction of serotypes using hyperimmune antisera raised against faecal virus (Table 4.3a) proved to be a better reagent than either the convalescent antisera (Table 4.2a) or the hyperimmune antisera raised to tissue culture virus (Table 4.4a). This could be due to the fact that faecal viruses contained more double shelled particles than the tissue culture adapted rotaviruses. The plaque reduction neutralisation assay would have been a feasible method for differentiating the serotypes as has already been shown for human rotavirus (Wyatt et al. 1982), but the plaques produced by the strains under investigation were not always as discrete as those described for the Lincoln strain of neonatal calf diarrhoea virus (Matsuno, Inouye and Kono, 1977) and SA-11 (Smith, Estes, Graham and Greba, 1979; Ramia and Sattar, 1979). Welch and Twiehaus (1973) found small and inconsistently produced plaques with strains of bovine rotavirus; and as noted above, these techniques (plaque reduction neutralisation, fluorescent reduction test and neutralisation test by cpe) are all essentially the same. Although subsequently, most bovine rotavirus isolates produced good cpe, the fluorescent focus reduction assay was used throughout. The use of this technique for typing field strains of bovine rotavirus was validated by showing that all the untypable strains when isolated in cell culture were indeed of distinct serotypes.

In addition to the serotypic variations in the human rotavirus a few rotaviruses from animals have been shown to share serotype specificity with mainly serotype 3 of human

rotavirus. These animal species include simian and porcine (Wyatt, Greenberg, James, Pittman, Kalica, Flores, Chanock and Kapikian, 1982), canine (Hoshino, Wyatt, Greenberg, Kalica, Flores and Kapikian, 1983a) and foal H-2 rotaviruses (Hoshino, Wyatt, Greenberg, Kalica, Flores and Kapikian, 1983b). However, there is another foal (H-1) virus that has been shown to be similar to three porcine strains but separate from any of the four human rotavirus serotypes (Hoshino, Wyatt, Greenberg, Kalica, Flores and Kapikian, 1983c).

Recently, 2 distinct serotypes of calf rotavirus with the possibility of a third were defined by the cytopathic serum neutralisation assay in Japan (Murakami, Nishioka, Hashiguchi and Kaniyasu, 1983) and by fluorescent focus reduction assay in USA (Woode, Kelso, Simpson, Gaul, Evans and Babiuk, 1983). In this present study, three bovine rotavirus serotypes were clearly distinguished. Although no comparison has been made between the calf rotavirus serotypes defined in this study and those from the USA and Japan, in each study the most commonly isolated rotavirus was serotypically related to the Lincoln strain of NCDV; so the characteristics of the bovine rotavirus serotype 1 would appear to be agreed.

A limited comparison of the bovine serotypes in this study has been made with the 4 serotypes of human rotavirus, and preliminary results show that no serotypic relationship exist between the serotypes 1 and 2 of bovine rotavirus and

any of the human serotypes (Snodgrass and Campbell, data to be published). These recent findings raise the question "What is a bovine, porcine, foal, simian or human rotavirus serotype?" It is possible that there is a limited number of serotypes from many species with certain serotypes predominating in a particular host species. However, nothing is known of the frequency of their occurrence; but it is possible to use a less pathogenic serotype from one species as a vaccine candidate to protect other species; as in the case of humans being protected against small pox infection with cow pox vaccine. This already is being tried out with simian rotavirus (Hoshino, Wyatt, Greenberg, Kalica, Flores Kapikian, 1983a), and calf rotavirus (Vesikari, Isolauri, Delem, D'Hondt, Andre and Zissis, 1983).

It has been reported that probably the only way of distinguishing rotaviruses from different species and indeed within species was by cross-neutralisation (Thouless, Bryden, Flewett, Woode, Bridger, Snodgrass and Herring, 1977; Sato, Inaba, Miura, Tokuhisa and Matumoto, 1982). But with the recent report of rotavirus crossing of species boundaries (Hoshino, Wyatt, Greenberg, Kalica, Flores and Kapikian, 1983a; 1983b; Wyatt, Greenberg, James, Pittman, Flores and Chanock. 1982), it becomes clear that some other means of differentiating rotavirus from different species must be considered.

The use of special ELISA techniques with highly specific reagents for the typing of human rotavirus has been reported (Thouless, Beards and Flewett, 1982) but, unfortunately, the adaptation of these methods to the present study did not give the desired results. This could be due to the different methods by which the reagents were prepared. However, the purpose of using this ELISA technique for serotyping human rotavirus was to show that it was possible, but not recommended, as a routine technique because of the laborious and time consuming method of absorbing the sera. For serotyping rotaviruses, neutralisation of faecal virus using the fluorescent focus reduction assay is a simpler method by comparison (Beards, personal communication). Nevertheless a better approach would have been the use of monoclonal antibodies in the derivation of the reagents for the typing ELISA. Monoclonal antibodies are more highly specific than the reagents derived from hyperimmune antisera and can be used for the comparison and classification of various virus strains. Thus Greenberg, McAuliffe, Valdesuso, Wyatt, Flores, Kalica, Hoshino and Singh (1983) found that two monoclonal antibodies directed against the 42K inner structural protein of rotaviruses were more efficient in detecting subgroup antigens than either convalescent or hyperimmune antisera. Presumably, the cross reactivity detected in hyperimmune antisera was due to the fact that the 42K protein has at least two antigenic sites, one of which is characterised by an antigenic region that is shared by all rotaviruses and the other site can detect subgroup specificities. If such

monoclonal antibodies are directed against the rotavirus protein (VP7) that is responsible for neutralising antibodies and infectivity, it will not only make it possible to determine the number of serologically different strains and their relative importance to calf rotavirus diarrhoea but also will help the rapid identification of new serotypes.

The PAGE with the silver staining technique has been a very useful tool for the rapid and unequivocal diagnosis of rotavirus. Although comparable in sensitivity to EM and ELISA (Herring, Inglis, Ojeh, Snodgrass and Menzies, 1982), it has the additional advantage over EM in the number of samples that can be handled without the high cost and sophistication associated with the EM. However its usefulness seemed limited in that it has not been helpful so far in the prediction of serotypes in calf or human rotaviruses (Thouless, Beards and Flewett, 1982; Gaul, Simpson, Woode and Fulton, 1982). Sabara, Deregt, Babiuk and Misra (1982) have shown that there is extensive variation among isolates of bovine rotavirus as revealed by PAGE analysis. Unfortunately, these variations have not been correlated with serotypic differences. In the comparison of the different electrophoretotypes (Chapter 5), no striking difference could be attached to the serotypes, except that strain 678 (serotype 2) had consistently shown segments 7,8 & 9 closely migrating, a feature not observed in any other bovine rotavirus in this study. The

significance of this is not known in the absence of other serotype 2 viruses. However, recent studies have shown that of the 4 serotypes of human rotavirus, serotypes 1 and 2 have had their gene-coding assignments thoroughly investigated but not serotype 3 because all the examples of this serotype studied so far have had the segments 7, 8 & 9 migrating together, thus making their analysis impossible (Greenberg, Wyatt, Kapikian, Kalica, Flores and Jones, 1982; Wyatt, Greenberg, James, Pittman, Kalica, Flores, Chanock and Kapikian, 1982; Greenberg, Flores, Kalica, Wyatt and Jones, 1983). This may be coincidental or basic to the characteristics of the serotypes. If the latter is the case then some prediction of serotype by PAGE will be possible. It will be interesting to isolate more of this bovine serotype 2 and compare their RNA segments 7, 8 & 9 complex.

The ligated gut loop technique did distinguish strains of calf rotavirus in apparent virulence but, in the absence of whole calf pathogenicity studies, it was difficult to relate these findings to pathogenicity. Certainly there was no clear relationship between ability to shorten villi and serotype. It might have been a more useful proposition first to check the in-vivo pathogenicity of the viruses and then repeat the study with more animals. Nevertheless the results obtained further confirmed and extended the findings of Carpio, Bellamy and Babiuk (1981), who reported differences in virulence among some Canadian bovine isolates. A situation in which subgroup II human rotaviruses have been associated with greater virulence

(severe diarrhoea) than those of subgroup I has been reported, which may explain also why the subgroup II virus was more frequently encountered and more readily isolated in cases of infantile gastroenteritis than the not-so-virulent subgroup I virus (Yolken, Wyatt, Zissis, Brandt, Rodriguez, Kim, Parrott, Urrutia, Mata, Greenberg, Kapikian and Chanock, 1978). Similar findings have also been reported for polioviruses in which serotype 1 viruses have greater neuropathogenicity than serotypes 2 and 3 (Melnick, 1976).

Control of viral diseases can be achieved by either active immunisation of susceptible hosts or, passively, by feeding them immune sera; or simply by vaccination of the dam in order to produce maternal immunity transmissible via the placenta or colostrum and milk. In the majority of cases, control by the former method is more practicable and vaccines are in the process of being developed for humans against rotavirus (Kapikian, Wyatt, Greenberg, Kalica and Chanock, 1980; Wyatt, Kapikian, Greenberg, Kalica and Chanock, 1981; Vesikari, Isolauri, Delem, D'Hondt, Andre and Zissis, 1983).

Experimental protection of calves from rotavirus neonatal diarrhoea by active immunisation has been reported (Woode, Bew and Dennis, 1978; Gaul, Simpson, Woode and Fulton, 1982; Woode, Kelso, Simpson, Gaul, Evans and Babiuk, 1983); but as passive immunisation via colostrum and milk is the most commonly practised method of immuno-prophylaxis in

calves (Navetat, 1982; Saif, Redman, Smith and Theil, 1983; Snodgrass, Fahey, Wells, Campbell and Whitelaw, 1980; Snodgrass, Nagy, Sherwood and Campbell, 1982; van Opdenbosch and Wellemans, 1982), the practical significance of rotavirus serotypes in calves is best studied in passive rather than active immunisation experiments.

One major contribution to the study of passive immunisation in bovine rotavirus in this thesis is the evidence that serotypes 1 and 2 of bovine rotavirus do not cross protect in passive immunisation studies. The results obtained from this study would suggest, just like active immunisation experiments, the need for a multivalent vaccine. However, as passive immunity has been shown to be broad and transferable between species (Bridger and Brown, 1981; Lecce, King and Mock, 1976), the same broad passive protection may occur among serotypes of bovine rotavirus under practical field conditions. On the other hand it could be argued that since the serotypic relationship of the porcine and bovine rotaviruses described by Bridger and Brown (1981) and Lecce, King and Mock (1976), was not established, especially in the light of recent findings of crosses between species serotype boundaries (Wyatt, Greenberg, James, Pittman, Kalica, Flores, Chanock and Kapikian, 1982; Hoshino, Wyatt, Greenberg, Kalica, Flores and Kapikian, 1983a; 1983b; 1983c), they might have belonged to the same serotype, hence their effective neutralisation of each other. Moreover the finding of Bridger and Brown (1981) that a strain of bovine rotavirus had a strong cross

relationship with a porcine rotavirus lends support to this argument. Nevertheless the fact that broad spectrum passive protection is possible has been shown when pregnant cows vaccinated with a monovalent vaccine produced a broad spectrum response when the sera were assayed against different serotypes of bovine and human rotaviruses (Snodgrass and Campbell, data to be published). All these findings notwithstanding, the possibility of serotypic variation needs to be borne in mind when investigating vaccination breakdowns in the field.

In conclusion, this thesis has shown that extensive variation exists among bovine rotaviruses serologically, in adaptability to cell culture, in virulence in calf gut loops and in genome dsRNA and polypeptide mobility patterns. On the other hand, all the strains studied were similar in their morphology and in sharing a common group antigen (with the exception of the group B and C rotaviruses mentioned in Chapter 6). All the strains multiplied in lambs and produced slight or inapparent infection, and infected the epithelial cells in the calf gut loops.

Are the variations observed dependent on each other? There is no evidence to suggest that they are. Certainly the serotypic variation was not linked to any other variable but, as the serological investigations depended on the adaptation to cell culture, any serotype unable to grow in-vitro would be missed.

The study of serotypic variations in bovine and other rotaviruses has generated a great deal of interest and will continue to be of great potential importance as shown by the passive immunisation experiments.

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PUBLICATIONS ARISING FROM THIS THESIS

HERRING, A.J., INGLIS, N.F., OJEH, C.K., SNODGRASS, D.R. and MENZIES, J.M. (1982).

Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. *Journal of Clinical Microbiology*. 16: 473-477.

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Evidence of serotypic variation among bovine rotaviruses. *Archives of Virology*. in press.

Rapid Diagnosis of Rotavirus Infection by Direct Detection of Viral Nucleic Acid in Silver-Stained Polyacrylamide Gels

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A rapid simple technique for the diagnosis of rotavirus has been developed based on the sensitive detection of rotavirus double-stranded RNA genome segments separated in polyacrylamide gels. The method utilizes a recently described ultrasensitive silver stain for polypeptides, which can also detect subnanogram amounts of nucleic acid. The sensitivity of the technique is comparable with that of electron microscopy or enzyme-linked immunosorbent assay.

Rotaviruses cause enteritis in a wide variety of species and have been intensively investigated for more than a decade (reviewed 5, 14). As members of the *Reoviridae* (13), they have a double-stranded RNA (dsRNA) genome consisting of 11 segments ranging in molecular weight from approximately 2.0×10^6 to 0.2×10^6 (9). Electrophoretic analysis has revealed major differences in the mobility of the genome segments between virus isolates from different host species and minor differences between individual isolates from the same species. Thus, genome electropherotyping has been the most common method for both taxonomic and epidemiological studies (9, 12, 18).

Recently, several methods have been reported which utilize silver staining for the ultrasensitive detection of polypeptides resolved by polyacrylamide gel electrophoresis (PAGE) (16, 17, 20). We used one of these methods (20) to monitor the purification of rotavirus from feces and found that the dsRNA bands were also stained with high sensitivity. This result was consistent with the well-described property of silver ions to form a stable complex with nucleic acids (7). Similar nucleic acid staining with another silver staining method has recently been reported by Somerville and Wang (22).

In this communication we describe a diagnostic test for rotavirus in feces based on this ultrasensitive detection of viral dsRNA, which has the advantages of simplicity, economy, and speed, and which simultaneously identifies the electropherotype.

MATERIALS AND METHODS

Fecal specimens. Infected and control fecal specimens were obtained from cattle and human sources. The bovine samples and two of the human samples

were examined for rotavirus by electron microscopy (EM) and by enzyme-linked immunosorbent assay (ELISA). The ELISA was performed essentially by the method of Yolken et al. (25), using a hyperimmune rabbit serum raised against tissue culture-grown bovine rotavirus, with a neutralization titer of 1:10,240 both to coat the wells and as a conjugate to detect antigen. Unconcentrated samples were examined by EM as described by Snodgrass et al. (21). The samples in the dilution experiment were coded and scored blind. The majority of the human specimens were kindly provided by the Edinburgh Regional Virus Laboratory. Rotavirus diagnosis had been carried out on these specimens by the cell culture method of Bryden et al. (2).

Nucleic acid extraction. Fecal samples were diluted 1:4 by weight with 0.1 M sodium acetate buffer (pH 5.0) containing 1% (wt/vol) sodium dodecyl sulfate; the normal sample size used was 0.25 g of feces, which provided enough extract for at least 10 separate analyses. An equal volume of a 3:2 (vol/vol) 'phenol'-chloroform mixture was added to the fecal suspension, and the sample was mixed for 1 min. ('Phenol' consisted of a mixture of 500 g of phenol, 70 g of *m*-cresol, and 200 g of water containing 0.5 g of 8-hydroxyquinoline.) The emulsified mixture was then centrifuged for 10 min at $1,200 \times g$, and the resulting clear upper aqueous layer was removed. A sample was then prepared for electrophoresis by the addition of 10 μ l of 25% (wt/vol) sucrose containing 0.2% bromophenol blue to 40 μ l of the aqueous layer.

Occasional samples failed to yield sufficient clear aqueous layer, but it was found that either further centrifugation for 3 min at $16,000 \times g$ in a microcentrifuge (Mechanika Precyzyjna, type 320a) or the addition of 0.5 ml of buffer followed by remixing and centrifugation at $1200 \times g$ gave an ample clear layer.

PAGE. The 50- μ l samples were loaded onto 5% polyacrylamide slab gels (acrylamide-to-bis-acrylamide ratio of 37.5:1) which were polymerized with 0.01% (vol/vol) *N,N,N',N'*-tetramethylethylenediamine and 0.05% (wt/vol) ammonium persulphate. The gel and electrode buffer was 0.036 M Tris-0.03 M

sodium dihydrogen phosphate-0.001 M EDTA (pH 7.8). Gel dimensions were 14-cm wide by 19-cm long and 0.15-cm thick. It should be noted that the gel thickness is critical with the silver staining technique (20). Deep sample wells (0.6 by 2.0 cm) facilitated loading without the transfer of sample to neighboring wells. Electrophoresis was performed at room temperature for 16 h at 20 mA and 70 V. In most experiments, one of the glass plates used to form the gel mold was treated with a 1% solution of Silane 174A in ethanol (Union Carbide Corp.) for 10 min, dried in air, rinsed in distilled water, and redried. This treatment caused the gel to adhere strongly to the plate and greatly simplified its handling during staining.

Silver staining. The gels were stained by using a slight modification of the method of Sammons et al. (20). The initial fixation steps described for protein staining were omitted, and the gels were washed with 10% ethanol-0.5% acetic acid for 30 min and then soaked in 0.011 M silver nitrate for 2 h. The gel was then rinsed briefly in distilled water, and the reduction step was performed with a solution of 0.75 M sodium hydroxide containing 0.1 M formaldehyde and 0.0023 M sodium borohydride. The bands appeared at this stage, and the reduction was continued until the bands were clearly visible for a maximum of 10 min. In our early experiments the gels were then placed in 0.07 M sodium carbonate, and the intensity of staining of both the bands and the background increased slightly in the 20 min or so after transfer. After 30 min, the gels were placed in fresh carbonate solution. However, it was found that, when using gels which were stuck to a glass plate with Silane 174A, an unacceptable degree of background staining sometimes developed when the gel was placed in the carbonate solution. This could be prevented by treating the gel with a 5% acetic acid solution for 30 min after the reduction and then transferring the gel to carbonate solution for storage. Gels have been successfully stored, sealed in polythene bags, for up to 6 months.

All of the solutions for the staining were made from single distilled water and, with the exception of the initial fixation solution, were degassed before use. The solutions were used in 200-ml volumes in a single plastic staining dish, and care was taken to avoid touching the gel surface with ungloved hands. Constant agitation of the solutions throughout the procedure was achieved with a rocking bed destainer. The gels were photographed by transmitted light, using a Wratten 85B filter.

Purification of virion dsRNA. Virus was purified from infected bovine feces essentially by the method described by Todd and McNulty (24), and the dsRNA was extracted with phenol and further purified by one cycle of CF11 cellulose chromatography (6) performed as described by Bevan et al. (1). The resulting dsRNA was quantified spectrophotometrically.

RESULTS

The results obtained by direct extraction of feces with phenol followed by gel analysis of the extract are shown in Fig. 1. The first nine samples were all from a herd affected with enteritis, and eight may be clearly seen to contain the characteristic dsRNA segments of bo-

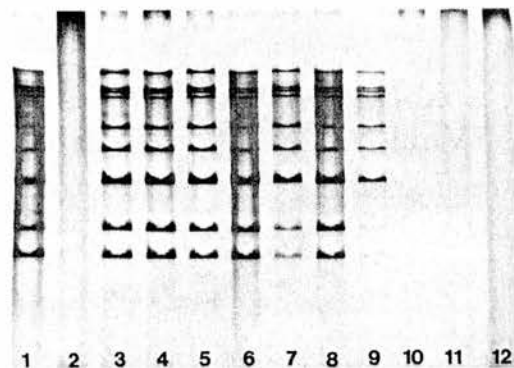


FIG. 1. Gel electrophoresis of fecal nucleic acid extracts. Tracks 1 through 9, extracts of fecal samples from diarrheic calves; tracks 10 through 12, extracts of feces from uninfected calves.

vine rotavirus; the other three control samples were negative. As would be expected, the samples from a single outbreak all showed the same electropherotype. Other bands were seen on the gels, especially near the origin where DNA forms a diffuse band, and occasional samples produced a continuous smear of stained material; but neither of these effects interfered with the detection of the dsRNA. The rotaviral genome segments could be identified by the characteristic sharpness and unique pattern of the dsRNA bands.

The results of a comparison between PAGE, ELISA, and EM are summarized in Table 1, together with the results of PAGE analysis on 24 human specimens which had been tested for rotavirus by cell culture. There was complete concordance between PAGE and ELISA results and only a single conflicting result in the PAGE and EM results. The level of virus in this one sample was clearly low, as the dsRNA bands were faint. The results with the human samples similarly showed just one conflicting result, which was positive by PAGE; this sample was obtained from a patient who also yielded other samples which were positive by cell culture.

The sensitivity of PAGE was investigated by dilution experiments. A positive sample judged to contain an average level of viral dsRNA was serially diluted with a negative sample to give a range of viral concentrations from 12.5 to 0.2% of that in the original sample, but with approximately the normal amount of contaminating non-viral material in each dilution. Extracts of these samples were analyzed by PAGE, and the results are shown in Fig. 2. Rotavirus dsRNA segments 1 through 4 were detected in dilutions down to 0.39%, but the lower-molecular-weight bands were not apparent at the higher dilutions. Figure 2 also shows the result of diluting the positive sample extract with electrophoresis

TABLE 1. Comparison of PAGE with other methods of rotavirus diagnosis

Specimen	No. of samples	Diagnostic method ^a				No. of samples in category
		PAGE	ELISA	EM	Cell culture	
Bovine	68	+	+	+	ND	41/68
		-	-	-	ND	26/68
Bovine	13	+	+	-	ND	1/68
		-	-	ND	ND	5/13
Human	2	+	+	+	ND	8/13
		-	-	-	ND	1/2
Human	24	+	ND	ND	+	18/24
		-	ND	ND	-	5/24
		+	ND	ND	-	1/24

^a +, Positive; -, negative; ND, test not performed.

buffer. All of the dsRNA segments were detected at a level of 0.39% of the original positive material.

A similar dilution series, using the same positive and negative feces and the appropriate buffers, was constructed and tested by ELISA and by EM (Table 2). EM was found to detect virus to a level of 1.56% of the original sample. The ELISA results are expressed as ratios of the positive and negative optical densities (P/N) as suggested by Yolken et al (25), who considered any value in excess of 2.1 to be rotavirus positive. Table 2 shows two values of P/N for each dilution. The first, and higher value, was based on the optical density given by the particular negative feces used for dilution, and the second is based on the optical density given by our standard uninfected feces. These data show the dilution series positive to the 0.39% and 1.56% levels, respectively. Thus, with the methods used, the sensitivities of PAGE, EM, and ELISA were approximately equal.

One further dilution series was investigated to determine the sensitivity of the silver staining method in absolute terms. Examination of gels loaded with a dilution series of purified dsRNA showed that the detection limit of the silver stain for a single band was 300 to 400 pg.

DISCUSSION

The diagnosis of rotavirus infection has been achieved by a variety of methods (reviewed in 10) based on either the direct visualization of the virion by EM or the detection of viral antigens by a wide diversity of immunological techniques, including the highly advanced and sensitive enzyme-linked fluorescence assay (26). The test described above is based on the direct extraction and detection of viral dsRNA. Two direct extraction methods have been reported previously, but both have been designed for epidemiological studies and genome analysis rather than for diagnosis. Clarke and McCrae (3)

described a method based on end labeling of total fecal nucleic acid followed by CF11 cellulose purification of the dsRNA and analysis by PAGE and autoradiography. This procedure could detect rotavirus dsRNA with very high sensitivity, but is too protracted and expensive to use as a routine diagnostic test. Theil et al. (23) recently reported a method for the bulk extraction of dsRNA from large fecal samples (6 ml), using CF11 cellulose as a batch procedure. Their results confirmed that directly extracted dsRNA was identical to virion dsRNA. It is an indication of the increased sensitivity of the silver staining method relative to ethidium bromide fluorescence that Thiel et al. (23) used the dsRNA from 0.4 ml of feces for a single analysis, whereas we routinely use the nucleic acid from only 0.01 ml of feces.

The greatest advantages of the PAGE and silver stain method are its lack of ambiguity and

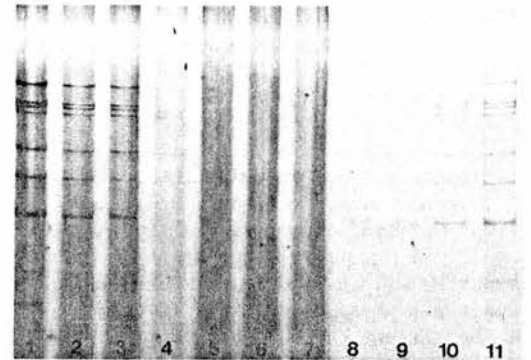


FIG. 2. Gel electrophoresis of fecal nucleic acid extracts. Tracks 1 through 7, extracts of a dilution series of bovine feces containing 12.5, 3.13, 1.56, 0.78, 0.39, 0.2, and 0% of positive sample, respectively; tracks 8 through 11, four dilutions in buffer of the nucleic acid extract of the positive feces containing 0.39, 0.78, 1.56, and 3.13% of the positive extract, respectively.

TABLE 2. EM and ELISA results on the dilution series of positive feces^a

Positive feces in dilution (%)	Test		
	EM	ELISA	
		P/N [1]	P/N [2]
12.5	+ [1]	9.12 (+)	4.8 (+)
6.25	+ [16]	5.55 (+)	2.92 (+)
3.13	+ [1]	4.79 (+)	2.52 (+)
1.56	+ [2]	4.25 (+)	2.23 (+)
0.78	-	3.53 (+)	1.85 (-)
0.39	-	2.28 (+)	1.20 (-)
0.20	-	1.86 (-)	0.98 (-)
0	-	1.0 (-)	0.53 (-)

^a The figures in brackets indicate the number of viral particles found in a standard 10-min search. Positive-to-negative (P/N) ratios are explained in the text. The test results (+, -) are shown in parentheses.

the fact that it provides information about viral electropherotype. Since the test detects the viral genome which has a unique number and pattern of dsRNA segments the results are unequivocal. None of the samples we have examined to date has given any spurious bands which could be confused with viral dsRNA. The only problem encountered in our early trials of the technique was the accidental transfer of sample to a neighboring well in the gel at the time of loading; certainly care is required at this stage, and accurate loading is facilitated by the use of deep sample wells. If confirmation of a weak positive result is required, the sample may be concentrated very simply by ethanol precipitation.

The unambiguous nature of a positive PAGE test contrasts with the difficulties in the interpretation of low-positive values in the ELISA. False-positive results have been reported to occur with ELISA (27), and it has been necessary to incorporate pretreatment of the samples with mild reducing agents (27) or additional controls with blocking antiserum (25), as is our practice.

The fact that the PAGE and silver stain method simultaneously produces an electropherotype is a feature which considerably enhances its value. In recent studies with a human virus the two distinct patterns which are seen for segments 10 and 11 appear to correlate with two major neutralization subgroups of the virus (4, 8). In addition, other major surveys of human viral genome electropherotypes have revealed considerable minor variations (12, 18), and one study has suggested that isolates from neonates may be distinct (18). Lourenco et al. (12) noted the limitation that their clinical samples were too small to allow multiple electrophoretic analyses. Silver staining should permit far more economical use of the dsRNA and has the added advan-

tage that the low loadings required enhance the resolution obtained. The adoption of the gel method for diagnosis should lead to a rapid increase in our understanding of rotavirus epidemiology. The method avoids the problems posed by the recent discoveries of rotaviruses without the group antigen (15, 19) and may also reveal whether the virus can cross species barriers as was recently suggested by McNulty et al. (15).

Most of the samples we have studied to date would be suitable for electropherotype analysis without further purification, but those which give high backgrounds could be conveniently purified by CF11 chromatography (1, 6). The gel system we describe above was selected to allow rapid staining of the gel after electrophoresis and not for maximum resolution of the dsRNA. Discontinuous buffer system gels (11) give the best resolution (18), but such gels require the full fixation and washing procedure described by Sammons et al. (20) to remove sodium dodecyl sulfate. Gels containing agarose cannot be stained by this method.

The method has several other minor advantages. The initial phenol extraction is both virucidal and bacteriocidal and thus eliminates the biohazard and much of the unpleasantness associated with fecal samples. The apparatus and chemicals employed are relatively inexpensive, and there is no dependence on immunological reagents which are variable and expensive to purchase or prepare.

The use of silver staining to detect nucleic acids in such low amounts should have considerable application in the study and diagnosis of other viruses, but it is particularly applicable to dsRNA because of the very sharp bands formed by this species in PAGE and because of the ease with which it may be purified from complex mixtures by CF11 cellulose chromatography (6). We have already found the method most useful for the detection of DNA in velocity sedimentation experiments and for the analysis of small restriction enzyme fragments.

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Evidence for serotypic variation among bovine rotaviruses

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Summary

Eight field strains of calf rotavirus from the U.K. were compared by neutralisation tests, using convalescent and hyperimmune antisera. Seven of these strains cross-reacted and were considered to be of one serotype, while the 8th was distinguished by a greater than 20-fold two-way difference in neutralisation titre suggesting a second serotype. Three widely-distributed reference strains (U.K., Northern Ireland and Lincoln) cross-reacted with the strains in the dominant serotype, as did 33 of 42 other field calf rotavirus strains. Nine field strains failed to cross-react with either serotype, suggesting the existence of other potential serotypes in the calf population.

Introduction

Rotaviruses are a major cause of diarrhoea in young animals and children (9, 18). Calf rotavirus is of worldwide distribution, and in our experience is the single most important infectious cause of diarrhoea in calves (31).

The antigenic relationships between rotaviruses from all species are complex and there are at least 3 groups of rotaviruses which share no common antigens (5, 20, 25, 27). Recent results obtained with reassortant viruses (12) and monoclonal antibodies (11) have helped clarify the situation within the most common antigenic group. It has been suggested that the term subgroup be used to describe antigens detected by broad serological reactions involving the major structural protein of the core, using assays such as complement fixation, enzyme-linked immunosorbent assay, and immune adherence haemagglutination (15, 39, 40). To date, two subgroups of human rotavirus have been demonstrated (15). The term serotype defines antigens involved in neutralisation reactions, at least one of which is associated with the surface glycoprotein of the outer shell (15). At least 4 and perhaps 5 human rotavirus serotypes exist (3, 36, 38).

Electrophoretic analysis of the eleven double-stranded RNA (ds RNA) viral genome segments has shown substantial variation in the pattern of segment mobilities (the electrophoretotype) both between and within virus from different species (7, 14, 26). Recent hybridisation and nuclease 'fingerprinting' studies have shown considerable sequence diversity even within dsRNA segments from strains of virus infecting one host species (27, 34). The non-cross-reactive groups of rotaviruses can be distinguished by electrophoretic analysis (5, 20, 25, 27); and the subgroups of human rotavirus can also be distinguished by their segment patterns (14).

Two serotypes of calf rotavirus distinguishable by neutralisation test have been reported from Japan (22), and some strains have been found distinct by haemagglutination inhibition test (33). Clearly serotype diversity is of great importance for potential vaccine development. The genome dsRNA pattern

of bovine rotaviruses shows extensive variation (26). In this paper we describe investigations into the relationships by neutralisation assay and dsRNA electrophoresis of strains of rotavirus in calves in the United Kingdom.

Materials and Methods

Viruses

Faeces from twelve 1-4 week old calves which were shown by ELISA (8) and by electron microscopy (EM) (32) to contain rotavirus were initially selected for study. The basis for selection was the widely separate geographical origins within the U.K. of the faecal samples. Subsequently 42 other strains from calf faeces submitted to this laboratory for diagnostic examination were studied. Reference tissue culture adapted rotavirus strains used were U.K. (Compton) (6) (cloned by Dr. R. G. Wyatt), Northern Ireland (19) and the Lincoln Nebraska Calf Diarrhoea Virus (21).

Infection of gnotobiotic lambs

Gnotobiotic lambs reared singly in plastic isolators were used for the multiplication of the different isolates as well as for the production of specific convalescent antisera.

A 10% suspension of each of the original faeces in 20 mM tris HCl buffer pH 7.5 (tris buffer) was blended with an equal volume of fluorocarbon (Arcton 113, ICI), centrifuged at 2000 g for 30 mins, and the aqueous phase filtered through a 0.45 μ m membrane. Three ml of filtrate was administered orally to each lamb 24 hrs after delivery. Faeces were collected and examined daily for rotavirus excretion by ELISA and EM. Twenty-one days after inoculation the lambs were bled for antisera.

Tissue culture

MA104 cells used throughout the study were grown in Eagles 59 medium containing 10% foetal bovine serum and maintained after inoculation in medium 199 containing 0.5% BSA and 2 μ g/ml trypsin (Sigma Chemicals Co. UK). All cultures were rolled after inoculation.

Tissue culture adapted strains of rotavirus were treated with 5 µg/ml of trypsin at 37°C for 30 mins before inoculation. Virus stocks were produced by harvesting infected cultures by 3 cycles of rapid freezing and thawing after 2-3 days or at maximum cytopathic effect (cpe).

To isolate rotavirus from field samples 0.5 ml of trypsin treated (10 µg/ml) faecal filtrate was inoculated to cell monolayers. Cultures were passaged at 3-day interval and examined daily for cpe and at each passage by immunofluorescence for rotavirus. Isolates were cloned by passaging thrice at terminal dilution from the 6th or 7th passage level.

Rotavirus purification

To prepare virus from the twelve gnotobiotic lamb faeces, samples were diluted in 3 volumes of tris buffer, extracted with Arcton, and centrifuged at 2000 g for 30 minutes. Ten per cent sodium dodecyl sulphate was added to the supernate to a final 1% v/v and incubated at room temperature for 5 mins. This initial step was omitted when tissue culture grown rotavirus was used. Preparations were then pelleted at 71,000 g for 45 mins. The pellets were homogenised in 1-2 ml tris buffer, layered onto a discontinuous gradient consisting of 2 ml of a solution containing 1.31 M CsCl and 1.58 M sucrose, overlaid by 2 ml 1.58 M sucrose in tris buffer and centrifuged at 154,400 g for 60 mins at 5°C. The opalescent band which appeared just below the interface was harvested, diluted four-fold and pelleted. Pellets were resuspended in 1-2 ml of tris buffer and layered onto a a 5-step CsCl/sucrose gradient to which 1.0 µg/ml ethidium bromide had been added and then centrifuged at 50,400 g for 18 hrs at 5°C. The gradient consisted of 1.66 M sucrose/1.49 M CsCl and 1.56 M sucrose/1.49 M CsCl at the extremities. An intermediate density was achieved by mixing equal volumes of the two extremes, and two further steps were achieved by mixing the intermediate solution with the two extremes. The virus band was located by fluorescence under ultraviolet light, harvested with a syringe, diluted in tris buffer, and pelleted. The pellets were examined by EM using negative staining with 1% ammonium molybdate (pH 6.0) and the proportion of complete virions estimated.

When tissue culture grown rotavirus was the starting material, virus was pelleted from the cleared supernate, and the resuspended pellet layered directly onto the 5-step CsCl gradient.

Hyperimmunisation of rabbits

Purified virus pellets containing greater than 99% complete virions were diluted to 1 ml in tris buffer with 2% Tween 80 added, and emulsified in incomplete Freund's adjuvant. Each rabbit (previously shown to be free of neutralising antibody to U.K. calf rotavirus) received a deep intramuscular injection of 1.0 ml of the emulsion at two different sites. The injections were repeated 14 days later and the rabbits were bled by cardiac puncture 7-10 days after the second injection.

Neutralisation test

With both tissue culture adapted rotavirus strains and faecal rotavirus, neutralisation of fluorescent focus production in MA104 cells was used, essentially as described by Beards et al (3). Titres (NT) are expressed as the reciprocal of the serum dilution reducing fluorescent foci by 50%.

In the serotyping of field strains of rotavirus in faeces with standard rabbit serotyping antisera, a constant serum-varying virus assay was used. Faeces were extracted with fluorocarbon, mixed with antibiotics and centrifuged at 200 g for 30 min. Half \log_{10} dilutions of the supernatant fluids were treated with trypsin at 10 $\mu\text{g}/\text{ml}$ for 1 hr at 37°C, then incubated with 4 antibody units of typing antiserum for 1 hr at 37°C (1 antibody unit was the amount of antibody neutralised by 100 TCID₅₀ of homologous rotavirus), and thereafter assayed for fluorescent foci on MA104 cells.

Polyacrylamide gel analysis (PAGE) of the dsRNA

Double stranded RNA was prepared from purified virions by phenol-chloroform extraction (13) and precipitation with alcohol and was fractionated in 7.5% polyacrylamide gels with no stacking gel. The Laemmli buffer system (16)

was used and electrophoresis was for 12 hr at a constant current of 0.1 mA/cm²; each gel track was loaded with 100-200 ng of dsRNA. After electrophoresis the gels were washed for 3-4 hr by gentle agitation in 5 changes of 1% acetic acid/10% ethanol and were then stained with silver as already described (13).

Results

Infection of gnotobiotic lambs

Rotavirus multiplication occurred in all 12 lambs, virus being excreted in faeces for at least 5 days. Coincident with virus excretion the faeces became loose and yellowish. A transient anorexia was noted in one lamb (678).

Examination of viral dsRNA by PAGE revealed that all 12 samples had different electrophoretic patterns. However, 4 of the samples (tracks 3, 7, 10 and 11) had more than 11 segments (Fig. 1). This was considered to reflect the presence of more than one strain of rotavirus in the original calf faeces, so subsequent studies were performed on the remaining 8 strains only.

Cell culture adaptation of faecal rotavirus

Two of the eight lamb-passaged calf rotavirus strains were selected for adaptation to cell culture, 639 as typical serologically of 7 of the strains, and 678 as a distinct strain (see results below).

By the 4th passage level both strains produced slight cpe after 2 days incubation, and by the 6th passage this had developed to a cpe involving complete destruction of the monolayers after 3 days incubation.

The two isolates were cloned by three passages at terminal dilution. Examination of virus genome by PAGE at stages from faeces to cloned virus showed a consistent migration pattern within each isolate (Fig. 2) making the possibility of strain cross contamination unlikely.

Neutralisation tests

1. Convalescent lamb antisera reacted with faecal virus.

All the lamb antisera possessed NT antibody to the homologous rotavirus strains, and also showed varying cross-neutralisation with other strains (Table 1). Within 6 of the strains 637, 639, 641, 649, 651 and 669, there was a high degree of cross-reactivity, with not more than an 8-fold variation between homologous and heterologous titres.

Antiserum to strain 678 showed 4 to 32 fold lower titres against all heterologous antigens, and 678 antigen was poorly neutralised by all other antisera. Strain 642 showed one-way cross-reaction with the group of 6 strains; 642 antigen was efficiently neutralised by other antisera, while 642 antiserum neutralised heterologous strains less efficiently.

2. Hyperimmune antisera to faecal virus reacted with faecal virus.

The 6 strains considered similar in their reactions with convalescent lamb antisera were also similar in their reactions with hyperimmune antisera, with not more than a 4-fold variation in titre between homologous and heterologous strains (Table 2). Strain 642 appeared to be more closely related to the 6 strains in these tests, with efficient neutralisation of 642 virus by other antisera, and up to a 16-fold variation in titre of 642 antiserum with heterologous virus strains. Once again strain 678 showed significant distinction from all 7 other strains, with greater than 20-fold differences of titre in both directions.

This evidence suggested that seven strains including 642 were of one serotype, with less than 20-fold difference in titre, and that strain 678 was a distinct serotype.

3. Hyperimmune antisera to tissue culture virus reacted with tissue culture virus.

The cell culture adapted U.K., Northern Ireland, and Lincoln strains were compared with 639 and 678 viruses isolated in cell cultures. The UK, Northern

Ireland and 639 strains appeared identical by cross neutralisation tests (Table 3). The Lincoln strain was less efficiently neutralised by antisera to these 3 strains, and thus showed slight one-way variation. However, the differences were never greater than 16-fold, so the Lincoln strain should still be considered as the same serotype.

Antiserum to 678 virus had heterologous titres in the range 64-512-fold less than the homologous titre although 678 virus was clearly distinguished by a greater than 20-fold titre difference by only 2 of the other antisera.

Serotyping faecal rotavirus strains

Due to the consistent distinction of 678 virus, it was considered to represent a separate serotype. Thus typing of field rotavirus strains was carried out using hyperimmune antisera to cloned 639 and 678 viruses. Allocation to serotype was on the basis of at least a 20-fold reduction in virus titre after incubation with the appropriate antiserum, compared with both the virus control titre and the titre with the other antiserum.

Sixty additional calf faeces samples containing rotavirus were examined. No typing of 18 strains was possible due to their failure to produce fluorescing foci in MA104 cells. Antiserum to virus 639 neutralised the virus in 33 samples, while virus in 9 samples was not neutralised by either antiserum.

dsRNA segment pattern

The PAGE analysis of the viral dsRNA (Fig. 1) showed that all strains examined varied in their migration patterns, with no clear relationship to serotype.

Discussion

This study demonstrates the existence of two serotypes of rotavirus in calves. Whether the field viruses not neutralised by either antiserum were mixtures of more than one strain or represent potential new serotypes is currently under investigation. These two serotypes were defined by a neutralisation assay using fluorescent focus reduction with a 20-fold or greater two-way difference in titre as criterion for distinction (10, 38). The fact that the majority of our field calf rotavirus strains as well as three

widely-used reference strains, all shared the same serotype, indicates that this serotype is at present the most common in the United Kingdom. This serotype, which we designated serotype 1, is probably similar to the serotype 1 proposed by Murakami et al (22), as both were found similar to the Lincoln strain. The reference strain for serotype 1 could be either Lincoln, or the cloned UK strain used in our studies.

The in vivo active and passive immune relationships between the two serotypes are as yet unknown. Passive immunisation by dam vaccination is currently the most favoured method of prophylaxis in cattle (23, 30, 37). However, as passive immunity has been shown to be heterogenous between species by the protection of piglets against pig rotavirus infections using bovine colostrum (4, 17), the same broad passive protection may occur among serotypes of bovine rotavirus. In any case, the predominant occurrence of a single serotype suggests that in most instances a monovalent vaccine may be effective.

The techniques for cell culture isolation of rotaviruses from faeces used for calf rotavirus (1, 2) and for human rotavirus (28) proved successful in this study. In addition to the two strains 639 and 678, four other strains that were not neutralised by antisera to either of the two serotypes have now been isolated and cloned. However, there was a degree of selection of suitable strains for culture, as 18 faecal samples containing rotavirus detected by EM and ELISA did not produce any fluorescent foci in MA104 cells. The serotyping of such viruses presents a difficult problem.

Examination of the rotavirus genome dsRNA segments by PAGE provided a means of quality control by isolate identification throughout the isolation and cloning of field strains. Identical dsRNA migration patterns in original faeces and cloned virus make laboratory contamination very unlikely due to the great diversity of patterns seen in rotavirus surveys (24, 26). PAGE examination also detected mixed strain rotavirus infections in 4 of our original 12 faeces samples.

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Table 1

Neutralising titres of antisera from convalescent gnotobiotic lambs, to 8 strains of calf rotavirus in faeces

Rotavirus	Antiserum to rotavirus							
	637	639	641	642	649	651	669	678
637	<u>128</u>	128	32	128	128	128	64	16
639	64	<u>128</u>	512	128	64	32	64	8
641	64	64	<u>64</u>	64	64	64	64	32
642	128	128	512	<u>1024</u>	128	128	128	32
649	128	128	32	ND	<u>128</u>	128	64	16
651	128	64	256	256	128	<u>64</u>	64	16
669	128	64	64	64	64	64	<u>128</u>	16
678	32	16	32	16	32	32	16	<u>256</u>

Homologous titres are underlined

N.D. not done

Table 2

Neutralising titres of hyperimmune rabbit antiserum to faecal rotavirus strains, to 8 strains of calf rotavirus in faeces

Rotavirus	Antiserum to rotavirus							
	637	639	641	642	649	651	669	678
637	<u>204800</u>	102400	51200	102400	409600	51200	25600	6400
639	204800	<u>102400</u>	102400	204800	819200	102400	25600	3200
641	102400	51200	<u>51200</u>	51200	204800	102400	25600	6400
642	409600	102400	204800	<u>819200</u>	12800	204800	51200	6400
649	204800	51200	204800	819200	<u>204800</u>	51200	51200	1600
651	51200	51200	204800	409600	819200	<u>102400</u>	51200	3200
669	102400	51200	51200	204800	204800	N.D.	<u>51200</u>	3200
678	3200	6400	6400	3200	6400	3200	3200	<u>102400</u>

Homologous titres are underlined

N.D. Not done

Table 3

Neutralising titres of hyperimmune rabbit antisera to rotavirus strains grown in tissue culture, to 5 strains of calf rotavirus in tissue culture.

Antiserum to rotavirus					
Rotavirus	UK	Northern Ireland	Lincoln	639	678
UK	<u>5120</u>	102400	409600	102400	3200
Northern Ireland	5120	<u>102400</u>	409600	51200	3200
Lincoln	320	51200	<u>409600</u>	6400	400
639	5120	102400	409600	<u>102400</u>	3200
678	160	3200	51200	25600	<u>204800</u>

Homologous titres are underlined

Legends

Fig. 1. Polyacrylamide-gel electrophoresis of dsRNA from 12 isolates.
Tracks 1: 637; 2: 639; 3: 646; 4: 641; 5: 642; 6: 651;
7: 657; 8: 669; 9: 649; 10: 663; 11: 666; 12: 678. Note
the existence of more than 11 segments in tracks 3, 7, 10 and 11.

Fig. 2. Comparison of the dsRNA of 639 and 678 after adaptation to tissue
culture with dsRNA from the original faeces.
Tracks 1 and 4: dsRNA of faecal 678 and 639 respectively; tracks
3 and 6: dsRNA of tissue culture adapted 678 and 639 respectively;
tracks 2 and 5: a mixture of the dsRNA from faecal and tissue
culture adapted virus.

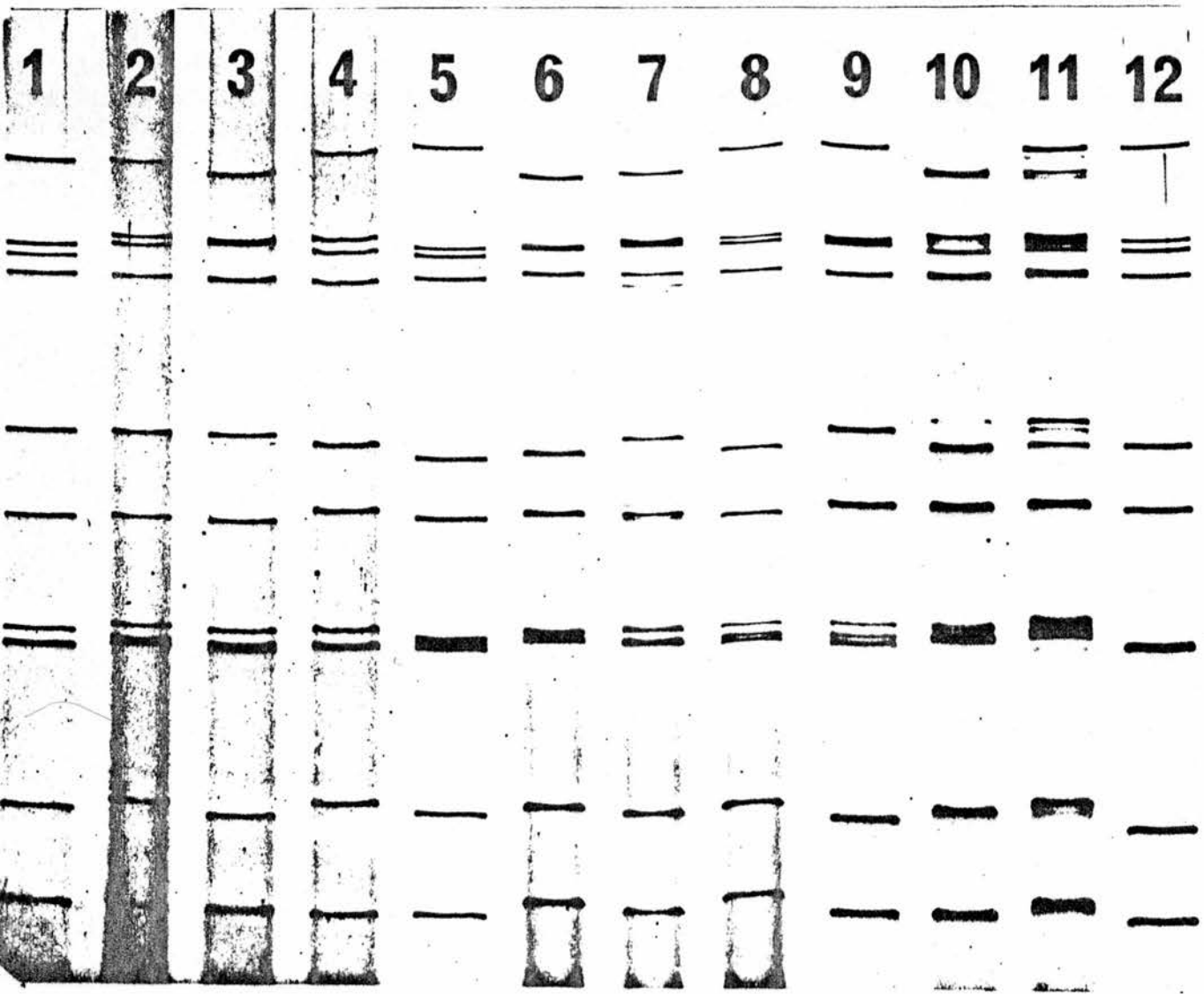


Fig 1

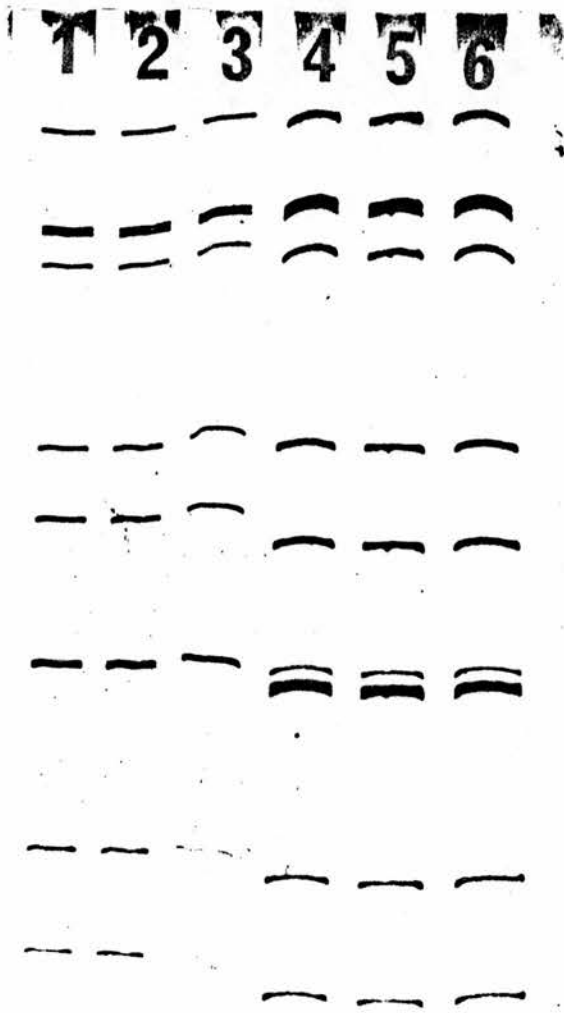


Fig 7.