STUDIES ON RESPIRATORY AND ENTEROPATHOGENIC VIRUS IN CATTLE

THESIS

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by

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To

MY AUNT Dr. (Mrs.) NARAINI, M.B.B.S., M.P.H. (U.S.A.)

With Reverence and Affection.

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This is to certify that the thesis entitled " Studies on respiratory and enteropathogenic virus in cattle " submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Veterinary Microbiology to the Tamil Nadu Agricultural University, Coimbatore, is a record of bonafide research work carried out by S.P. Anbumani, under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.

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Chapter I

PLAN OF STUDY

The countrywide implementation of various livestock development and production programmes on an intensive manner especially in cattle is seriously impaired by an increase in the incidence of morbidity and mortality among young calves. This malady due to various causes poses series of problems not only in rearing cross-bred and buffalo calves but also on the economy of the livestock breeders.

The involvement of various viruses as aetiological factors causing calf diarrhoea leading on to morbidity and mortality has not been fully investigated into. Some viral diseases like rinderpest and foot-and-mouth are easily recognised by their pathognomonic symptoms while other viral infections of respiratory and alimentary systems have not been fully identified probably because of absence of clear-cut disease symptoms.

Despite several reports of viral involvement in this malady of calf diarrhoea from other countries, not much work has been done in India. In U.S.A. Olafson *et al.*, (1946) reported on a highly contagious disease of cattle which was called virus diarrhoea. Similar reports were made by

Pritchard et al., (1956), Ramsey and Chivers (1953) in U.S.A. and Correa et al., (1972) in Brazil.

Darbyshire et al., (1965) studied Adenoviruses affecting respiratory and alimentary systems inducing diarrhoea and pneumonia in calves. More investigation into diarrhoea disease of calves was encouraged by the recognition of Rotaviruses as a cause of gastro-enteritis in calves (Almeida et al., 1978). It was reported that Rotavirus in the presence of other viruses and Escherichia coli (E. coli) causes serious scouring in neonatal calves.

Further proof goes to show that reovirus-like particles were reported to cause diarrhoea in newborn calves in Nebraska in U.S.A. (Flewett et al., 1974). A polyarthritic disease of newborn calves in U.S.A. known as weak calf syndrome was described and Bovine viral diarrhoea (BVD) virus, Bovine adeno type 5 (BA) were isolated from such ailing calves (McClurkin and Coria, 1975).

Neonatal calf diarrhoea was extensively investigated since it posed health problems (Morin et al., 1974). Though E. coli was considered to be the main cause of neonatal calf diarrhoea for several years, other agents like Chlamydia and viruses such as BVD, IBR, Adenovirus, Parvovirus and Enteroviruses were suggested as possible causes of the disease (Morin et al., 1974).

In India, Chandrasekaran and Balasubramaniam (1962) reported on mucosal disease complex affecting buffalo calves in Madras. Sapre (1962) reported that mucosal disease occurred in cattle and buffaloes in all age groups irrespective of sex causing low morbidity with high mortality. Similar observations were made by Parnaik et al., (1964) in a dairy farm in Bombay. Enterovirus infections were identified as mucosal disease (MD) in Maharashtra, Himachal Pradesh and Gwalior region (Nilakanian, 1963). Parainfluenza type 3, Adenoviruses, Reovirus type 2, Entero and Rhinoviruses were isolated from cattle (Datt and Raghavan, 1969; Raghavan and Datt, 1973). The above reports clearly indicatedthat the viruses as aetiological agents played significant role in causing pneumonia and diarrhoea in young calves.

In order to gain more insight into the aspect of viral aetiology of calf morbidity and mortality, this work was undertaken by attempting to isolate the viruses from the throat and rectal swabs and dung samples collected from diarrhoeic and non-diarrhoeic, pneumonic and non-pneumonic calves, in tissue culture system using bovine calf kidney and to identify and characterise them by applying various techniques.

Chapter II

REVIEW OF LITERATURE

Some of the enteric viral diseases in cattle like rinderpest, foot-and-mouth disease are easily recognised by their pathognomonic syndromes and necessary control measures are already available to contain them. The agents causing Bovine viral diarrhoea (BVD)/mucosal disease were not involved in the calf scour syndrome (Woode and Bridger, 1975). Calves under 3 months of age were not susceptible to BVD (Olafson et al., 1946; and Olafson and Rickard, 1947). But there are other viral infections of respiratory and alimentary systems especially in neonatal calves whose involvement as aetiological agents have not been fully and properly identified and hence these aetiological agents of these infections are not in the lime light as to their role in the causation of the disease. Neonatal calf diarrhoea or calf scour and other viral syndromes of neonates involving respiratory and enteric tracts leading to weak calf syndrome and calf mortality had been recognised as important cause of economic loss (Mills, 1976). The economic loss is not only due to mortality which varies between 0 to 30 per cent but also to medical costs and poor growth of calves (Woode and Bridger, 1975).

1. Neonatal calf diarrhoea

(i) History and disease syndrome: Neonatal calf diarrhoea (NCD) or calf scour is an important disease syndrome causing economic loss both in beef and dairy herds (Morin et al., 1974; Woode and Bridger, 1975; England, 1977) and the discussion here is limited to the roles of the neonatal calf diarrhoea viruses, viz. Rotavirus and Coronavirus. NCD accounted for a greater toll of very young calves from birth to 2 weeks of age (Amstutz, 1965; White et al., 1970). Although many microbial organisms had been associated with the disease there is doubt concerning their role as causative agents and the role of viruses has gained much importance in recent years.

For several years E.coli was considered to be the main cause of the disease (White et al., 1970; Morin et al., 1974; Woode and Bridger, 1975), but the difficulty in reproducing the disease with various E.coli isolates by various workers like Lambert and Fernelius (1968), White et al., (1970), Mebus et al., cited by Woode and Bridger (1975), made it doubtful as to their role in all outbreaks of calf scours.

Other agents like Chlamydia and six viruses, viz. Reovirus, Coronavirus, Bovine Viral Diarrhoea (BVD) virus, Parvovirus, Enterovirus, Adenovirus, were suggested as the

potential agents in the neonatal calf diarrhoea causing mixed infection (Fernelius, 1973; Morin et al., 1974; McClurkin, 1977). Even among the viruses reovirus-like agents, calf Coronavirus (Morin et al., 1974; Woode and Bridger, 1975) and Bovine Viral Diarrhoea Virus were considered to be the primary viruses associated with neonatal calf diarrhoea, since they were the most often isolated ones (England, 1977).

In recent years reovirus-like agents and calf Coronavirus were shown to be the primary cause of neonatal calf diarrhoea (Morin et al., 1974; Woode and Bridger, 1975; Holmes et al., 1974; Pastoret and Schoenaers, 1977; Almeida et al., 1978; Marsolais et al., 1978).

The symptoms observed were rapid onset, depression, watery yellow or yellowish green faeces (White et al., 1970; Morin et al., 1974) and white to yellow coloured diarrhoeic faeces (Woode and Bridger, 1975), the small intestine, caecum, colon and rectum were distended by a watery yellow content with large amount of mucosal debris free in the intestinal contents and with white membranes attached to several areas of the intestinal mucosa (Morin et al., 1974).

Histopathologically diminution of small intestinal villi, progressive replacement of epithelium with squamous and cuboidal cells that lack brush borders were observed.

Several villi were blunt and covered with vacuolated columnar epithelial cells. The calf Coronavirus caused more severe lesions with a greater dwarfing of the villi and lesions were also observed in the colon and mesenteric lymph nodes (Morin et al., 1974; Mebus et al., 1973a; Woods and Bridger, 1975; Dubourguier et al., 1978).

(ii) Incidence: Calves with an acute febrile illness accompanied by rhinorrhoea and diarrhoea had been recorded in Oregon, U.S.A. in naturally occurring infection of calves (Mattson, 1973). Ranches in Nebraska and surrounding States of America were troubled with diarrhoea in neonatal calf for several years causing 90 -100 per cent morbidity with a mortality rate upto 50 per cent, the causative agent being a virus detected in 135 of 237 faecal samples by fluorescent antibody technique (White et al., 1970). Electron microscopic examination revealed Coronavirus like agents (Stair et al., 1972).

A reo-like neonatal calf diarrhoea virus was demonstrated in Denmark by fluorescent antibody staining in the intestinal smear that died of diarrhoea (Meyling, 1974) and Corona-viruses were also implicated in Great Britain and Denmark (Bridger et al, 1978). Neonatal calf diarrhoea which was a consistent problem of neonatal calves for two years was

recorded in a Quebec dairy herd causing 100 per cent morbidity with 25 to 45 per cent mortality. The cause was due to two Nebraska NCD virus (Morin et al., 1974). Cases of acute gastroenteritis in young children reported in Australia and United Kingdom and outbreaks of diarrhoea in newborn calves that occurred in Nebraska and Berkshire were caused by human infantile diarrhoea virus and Neonatal calf diarrhoea reolike (NCDR) virus respectively and these viruses were found to be indistinguishable from each other (Flewett et al., 1974; Albrey and Murphy, 1976). Outbreaks of diseases in cattle with respiratory and diarrhoea symptoms in Japan were reported to have been caused by new serotypes of Reovirus (Kurogi et al., 1974).

There were three serious outbreaks of enteritis in newborn calves since 1975 in North-Eastern Italy, mortality recurring in each outbreak; and of the 256 serum samples collected in the area which had shown episodes of gastro-enteritis, 214 were positive for reo-like virus (Cancellotti et al., 1976).

Diarrhoea in neonatal calves were reported in France in which reo-like virus (calf Rotavirus) was shown to be the causative agent (Scherrer et al., 1976). Similar reports of neonatal calf diarrhoea were reported in Federal Republic of

Germany in 1977 which were caused by Rota and Corona viruses as reported in U.S.A., Belgium, Canada, England and New Zealand (Dirksen and Bachmann, 1977; Frey et al., 1979). Fiftyeight calves and yearlings were affected with diarrhoea in New Zealand and Enteroviruses and reolike viruses were responsible for this ailment (Durham and Burgess, 1977). Yearlings and heifers were affected with scours in New Zealand and Adenovirus was reported to be responsible for such scourings (Thompson, 1977).

Outbreaks of neonatal calf diarrhoea among 134 calves were reported in Canada; Fluorescent antibody staining technique (FAT) and Electron microscopic (EM) study revealed Nebraska viruses as caussk agents (Marsolais et al., 1978).

(iii) Age and breed: Neonatal calf diarrhoea was reported to have occurred in Nebraska and surrounding States affecting the calves from 12 hours to several weeks of age (White et al., 1970). Neonatal calves in 19 herds in Western Nebraska were affected with neonatal calf diarrhoea in which corona virus-like agent was detected (Stair et al., 1972). Occurrence of neonatal calf diarrhoea in 3 day old calves were reported in Quebec dairy herd. Necropsy findings indicated the presence of rem-like and corona-like viruses (Morin et al., 1974). Rotavirus was isolated from 6 of 16 faecal specimens

from newborn calves with gastroenteritis (scours) and a morphological similarity to infant Rotavirus were described (Albrey and Murphy, 1976). Calf diarrhoea was observed in a farm in New Zealand and all calves commenced scouring when they were 1-4 days old (Burges and Simpson, 1976). Of the 40 calves studied, 30 per cent of the calves developed diarrhoea before 10 days of age (Acres et al., 1975). Outbreaks of virus diarrhoea affecting 3-6 months old were reported (Kharlambiev et al., 1976; Galiev et al., 1978).

(iv) Season: Infectious calf diarrhoea, a major problem in beef and dairy herds was observed to affect calves during all seasons but it was most prevalent during winter months (Amstutz, 1965). A field outbreak of diarrhoea in North Central Nebraska had occurred in March 1969 affecting the young calves (White et al., 1970). In September and October 1971, there were outbreaks of viral diarrhoea in cattle herds in Brasil causing 20 per cent mortality among affected cattle (Correa et al., 1972).

(v) Characteristics of aetiological agent.

A. Rotaviruses

a) Physico-chemical features: The nucleic acid of calf Rotavirus showed to contain RNA initiated by the inability of 5-iodo-2-deoxy uridine to inhibit the multiplication of the

virus at a concentration upto 500 ug/ml and also by actinomycin D (Welch and Thompson, 1973) the genome was double stranded and segmented with a total mol. wt. of 11-12 x 10^6 (Newman et al., 1975). Polyacrylamide gel electrophoretic analysis of purified preparation of human and calf diarrhoea virus indicated eight to nine polypeptide components (Rodger et al., 1975). Rotaviruses occupy a position between Reovirus and Orbivirus in their susceptibility to various inactivating agents. All the three viruses were resistant to the action of chloroform and ether. Reovirus and Rotaviruses were stable at pH 3 and stabilised by Mg ions for heat inactivation whereas Orbiviruses were not (Welch and Thompson, 1973; McNulty, 1978). Calf Rotavirus was inactivated when heated at 50°C in the presence of 1 M-$MgCl_2$ whereas reovirus was not (Estes et al., 1979).

Preparation of calf Rotavirus agglutinated erythrocytes from a variety of animals. A component on the outer capsid layer of the virus was found to be responsible for haemagglutination (Inaba et al., 1977; Fauvel et al., 1978). Double shelled particles agglutinated erythrocytes, but single-shelled particles did not (Fauvel et al., 1978). Further it was reported that development of inhibitors in cell culture closely correlated with the decline in haemagglutinin titre

between days 2 to 7 rendering a tissue culture isolate harvested during the above period to an undetectable level of haemagglutination activity (Inaba et al., 1977).

b) Biological and serological features: Experimentally diarrhoea was produced in colostrum deprived calves by inoculation of faecal material from field cases. Bacteria cultured from the faeces of diarrhoeic calves did not cause the disease, whereas bacteria-free faecal filtrate did. Further characterisation showed that it was similar to Reovirus in morphology but was serologically unrelated to Reovirus types 1 and 3 and occupied a position intermediate between Reovirus and Orbiviruses named as Rotavirus (McNulty, 1978; Newman et al., 1975, Derbyshire and Woode, 1978).

A review of aetiology, diagnosis and treatment of viral neonatal diarrhoea in the calves suggested that Rotavirus and Coronavirus play the leading role (Pastoret and Schoenaers, 1977). Rotavirus infection occurred characteristically in young animals and Rotavirus associated diarrhoea occurring in the first week of life had been described in calves (Woode and Bridger, 1975; Holmes et al., 1974).

Comparative studies had confirmed that the Rotaviruses of human infants, calves, pigs and foals were morphologically indistinguishable from each other and from Epizootic

diarrhoea of infant mice (EDIM) virus, S.A.11 virus and the 'O' agent (Kapikian et al., 1975; Flewett et al.,1974; Holmes et al., 1974; Sharpee and Mebus, 1975) and it was suggested that it might be possible to immunise against the human disease utilising the calf virus if the calf virus is proved capable of infecting man without causing disease (Kapikian et al., 1975).

c) Electron microscopic demonstration of virus: Electron microscopic study of the virus revealed that intact viral particles were 60 nm to 66 nm in diameter. They consisted of an electron dense centre about 36-38 nm in which short cylindrical capsomeres radiated outwards. Attached to the ends of these capsomeres an additional outer layer of capsomeres gave an appearance of a sharply defined rim attached to short spokes upon a wide hub suggestive of a wheel. The Rotavirus differed from Reovirus and Orbivirus which did not possess the well defined circular outline (Flewett et al., 1974).

Electron microscopic study to implicate the presence of virus as aetiological agent in a widely disseminated cases of calf diarrhoea was conducted which revealed the presence of reovirus-like particles from faeces and intestinal mucosa of experimentally infected calf (Welch, 1971; Turner et al.,1973;

Wyn-Jones et al., 1978). Sixtytwo specimens from sixty patients most of them below 5 years of age, were examined for the presence of virus by electron microscopy. 66 nm Rotavirus particles were demonstrated in 19 out of 35 patients with acute gastroenteritis and from none of 25 patients with other diseases. Echovirus type 7 and Adenovirus were detected along with Rotavirus. Based on the demonstration of virus particles in 54 per cent of the patients, it was concluded that the Rotavirus may be an aetiological agent in infantile acute gastroenteritis (Orstavik et al., 1974; Paniker et al; 1977)

Electron microscopic examination of the faeces of calves with diarrhoea revealed in 33 of 35 faeces samples reovirus-like particles, corona virus-like particles and also the presence of myxovirus and mycoplasma-like particles (McNulty et al., 1975, 1976a). Of the 74 faecal samples examined from natural cases in field outbreak of neonatal calf diarrhoea and from experimental animals by immune electron microscopy and immunofluorescence, 98 per cent of the reports were in agreement in diagnosing the presence of virus (Bridger and Woode, 1975; Burges and Simpson, 1976; England et al., 1976).

A reolike-virus (calf Rotavirus) was shown to be associated with cases of neonatal calf diarrhoea in France,

By electron microscopy and immunofluorescent studies virus could be detected in more than 50 per cent diarrhoeic faecal samples, which was closely related or identical to the agent isolated in the United States (Scherrer et al., 1976). Direct virological examination of faeces in calves of two herds in the Federal Republic of Germany revealed the occurrence of both Rota and Coronaviruses akin to the viruses described in U.S.A., Belgium, Canada, England and New Zealand. Rotavirus infection was fatal in one of the 14 calves, Coronavirus infection caused sickness in 10 calves and death in 4 calves (Dirksen and Bachmann, 1977).

One hundred and thirty four samples were examined by electron microscopy and fluorescent antibody technique for the detection of viruses associated with neonatal calf diarrhoea. In 107 cases (80%) Nebraska virus had been demonstrated evidencing their role in the causation of calf scouring (Marsolais et al., 1978; Almeida et al., 1978; Frey et al., 1979).

d) Fluorescent microscopic demonstration of virus: The virus which was capable of causing diarrhoea was detected by the fluorescent antibody technique in 135 of 237faecal samples collected on ranches from calves with diarrhoea and in intestinal contents from 14 of 22 dead calves (White et al.,1970).

Two Nebraska NCD virus were detected in the cytoplasm of many absorptive cells of the small intestine from a calf submitted for necropsy and the disease was reproduced experimentally in 2 day-old colostrum deprived calf with a bacteria-free intestinal homogenate obtained from the naturally infected calf (Morin et al., 1974; Blackmer, 1976), in Denmark by Meyling (1974), in Italy by Cancellotti et al., (1976).

In an area where diarrhoea was causing heavy calf losses, Reovirus and Coronavirus were demonstrated by fluorescent antibody tests on sections of small intestine and colon from dead animals. The results of vaccination trial to which all calves born in a period were dosed with virus followed by a period in which calves were not treated, gave more impressive results, only 3 per cent mortality upto three weeks of age in the treated group compared with 22 per cent in the untreated group (Blackmer, 1976).

Fluorescent virus precipitin test conducted on faeces of thirty four calves and five infants for the presence of neonatal calf diarrhoea virus and reovirus-like infantile diarrhoea agent had revealed that fourteen calf stools contained detectable neonatal calf diarrhoea (NCD) virus and four of the five infants' stools contained the reovirus-like human agent (Peterson et al., 1976).

e) Immunodiffusion demonstration of virus: Immunodiffusion test with polyethylene glycol (PEG) appeared to be more sensitive than electron microscopy which allowed quick and early detections of Rotavirus antigen in faecal materials and in organs of calves that died of digestive troubles (Van Opdenbosch et al., 1979).

Sera from 266 infants and children were tested for Rotavirus specific antibody in counter immunoelectrophoresis. A dilute suspension of Rotavirus rich human faeces was used as antigen. It reacted with rabbit antibody to a calf Rotavirus but not with normal rabbit serum. A total of 182 sera (68.4%) were found to be positive. The result indicated that Rotaviruses were very common infectious agents in infants and preschool children (Jesudoss et al., 1978; Mohammed et al., 1978).

f) Cell culture demonstration of virus: Attempts to isolate the reovirus-like particles in cell cultures were not successful (Orstavik et al., 1974; McNulty et al., 1975). However, attempts were made to produce cytopathogenic virus on calf embryo kidney cells, secondary bovine kidney cultures and Madin-Darby Bovine kidney (MDBK) cells. The virus grew in all these cells and CPE was produced consistently only in MDBK cells consisting of cytoplasmic vacuolation,

eosinophilic intracytoplasmic inclusions, degeneration and detachment of cells from the monolayer (McNulty et al., 1977; Lemeshev and Pilui, 1977; Welch and Twiehaus, 1973).

Rotavirus generally difficult to isolate and culture in vitro was made possible by treatment of faecal samples containing Rotavirus with trypsin which enhanced the infectivity of the virus resulting in increased virus spread and in virus yield (Babiuk et al., 1977). Passaging the virus in the culture in the presence of 5 ug/ml trypsin retained the infectivity of the calf Rotavirus (Babiuk and Mohammed, 1978) and pig Rotavirus (Theil et al., 1978). The use of traces of trypsin at a concentration of 1 ug/ml was found to increase the growth by two log 10 in excess of 10^8 immunofluorescent units per ml. Such a technique was used favourably for isolating Rotavirus in continuous line of bovine kidney cells (MDBK), LLC-MK2, and He La cells (Clark et al., 1979).

g) Demonstration of virus by experimental reproduction: Ranches in Nebraska and surrounding States had been troubled with diarrhoea in neonatal calves for several years causing 90-100 per cent morbidity with a mortality rate upto 50 per cent. Death occurred in some calves before diarrhoea was evident. Using the virus that was isolated from a field outbreak of diarrhoea in bacteria-free calves typical neonatal

calf diarrhoea was reproduced characterised by yellow and liquid faeces (White et al., 1970).

Newborn colostrum deprived calves inoculated by live vaccine prepared with a reovirus-like calf diarrhoea agent resisted to the challenged virulent virus given 48-72 hours after vaccination. Vaccination of the calves reduced the frequency of diarrhoea, suggesting that exposure of small intestinal epithelium to virus was more important than circulating antibody in producing resistance to the infection (Mebus et al., 1973b; Woode et al., 1978).

A virus isolated from calves with diarrhoea in England was inoculated in colostrum deprived and gnotobiotic calves. All the calves developed diarrhoea with reisolation of the agent. On the basis of morphology, serology and pathogenesis, it was found as reovirus-like agent (Woode et al., 1974; Reves et al., 1977).

Neonatal calf diarrhoea induced with several agents of infection was studied by scanning electron microscopy. Infection with E.coli (pathogenic strain) produced lesion principally of the distal, ileum, small intestine causing desquamation and puffiness of microvilli, whereas Rotavirus caused stunting of and fusion of villi seen principally in the

proximal and middle small intestine. The abomasum was covered with abundant mucus film and appeared to be desquamated (Dubourguier et al., 1978; Pearson et al., 1978).

B. Coronavirus

a) Physico-chemical features: In cell culture the virus was found to produce CPE in embryonic tissues of trachea. In negatively stained preparations the viruses were seen to be pleomorphic, enveloped particles of the size of 120 nm in diameter with large petal-shaped peplomers 15-20 nm long (Fenner et al., 1974a). The fringe of projections resembled the solar corona. The genome consisted of single stranded RNA with a lipoprotein envelope multiplying in the cytoplasm as evidenced by the presence of cytoplasmic inclusions (Fenner et al., 1974a).

b) Biological and serological features: Neonatal calf diarrhoea (NCD) has been extensively investigated since it is known to be a major health problem in young calves causing morbidity approaching 100 per cent and mortality varying from 20 to 45 per cent. Generally diarrhoea appeared at three days of age. By the fluorescent antibody tissue section technique, reo-like and corona-like viruses were detected in the cytoplasm of many absorptive cells of the small intestine. Reo-like

virus antigen was not detected in the absorption cells and crypt cells of the colon but Coronavirus antigen was present (Morin et al., 1974; Woode and Bridger, 1975). The isolates on inoculation in gnotobiotic colostrum deprived calves produced diarrhoea in all the calves with recovery of the agent in the faeces (Stair et al., 1972). Lesions induced by a bovine Coronavirus in gnotobiotic and colostrum fed calves were studied histologically and electron microscopically. Lesions in gnotobiotic calves were present in the colon, mesenteric lymph nodes and all segments of the small intestine. Calves killed at 4 hours after the onset of diarrhoea had immunofluorescent epithelial cells on the villi of the small intestine and surface of the colon. Calves killed at 44 hours had shortened intestinal villi and cuboidal epithelial cells. The villi to crypt ratio averaged 1.0 compared with 5.3 in the control calf (Mebus et al., 1973b).

Serum samples and colostrum obtained from 45 cows and serum obtained from 20 fattening cattle, 15 suckling calves of 10 days age, and 10 fattening calves aged 4-5 months were screened for the presence of antibodies to Coronavirus. Antibody to Coronavirus were found in all the cows, fattening cattle and calves aged 4-5 months and in all but one suckling

calves. Colostrol titres were correlated with serum titres. Antibody to reo-like virus was found in 93 per cent of the cows and 70 per cent of the fattening cattle but only one of suckling calves. It was concluded that mixed infection with these two viruses were common but infection could occur with Coronavirus alone (Zygraich et al., 1975).

c) Electron microscopic demonstration of virus: Under electron microscope the viruses were found to be pleomorphic enveloped particles about 120 nm in diameter with large petal-shaped peplomers 15-20 nm long and quite narrow at the base. No evidence of a spherical or tubular nucleocapsids inside the shell had been demonstrated (Fenner et al., 1974a).

Coronaviruses were demonstrated by electron microscope examination of the faeces and intestinal contents. In neonatal calves with diarrhoea in Great Britain and Denmark, serially passaged samples in gnotobiotic calves produced acute diarrhoea and examination of large and small intestines under EM revealed the presence of Coronavirus which were morphologically and antigenically similar to bovine Coronavirus isolated in U.S. (Bridger et al., 1978). Samples of faeces from calves aged 2-9 days with diarrhoea were homogenised, centrifuged and the supernatant was centrifuged on saccharose gradient. EM revealed virus particles resembling Reovirus,

Coronavirus and Enterovirus in 5 of 8 samples (Bogatyrenko et al., 1976). Electron microscopy along with fluorescent antibody technique were used to detect Rotavirus and Coronavirus in faeces from calves with diarrhoea. Of 134 samples examined, Rota and Corona viruses were detected in 107 (80%) (Marsolais et al., 1978).

d) Fluorescent microscopic demonstration of virus: A corona-like virus was demonstrated in 3 of the 15 calves infected with feovirus-like virus and a nonpathogenic strain of bovine virus diarrhoea virus (Acres et al., 1975). Comparison of results obtained by different procedures showed that FAT was specific and would be a useful addition to available methods for identifying infections serologically (Monto and Rhodes, 1977).

FAT was favourably employed to demonstrate the presence of Coronavirus in the absorptive cells of small intestine (Morin et al., 1974; Woode and Bridger, 1975) and in the epithelial cells on the villi of the small intestine and surface of the colon (Mebus et al., 1973a).

e) Haemadsorption-elution-haemagglutination demonstration: Bovine Coronavirus was detected in calf faeces by employing haemadsorption-elution-haemagglutination assay. The method consisted of adsorption of virus onto mouse erythrocyte

at 4°C, removal of unadsorbed material and elution of adsorbed viral material at 37°C. The elute was then used in a haemagglutination test. The sensitivity of the test appeared to be better than that of electron microscopy (Vanbalken et al., 1979).

C. Other viruses

Electron microscopic study of faeces of 17 crossbred calves with scour during the winter months for several years had revealed that only one calf of 17 showed the presence of virus. In addition to Rota and Corona, a third type of virus was present. This was small cubic virus of 30 nm in diameter designated as Astro and Calici viruses (Almeida et al., 1978; Woode and Bridger, 1978). Norwalk type, Small Round Virus (SRV) were also found (Madeley, 1979).

Electron microscopic examination of faecal sample from 17 diarrhoeic lambs revealed the presence of 30 nm viral particles in 8 lambs showing their surface structure arranged in the form of six pointed star referred to as Astrovirus (Snodgrass and Gray, 1977).

2. Weak calf syndrome

Besides neonatalcalf diarrhoea, an idiopathic polyarthritis popularly known as weak calf syndrome (WCS) could bring about a heavy loss in calf rearing enterprises.

i) History and disease syndrome: Weak calf syndrome (WCS), a disease condition of neonatal calves, was first observed in 1964 in the Bitterroot valley of Montana and more recently the weak calf syndrome has been seen in Idaho, Utah, Oregon, Colorado and Nevada (Ivanoff and Renshaw, 1975; McClurkin and Coria, 1975) and had been known to exist in the eastern Idaho, South-Western Montana area for 6 to 10 years (Cutlip and McClurkin, 1975).

It was suggested that the cause of weak calf syndrome was due to adverse environmental factors, nutritional deprivation of the pregnant cow and/or microbial agents (Ivanoff and Renshaw, 1975) but the study on naturally occurring infection of newborn calves revealed the presence of bovine Adenovirus type 3 (Mattson, 1973) and the isolation of bovine Adenovirus type 5 from newborn calves with polyarthritic disease (McClurkin and Coria, 1975). Experimental reproduction of the disease with bovine Adenovirus type 5 (Cutlip and McClurkin, 1975; McClurkin

and Coria, 1975) with bovine Adenovirus type 7 (Stauber and Card, 1978) had revealed that the causative agents were bovine Adenoviruses.

The disease syndrome associated with WCS infected with bovine Adenovirus type 5 was that the affected calves were usually weak at birth, often unable to get up and nurse, marked pyrexia, mild diarrhoea and sore leg joints (McClurkin and Coria, 1975). The disease was characterised by haemorrhages and oedema of subcutaneous tissues of the knee and hock joints, with blood tinged synovial fluid (Ivanoff and Renshaw, 1975; McClurkin and Coria, 1975). Haemorrhages and oedema of the subserosa of the gastrointestinal tract was present and microscopically haemorrhages were evident in the kidneys and adrenal glands. There were multiple foci of necrosis and cellular response with intranuclear inclusions in perivascular cells in the kidneys and adrenal glands (Cutlip and McClurkin, 1975; Bulmer et al., 1975).

The clinical signs included in the naturally occurring infection of newborn calves with bovine Adenovirus type 3 were excessive occular and nasal discharge, tympanitis, colic and diarrhoea (Mattson, 1973), dyspnoea, diarrhoea with pyrexia; at necropsy, areas of consolidation of lungs, lymph-adenitis were reported (Darbyshire et al., 1965).

Experimental infection of three, third trimester foetuses with Adenovirus type 7 associated with weak calf syndrome resulted in preterm delivery of clinically ill-calves characterised by weakness, severe depression and inability to stand and nurse. Postmortem lesions were petichael and echymotic haemorrhages and oedema of the gastro-intestinal tract. Histological lesions included necrosis of the mucosa of the stomach, acute non-suppurative focal necrosis of liver, kidney and adrenal glands (Stauber et al., 1976; Stauber and Card, 1978).

(ii) Incidence: Weak calf syndrome, a disease condition of neonatal calves was first observed in 1964 in the Bitterroot Valley of Montana (Ivanoff and Renshaw, 1975).

Study of naturally occurring infection of newborn calves over a period of 4 years in Oregon had revealed that the causative agent was a bovine Adenovirus type 3 (Mattson, 1973). More recently weak calf syndrome (WCS) was observed in Idaho, Montano (McClurkin and Coria, 1975), Urah, Oregon, Colorado and Nevada affecting 20 per cent of the calves (Ivanoff and Renshaw, 1975) causing morbidity in 6 to 15 per cent of the calves in a herd and mortality 60 to 80 per cent of the untreated calves (Outlip and McClurkin, 1975).

A viral agent designated Idaho-1 (Id-1) was isolated from the buffy coat of a calf with weak calf syndrome, resembled Adenovirus type 7 (Stauber et al., 1976). Experimental infection of calves with bovine Adenovirus type 5 isolated from weak calf syndrome closely resembled lesions seen in naturally occurring weak calf syndrome of Eastern Idaho and South-Western Montana (Cutlip and McClurkin, 1975; McClurkin and Coria, 1975). Isolation of Adenovirus from scouring and ill-thrifty calves in New Zealand had been reported (Thompson, 1977).

(iii) Age and breed: Serum samples from 919 (51.8%) of the 1,771 grazing cattle were positive for Adenovirus type 6 infection(Sato et al., 1969). Naturally occurring infection of newborn calves in a large beef herd which was due to bovine Adenovirus type 3 had affected calves between 1 to 4 week old (Mattson, 1973). Observation on two week old calves with pyrexia, diarrhoea and subsequent dehydration had revealed that the causative agent was a bovine Adenovirus (Bulmer et al., 1975). Calves became affected with weak calf syndrome at birth to 10 days of age (Cutlip and McClurkin, 1975) had developed diarrhoea a few days after birth (McClurkin and Coria, 1975), most calves had shown clinical signs at birth and the majority had signs between 3 and 7 days

of age (Ivanoff and Renshaw, 1975). Observation of Adenovirus infection in yearlings of Friesian heifers were reported in New Zealand (Thompson, 1977).

(iv) Seasons: The study of Mattson (1973) on infection of newborn calves over a period of 4 years had revealed that the disease began during the calving season and the incidence washigh in calves during epizootics.

The percentage of weak calf syndrome affected calves were high during inclement weather conditions (Ivanoff and Renshaw, 1975) and cold and wet weather were believed to influence the occurrence and severity of the disease complex (McClurkin and Coria, 1975).

(v) Characteristics of aetiological agents.

Adenoviruses

The virus isolated from infection of newborn calves was serologically identical to bovine Adenovirus type 5, strain WBRI (Mattson, 1973). The virus isolated from calves with naturally occurring weak calf syndrome and experimentally induced illness was identified by neutralisation technique using homologous serum on Adenovirus type 5. Necropsy findings indicated multiple intranuclear inclusions in the interstitial cells of lamina propria of duodenum (McClurkin and Coria, 1975; Cutlip and McClurkin, 1975), numerous large

amphophilic intranuclear inclusions in endothelial cells of blood vessels in abomasum, endothelial cells of adrenal cortical sinusoids and glomeruli (Bulmer et al., 1975; Stauber and Card, 1978). The viral agent isolated from the buffy coat of a calf suffering from weak calf syndrome, on culture on bovine salivary gland caused a CPE characterised by rounding and clumping of cells. Stained preparations of infected monolayers revealed multiple intranuclear inclusions, resistant to chloroform, ether and sensitive to a temperature of 70°C and was neutralised by Adenovirus type 7 antiserum, and the genome consisted of DNA (Stauber et al., 1976).

3. Calf mortality

Calf mortality is an important cause of economic loss and the death of young calves may markedly increase the production costs of both dairy and dairy-beef products. Though factors such as management and housing, adverse climatic conditions and/or nutritional status of the calf are attributed to the cause of morbidity and mortality, microbial agents play a vital role in causing calf mortality by causing infection of either respiratory or alimentary system or both. Among the microbial agents the most common virus that affected respiratory system causing great loss was Infectious Bovine Rhinotrachitis (IBRT) virus (Bartha et al., 1974) and

infection of parainfluenza virus 3 (P1-3) (Zebrowski et al., 1973). The most common virus that affect enteric system other than reo-like and Coronavirus which are cited elsewhere were Bovine Viral Diarrhoea (BVD) virus (Lambert and Fernelius, 1968; Nilakantan, 1963) and Enteroviruses (Dunne et al., 1974).

Adenovirus affected both respiratory and alimentary system causing pneumoenteritis (Darbyshire et al., 1965; Mattson, 1973).

(i) History and disease syndrome: Severe loss in herds with febrile disease with respiratory symptoms caused by Bovine Rhinotracheitis (IBR) were reported (Bartha et al., 1974). Death of 15 animals were reported in an outbreak due to IBR (Gibs et al., 1975) in Cyprus. The disease was characterised by fever upto 41°C to 42°C, listlessness, serous nasal discharge, foamy salivation, dyspnoea, dry cough (Bartha et al., 1974), lacrimation, rhinitis (Grandel, 1974), attaxia, blindness, respiratory symptoms and kerato-conjunctivitis (Gibs et al., 1975).

Outbreaks of diarrhoea in a herd caused 50 per cent morbidity with 20 per cent mortality in Brazil and the causative agent was Bovine viral diarrhoea virus (Correa et al., 1972), viral diarrhoea affected 85 of 170 calves causing death of 47

calves (Kharalambiev et al., 1976). The disease syndrome included were rise of temperature upto 40°C, loss of appetite, nasal discharge, blood stained faeces, mild enteritis, ulceration of oral mucosa (Sapre, 1962; Nilakantan, 1963), leucopenia (Parnaik et al., 1964), loss of appetite, profuse diarrhoea (Schipper, 1973, Correa et al., 1972), erosions of the digestive tract (Lambert et al., 1974). The viral aetiology was shown by isolating a cytopathogenic virus neutralised by serum against Bovine diarrhoea virus (Kharalambiev et al., 1976) and by necropsy findings (Correa et al., 1972).

Calves dying from diarrhoea was reported to be due to bovine Enterovirus which was isolated in bovine kidney cultures (Krasnikov and Belokonov, 1970).

Experimental infection of bovine Enterovirus into colostrum deprived calves had resulted in the death of calves (Dunne et al., 1974), the symptoms included were diarrhoea, fever and leucopenia (Dunne et al., 1974).

(ii) Incidence: A febrile disease due to Infectious Bovine Rhinotracheitis causing severe loss in herds had been reported to have occurred in Hungary (Bartha et al., 1974) in Cyprus (Gibs et al., 1975).

There were outbreaks of viral diarrhoea in herds in Sao Paulo State causing morbidity of 50 percent with 20 per cent mortality (Correa et al., 1972). Virus diarrhoea affected 85 of 170 calves causing death of 47 calves (Kharalambiev et al., 1976).

In India the disease similar to the mucosal disease as observed in U.S.A., Australia, England, France, Germany, Sweden, Denmark, etc. had been reported to cause mortality of 86 per cent in young stock in Uttar Pradesh, Madhya Pradesh, Maharashtra, Himachala Pradesh (Nilakantan, 1963). A disease similar to British mucosal disease had been reported in a farm in Bombay (Parnaik et al., 1964).

There were disease outbreaks in cattle within Poland and Enterovirus was isolated from 20-70 per cent of faecal samples (Zebrowski et al., 1973).

(iii) Age and breed: Severe loss in herds of 1 to 6 months old rearing calves due to IBR had been reported in Hungary, affecting less often growing cattle and breeder cows (Bartha et al., 1974) and beef lots in Cyprus (Gibs et al., 1975).

Mucosal disease affected cattle and buffaloes in all age groups (Sapre, 1962), the disease was severe in 4 to 12 month old calves causing 86 per cent mortality than in adult cattle

20.9 per cent (Nilakantan, 1963), 3-6 months old calves were affected by Bovine Viral Diarrhoea Virus (Kharalambiev et al., 1976).

Experimental infection of Bovine Enterovirus in the one day old to 8 week old calves deprived of colostrum resulted in death of 50 per cent of the calves (Dunne et al., 1974).

(iv) Season: In Sao Paulo State the 8 outbreaks of viral diarrhoea in cattle herds had occurred in September and October 1971 causing 20 per cent mortality (Correa et al., 1972). Time series studies indicated that the death loss in calves increased in winter months being 20 per cent than in summer (Martin et al., 1975).

(v) Characteristics of aetiologic agents.

A. Infectious Bovine Rhinotracheitis: Infectious Bovine Rhinotracheitis virus (IBR) was propagated in culture of calf kidney or rabbit spleen cells (Armstrong et al., 1961), in bovine kidney cells (Gillespie et al., 1957; Darbyshire et al., 1962). The virus produced characteristic cytopathogenic effect (CPE) with intranuclear inclusion of Cowdry type A (Armstrong et al., 1961); eosinophilic inclusions was reported by Darbyshire et al., (1962). The virus was ether sensitive, in tissue cultures intranuclear virus particle each with one membrane with a diameter of 115 to 150 mu were observed

(Armstrong et al., 1961; Darbyshire et al., 1962), DNA virus, multiplied in the nucleus and matured by budding at the nuclear membrane (Fenner et al., 1974b).

B. Parainfluenza - 3 had been reported to be having lipoprotein envelope 100 to 300 nm in diameter, with specific haemagglutinin and neuraminadase. The virus, a RNA virus, multiplied in cytoplasm and matured in the cytoplasmic membrane or cytoplasm (Fenner et al., 1974c). The virus was shown to grow in bovine kidney cell cultures (Reisinger et al., 1959) and shown to possess haemadsorption property to red cells of guinea pigs, cattle, human and avian (Andrewes and Pereira, 1972).

C. Bovine Viral Diarrhoea virus: As per detailed study of the virus, the virus was reported to be RNA virus, sensitive to lipid solvents and trypsin; approximately 40 nm in diameter. The virus was ether sensitive and spherical and possessed envelope with surface projection (Galiv et al., 1978). The virus was propagated in bovine kidney cells which produced cytopathogenic effect (CPE) in 6 to 8 days, the affected cells became rounded and gradually detached from the sheet (Gillespie et al., 1957).

D. Bovine Enterovirus: Enteroviruses were RNA virus, the size of the viral particle were 22-25 nm in diameter, inactivated at 50°C but stabilised by molar Mg Cl_2, insensitive to the action of chloroform, acid pH (Raghavan and Datt, 1975; Taylor et al., 1974; Mattson and Reed, 1974; Durham and Burgess, 1977). Enterovirus were readily propagated in bovine kidney cells with ease causing of rounding of cells and detachment from the sheet (Datt and Raghavan, 1969).

Chapter III

COLLECTION OF SAMPLES AND PROCESSING IN TISSUE CULTURE FOR VIRUS ISOLATIONS

1. Introduction

With the recent advances in cell culture technology and its increasing use in virological field, it had become possible to uncover a wide variety of viruses affecting animals and also to undertake systematic characterization of the viruses. This had been particularly so in the case of viruses inhabiting the respiratory and alimentary tract of animals. Information on certain obscure types of respiratory/alimentary ailments in animals had been forthcoming by isolation of viruses in tissue culture system since the use of kidney and lung cultures of appropriate host species were said to be ideal for isolation of respiratory/entero viruses (Datt and Raghavan, 1969). Viruses that produce visible and characteristic cytopathogenic effect (CPE) in cell cultures like Adenovirus (Datt and Raghavan, 1969; Stauber et al., 1976), Entero, Rhino viruses (Datt and Raghavan, 1969), Infectious Bovine Rhinotracheitis (IBR) virus (Gillespie et al., 1957; Armstrong et al., 1957), Bovine Viral Diarrhoea (BVD) virus (Gillespie et al., 1957) and other CPE producing viruses were isolated, purified and characterised by

different titrations, assays in tissue culture systems like bovine kidney, testis, foetal bovine kidney and lung cells (Datt and Raghavan, 1969), salivary glands (Stauber et al., 1976) and other susceptible cell lines.

In the present work, calf kidney cell cultures were used for isolation and further studies with materials collected from ailing and apparently healthy calves.

2. Materials and Method

i) Collection of samples: Rectal swabs and throat swabs were collected in accordance with the procedure adopted by Datt and Raghavan (1969) and Masillamony (1972) with slight modifications.

Rectal swabs and throat swabs were collected from calves generally below 3 months of age that were ailing from respiratory/enteric affections as well as from calves that were apparently healthy. The materials were collected from different farms in the State during different seasons of the year.

ii) Method of collection.

A. Rectal swab: Specially designed glass tampon tubes measuring 20 cm by 1 cm to hold 13 ml of the medium was used for collecting rectal swabs. Cotton swabs were made at one end of a aluminium rod measuring 28 cm forked at the tip for keeping

the swab in position while collecting the sample. The rectal samples were collected by inserting the sterile absorbent tampon moistened with serum-free maintenance medium (MM) sufficiently inside the rectal wall avoiding dung mass with a gentle rotatory action. The swabs were collected in a tampon tube containing 5 ml serum-free maintenance medium and placed at 4°C for 20 minutes and then squeezed with sterile forceps. The extracted fluids were transferred to sterile tubes which were transported to the laboratory by liquid nitrogen container at -196°C. The samples were centrifuged at 3000 rpm for 30 minutes at 4°C and the supernatant collected, labelled and stored at -20°C for further processing.

B. Throat swab: By carefully securing the calf, throat swabs were collected by inserting the swab well inside the pharyngeal region without touching the tongue and upper palate and immediately soaked in 5 ml of the serum-free maintenance medium (MM) which was transferred to the tampon at the time of collection. After a brief storage at 4°C the swabs were squeezed with forceps and fluid transferred to sterile tubes. These samples were transported in a liquid nitrogen container at -196°C and the swab extracts were centrifuged at 3000 rpm for 30 minutes at 4°C and the supernatant collected, labelled and stored at -20°C for further processing.

iii) Primary calf kidney cell culture.

A. Kidney dissection: Healthy male calves less than 15 days old brought on the day prior to culturing kidney cells were slaughtered and kidneys were collected in a sterile beaker containing chilled Hanks' BSS (Basal salt solution) with antibiotics. The kidneys were taken to the tissue culture laboratory provided with laminar flow. After removing the renal fat and capsule, the kidneys were washed twice or thrice with Hanks' buffer. The fragments of kidney cortex alone were dissected by means of sterile scissors and placed in a sterile 250 ml beaker containing Banks' BSS. The tissue was minced into small pieces with the help of scissors, washed 3 or 4 times and finally transferred to a trypsinisation flask containing magnetic pellet (Parker, 1961).

B. Cell dispersion: In general multiple extraction procedure of Tyrrell et al., (1960) was followed. Sufficient quantity of prewarmed 0.25 per cent trypsin solution at pH 7.8 containing antibiotics was added to the trypsinising flask containing minced kidney tissue and agitated for 15 minutes at 37°C using a magnetic stirrer geared to about 200 rpm and the supernatant was discarded to remove dead cells and toxic factors. Fresh trypsin solution was added and trypsinised for 45 minutes at 37°C. The supernatant cell suspension was

collected in a beaker. The procedure was repeated and the cell suspension pool was filtered through sterile muslin cloth to remove sediments and coarse particles. The filtrate was transferred to 30 ml centrifuge tube and centrifuged in a refrigerated centrifuge at 600 rpm for 10 minutes. The supernatant was discarded and the sedimented cell clumps were vigorously pipetted with a few ml of growth medium. The cells were diluted with growth medium containing 10 per cent inactivated goat serum, antibiotics, Penicillin 500 I.U., Streptomycin 250 ug and Mycostatin 50 units per ml (Datt and Raghavan, 1969) to contain 1.5 to 3 x 10^5 cells per ml.

C. Standardisation of cell suspension: Cell suspensions were pooled in 10 ml of growth medium, 1 ml of cell suspension was taken to which 2 ml of citric acid-crystal violet solution was added, mixed and kept at room temperature for 15 minutes. This stained cell suspension was carefully filled in both chambers of haemocytometer and cells were counted in W.B.C. counting chamber. Cells with intact boundaries with clear nucleus were counted and cell clumps were counted as one cell and the number of cells calculated as follows.

$\frac{\text{Number of cells in 4 corner squares}}{4}$ X 10000 x 3 = number of cells per ml of pooled suspension.

The suspension was suitably diluted to contain 1.5 to 3 x 10^5 cells per ml (Merchant et al., 1964).

D. Seeding of cells in culture tubes and bottles: The diluted suspension was mixed uniformly and 1 ml transferred to culture tubes and 10 ml to culture bottle, plugged with sterile rubber cork and kept in slanting position in racks and trays and placed in the incubator at 37°C.

E. Change of growth medium: Depending on the rate of growth and rapidity of fall of pH, fresh growth medium was added after 4 or 5 days and incubated at 37°C.

F. Change to maintenance medium: One to two days after the change of growth medium when confluent sheet had formed growth medium was replaced by serum-free maintenance medium pH 7.8 to 8 and kept for one day before inoculation of the samples.

iv). Inoculation of sample and virus isolation technique: 0.2 ml of each sample was inoculated into 4 tubes with good sheet leaving one tube as control and incubated at 37°C. Each day the tubes were examined for characteristic cytopathogenic effect (CPE) for 14 days. On the day when the tubes that

showed a 4+ CPE were harvested and stored at -20°C. The samples that did not show any CPE were harvested after 14th day and frozen. The tubes were frozen and thawed three times to release the virus from the cells. Each sample was pooled and centrifuged at 2000 to 3000 rpm for 10 minutes, the supernatant was collected, labelled as first passage and stored at -20°C.

The first passage samples irrespective of whether they showed CPE or not were passed for a second time in calf kidney cell cultures to rule out negatives and to establish positives. The negative ones were discarded and the positive ones were harvested and given an isolate number and stored at -20°C for further characterisation.

3. Results and Discussion

From the nature of CPE produced by the agents in cell culture it was possible to classify the agents broadly into groups (Datt and Raghavan, 1969). Three types of CPE were recognised in the present study.

A. Adenovirus Type CPE: It was characterised by refractile rounded cells, appearing on the 2nd day and forming clusters and bunches in 24 hours to 48 hours and occupying the whole sheet in 48 to 72 hours, resulting in falling off the cells thereafter.

B. Enterovirus Type CPE: The changes start appearing by 24-48 hours as small refractile round cells occurring individually strewn all over the sheet. In 48-72 hours the whole sheet was observed with these cells. In some areas, destruction of sheet was observed which progressed to the entire sheet resulting in falling off the sheet from the glass.

C. Rotavirus Type of CPE: This type of CPE was observed to occur in 36-72 hours, also occurring as refractile rounded cells individually followed by the appearanceof granularity in the cell sheet with destruction of sheet from 48-72 hours onwards. Some of the cells were seen floating with long processes attaching to the glass surface. The entire sheet gave a moth-eaten appearance (McNulty et al., 1976b).

Two hundred rectal and throat swabs collected from calves generally below 3 months of age, from different farms in the State, in different periods were screened in calf kidney tissue culture for the presence of viruses. Particulars regarding the samples collected were tabulated chronologically, giving a separate serial number. Place of collection, breed, sex, age and condition of the animal at the time of collection were obtained carefully and furnished in Table 1.

Table 1

Particulars of Samples Collected and Processed in Tissue Culture

S.No 1.	Date of collection 2.	Place of collection 3.	Specimen number 4.	Animal No. 5.	Breed 6.	Sex 7.	Age 8.	Kind of sample RS/TD/DS 9.	Condition of the animal 10.	Types of TC cytopathogenic agents 11.
1.	26.8.78	Model Dairy, Madras	A001	MD 65	Cross	BC	3 M	RS	DIA, DE	Enterovirus
2.	do		A002	MD 67	do	HC	2½ "	RS	SEMI, GD	Enterovirus
3.	do	Vety College	A003	MD 68	do	BC	1½ "	RS	GD	Enterovirus
4.	do		A004	MD 69	do	HC	1 "	RS	GD	Enterovirus
5.	17.11.78	Large Animal Clinic, Madras	A005	LAC 11704	Buffalo	HC	3 "	RS	DIA, DE RESP	-
6.	do		A006	do	do	HC	3 "	TS		-
7.	do	Vety College	A007	LAC 11477	do	HC	10 "	RS	DIA, DE	-
8.	do	,,	A008	do	do	HC	10 "	TS		-
9.	do	,,	A009	LAC 11630	Cross bred	BC	4 "	RS	DIA, DE	-
10.	do	,,	A010	do	do	BC	4 "	TS		-
11.	do	,,	A011	LAC 12010	do	BC	8 "	RS	RESP	-
12.	do	,,	A012	do	do	BC	8 "	TS		-
13.	do	,,	A013	LAC 11931	do	BC	1½ "	RS	DIA, DE	-
14.	do	,,	A014	do	do	BC	1½ "	TS		-
15.	do	,,	A015	LAC 12135	Cross	HC	1 "	RS	DE	-
16.	do	,,	A016	do	do	HC	1 "	TS		-
17.	do	,,	A017	LAC 12114	do	HC	3 "	RS	DE, RESP	-
18.	do	,,	A018	do	do	HC	3 "	TS		-

Table 1 (contd)

S.No 1.	Date of collection 2.	Place of collection 3.	Specimen number 4.	Animal No. 5.	Breed. 6.	Sex 7.	Age 8.	Kind of sample RS/TD/DS 9.	Condition of the animal 10.	Types of TC cytopathogenic agents 11.
19.	30.1.79	District Livestock Farm, Pudukottai.	A019	T 939	Jersey cross	BC	3 M	RS	GD	-
20.	do		A020	do	do	BC	3 "	TS		-
21.	do		A021	T 940	Buffalo	BC	2½ "	RS	SOL, GD	-
22.	do	,,	A022	do	do	BC	2½ "	TS		Adenovirus
23.	do	,,	A023	T941	do	BC	2½ "	RS	SEMI, GD	Adenovirus
24.	do	,,	A024	do	do	BC	2½ "	TS		-
25.	do	,,	A025	T 944	do	HC	2 "	RS	DIA, GD	-
26.	do	,,	A026	do	do	HC	2 "	TS		-
27.	do	,,	A027	T 945	do	HC	2½ "	RS	SEMI, DE	-
28.	do	,,	A028	do	do	HC	2½ "	TS		-
29.	do	,,	A029	T 953	do	BC	2 "	RS	SOL, GD	-
30.	do	,,	A030	do	do	BC	2 "	TS		-
31.	do	,,	A031	T 956	do	BC	2 "	RS	SOL, GD	-
32.	do	,,	A032	do	do	BC	2 "	TS		-
33.	do	,,	A033	T 957	Jersey cross	BC	2½ "	RS	DIA, DE	-
34.	do	,,	A034	do	do	BC	2½ "	TS		-
35.	do	,,	A035	T 9	do	HC	2 "	RS	DE	Enterovirus
36.	do	,,	A036	do	do	HC	2 "	TS		-
37.	do	,,	A037	T 966	Buffalo	HC	1 "	RS	SOL, GD	-
38.	do	,,	A038	do	do	HC	1 "	TS		-

Table 1 (contd)

S.No. 1.	Date of collection 2.	Place of collection 3.	Specimen number 4.	Animal No. 5.	Breed 6.	Sex 7.	Age 8.	Kind of sample RS/TD/DS 9.	Condition of the animal 10.	Types of TC cytopathogenic agents 11.
39.	30.1.79	District Livestock Farm, Pudukottai.	A039	T 942	Buffalo	HC	3 M	RS	SOL, GD	Enterovirus
40.	do		A040	T 949	Jersey	BC	1½ "	RS	SEMI, GD	Adenovirus
41.	do		A041	T 954	Buffalo	HC	2 "	RS	SOL, GD	Adenovirus
42.	do	,,	A042	T 963	Jersey cross	HC	1½ "	RS	DIA, DE	Adenovirus
43.	do	,,	A043	do	do	HC	1½ "	TS		Adenovirus
44.	do	,,	A044	T 967	Buffalo	HC	1 "	RS	SOL, GD	Enterovirus
45.	do	,,	A045	T 972	do	HC	1 "	TS	Mushy, GD	-
46.	do	,,	A046	T 969	Jersey cross	HC	1 "	TS	SOL GD	-
47.	do	,,	A047	do	do	HC	1 "	RS		-
48.	do	,,	A048	T 981	do	HC	15 D	TS	SOL DE	-
49.	do	,,	A049	do	do	HC	15 "	RS		-
50.	do	,,	A050	T 975	Buffalo	BC	1 M	TS	SOL, GD	-
51.	do	,,	A051	do	do	BC	1 "	RS		-
52.	do	,,	A052	T 978	Jersey cross	BC	1 "	TS	SOL, GD	-
53.	do	,,	A053	do	do	BC	1 "	RS		-
54.	do	,,	A054	T 976	Buffalo	HC	1 "	TS	SEMI, GD	-
55.	do	,,	A055	do	do	HC	1 "	RS		-
56.	do	,,	A056	T 980	Jersey cross	HC	15 D	TS	DIA, Fair	-
57.	do	,,	A057	do	do	HC	15 "	RS		-

Table 1 (contd)

S.No. 1.	Date of collection 2.	Place of collection 3.	Specimen number 4.	Animal No. 5.	Breed 6.	Sex 7.	Age 8.		Kind of sample RS/TD/DS 9.	Condition of the animal 10.	Types of TC cytopathogenic agents 11.
58.	16.3.79	Private Dairy Units, Coimbatore.	A058	B1	Cross bred	BC	15	D	TS	Mushy, Fair	-
59.	do		A059	do	do	BC	15	"	RS		-
60.	do		A060	B2	do	BC	20	"	TS	SEMI, GD	-
61.	do	,,	A061	B3	do	BC	21	"	RS	GD	-
62.	do	,,	A062	B5	do	BC	2½	M	TS	SOL, GD	-
63.	do	,,	A063	B7	do	HC	22	D	RS	GD	-
64.	do	,,	A064	V1	do	HC	2½	M	RS	SOL, GD	-
65.	do	,,	A065	do	do	HC	2½	"	TS		-
66.	do	,,	A066	V2	do	HC	2½	"	TS	SOL, GD	-
67.	do	,,	A067	do	do	HC	2½	"	RS		-
68.	do	,,	A068	V3	do	BC	25	D	TS	DIA, DE, RESP	-
69.	do	,,	A069	do	do	BC	25	"	RS		-
70.	do	,,	A070	V4	do	BC	20	"	TS	SOL, GD	-
71.	do	,,	A071	do	do	BC	20	"	RS		-
72.	do	,,	A072	J1	do	HC	2	M	TS	SOL, GD	-
73.	do	,,	A073	do	do	HC	2	"	RS		-
74.	29.7.79.	Government Dairy Farm, Madhavaram	A074	K111	Cross bred	HC	3	"	TS	SOL, GD	-
75.	do		A075	do	do	HC	3	"	RS		-
76.	do	,,	A076	K114	do	HC	3	"	TS	SOL, GD	-
77.	do	,,	A077	do	do	HC	3	"	RS		-

Table 1 (contd)

S.No 1.	Date of collection 2.	Place of collection 3.	Specimen number 4.	Animal No. 5.	Breed 6.	Sex 7.	Age 8.	Kind of sample RS/TD/DS 9.	Condition of the animal 10.	Types of TC cytopathogenic agents 11.
78.	29.7.79.	Govt. Dairy Farm	A078	K115	Cross bred	HC	3 M	TS	DIA, GD	-
79.	do	Madavaram	A079	do	do	HC	3 "	RS		
80.	do	,,	A080	K118	do	HC	2½ "	TS	SOL, GD	-
81.	do	,,	A081	do	do	HC	2½ "	RS		Rotavirus
82.	do	,,	A082	K119	do	HC	2½ "	TS	SEMI, GD	-
83.	do	,,	A083	do	do	HC	2½ "	RS		-
84.	do	,,	A084	N119	do	BC	9 D	TS	Mushy, W	-
85.	do	,,	A085	do	do	BC	9 "	RS		-
86.	do	,,	A086	K122	do	HC	2½ M	TS	GD	-
87.	do	,,	A087	do	do	HC	2½ "	RS		-
88.	do	,,	A088	K124	do	HC	2½ "	TS	SOL, GD	-
89.	do	,,	A089	do	do	HC	2½ "	RS		-
90.	do	,,	A090	K125	do	HC	2½ "	TS	SOL, GD	-
91.	do	,,	A091	do	do	HC	2½ "	RS		-
92.	do	,,	A092	K123	do	HC	2½ "	TS	SOL, GD	-
93.	do	,,	A093	do	do	HC	2½ "	RS		-
94.	do	,,	A094	K124	do	HC	2½ "	TS	SOL, GD	-
95.	do	,,	A095	do	do	HC	2½ "	RS		-
96.	do	,,	A096	K127	do	HC	2 "	TS	SOL, GD	-
97.	do	,,	A097	do	do	HC	2 "	RS		-

Table 1 (contd)

S.No. 1.	Date of collection. 2.	Place of collection. 3.	Specimen number 4.	Animal No. 5.	Breed 6.	Sex 7.	Age 8.	Kind of sample RS/TD/DS 9.	Condition of the animal 10.	Types of TC cytopathogenic agents 11.
98.	29.7.79.	Govt. Dairy Farm Madhavaram.	A098	K133	Cross bred	HC	2 M	TS	SOL,GD	-
99.	do		A099	do	do	HC	2 "	RS		-
100.	do		A100	DN200	do	BC	7 D	TS	Mushy, DE	-
101.	do	,,	A101	do	do	BC	7 "	RS		-
102.	do	,,	A102	DN228	do	HC	7 "	TS	Mushy, DE	-
103.	do	,,	A103	do	do	HC	7 "	RS		-
104.	do	,,	A104	DN279	do	HC	20 "	TS	Mushy, DE	-
105.	do	,,	A105	do	do	HC	20 "	RS		-
106.	do	,,	A106	E494	do	HC	15 "	TS	SOL, GD	-
107.	do	,,	A107	do	do	HC	15 "	RS		-
108.	do	,,	A108	B803	Buffalo	BC	1 M	TS	Mushy, GD	-
109.	do	,,	A109	do	do	BC	1 "	RS		-
110.	do	,,	A110	DX850	Cross bred	HC	15 D	RS	SOL, GD	-
111.	20.2.79	Central Cattle Breeding Farm, Alamadi	A111	1/79	Buffalo	HC	1½ M	TS	DIA,GD	-
112.	do		A112	do	do	HC	1½ "	RS		Rotavirus
113.	do		A113	2/79	do	HC	1½ "	TS	SEMI,GD	-
114.	do		A114	do	do	HC	1½ "	RS		-
115.	do	,,	A115	4/79	do	BC	1½ "	RS	SEMI, GD	-
116.	do	,,	A116	13/79	do	BC	20 D	TS	GD	-
117.	do	,,	A117	do	do	BC	20 "	RS		-

Table 1 (contd)

S.No. 1.	Date of collection 2.	Place of collection. 3.	Specimen number 4.	Animal No. 5.	Breed. 6.	Sex. 7.	Age. 8.	Kind of sample RS/TD/DS 9.	Condition of the animal 10.	Types of TC cytopathogenic agents 11.
118.	20.2.79	Central Cattle Breeding Farm, Alamadi.	A118	14/79	Buffalo	HC	20 D	TS	GD	-
119.	do		A119	do	do	HC	20 "	TS	GD	-
120.	do		A120	17/79	do	HC	13 "	RS	GD	-
121.	do		A121	21/79	do	HC	10 "	TS	DIA,DE	-
122.	do	,,	A122	22/79	do	HC	8 "	RS	DIA, DE	-
123.	do	,,	A123	23/79	do	HC	7 "	RS	SEMI,GD	-
124.	do	,,	A124	24/79	do	BC	3 "	RS	SEMI,GD	-
125.	21.3.79	Govt.Dairy Farm, Madhavaram	A125	K143	Cross bred	HC	2 M	TS	DIA, DE	-
126.	do		A126	do	do	HC	2 "	RS		Rotavirus
127.	do	,,	A127	K145	do	HC	1½""	TS	DIA, DE	-
128.	do	,,	A128	do	do	HC	1½ "	RS		-
129.	29.6.79	District Livestock Farm,Orathanad.	A129	0.514	Jersey cross	BC	3 M	TS	DIA,DE	-
130.	do		A130	do	do	BC	3 "	RS		Adenovirus
131.	do		A131	0.515	Sindhi	HC	3 "	TS	SOL,DD	-
132.	do	,,	A132	do	do	HC	3 "	RS		Rotavirus
133.	do	,,	A133	0.516	Buffalo	HC	3 "	TS	SEMI,GD	-
134.	do	,,	A134	do	do	HC	3 "	RS		-
135.	do	,,	A135	0.517	Brown Swiss	HC	3 "	TS	SOL, W	-
136.	do	,,	A136	do		HC	3 "	RS		-

Table 1 (contd)

S.No. 1.	Date of collection. 2.	Place of collection. 3.	Specimen number 4.	Animal No. 5.	Breed. 6.	Sex. 7.	Age. 8.	Kind of sample RS/TS/DS 9.	Condition of the animal 10.	Types of TC cytopathogenic agents 11.
137.	29.6.79	District Livestock Farm, Orathanad.	A137	0.519	Jersey cross	BC	3 M	TS	SOL, GD	-
138.	do		A138	do	do	BC	3 "	RS		-
139.	do		A139	0.521	Buffalo	HC	3 "	TS	SRMI, GD	-
140.	do	,,	A140	do	do	HC	3 "	RS		-
141.	do	,,	A141	0.529	Jersey cross	BC	2½ "	TS	SOL, GD	-
142.	do	,,	A142	do	do	BC	2½ "	RS		Enterovirus
143.	do	,,	A143	0.531	do	HC	2½ "	TS	CON, GD	-
144.	do	,,	A144	do	do	HC	2½ "	RS		-
145.	do	,,	A145	0.535	do	HC	2 "	TS	SOL, GD	Enterovirus
146.	do	,,	A146	do	do	HC	2 "	RS		-
147.	do	,,	A147	0.538	Sindhi	BC	2 "	TS	GD	-
148.	do	,,	A148	do	do	BC	2 "	RS		-
149.	do	,,	A149	0.539	do	BC	2 "	TS	GD	-
150.	do	,,	A150	do	do	BC	2 "	RS		-
151.	do	,,	A151	0.540	do	BC	1½ "	TS	GD	-
152.	do	,,	A152	do	do	BC	1½ "	RS		-
153.	do	,,	A153	0.543	Jdrsey cross	HC	1½ "	TS	DIA, DE	-
154.	do	,,	A154	do	do	HC	1½ "	RS		-
155.	do	,,	A155	0.543	Buffalo	BC	1½ "	TS	SOL, GD	Adenovirus
156.	do	,,	A156	do	do	BC	1½ "	RS		Rotavirus

Table 1 (contd)

S.No 1.	Date of collection. 2.	Place of collection. 3.	Specimen number 4.	Animal No. 5.	Breed. 6.	Sex. 7.	Age 8.	Kind of sample RS/TS/DS 9.	Condition of the animal 10.	Types of TC cytopathogenic agents 11.
157.	29.6.79	District Livestock Farm, Orathanad.	A157	0.544	Sindhi	HC	1 M	TS	SOL, GD	Enterovirus
158.	do		A158	do	do	HC	1 "	RS		Enterovirus
159.	do		A159	0.545	Buffalo	BC	1 "	TS	SOL, GD	-
160.	do	,,	A160	do	do	BC	1 "	RS		Enterovirus
161.	do	,,	A161	0.547	Sindhi	HC	1 "	TS	CONS, GD	Adenovirus
162.	do	,,	A162	do	do	HC	1 "	RS		-
163.	do	,,	A163	0.548	Buffalo	HC	25 D	TS	SOL, GD	-
164.	do	,,	A164	do	do	HC	25 "	RS		-
165.	do	,,	A165	0.549	Jersey cross	BC	25 "	TS	SOL, GD	-
166.	do	,,	A166	do	do	BC	25 "	RS		Adenovirus
167.	do	,,	A167	0.550	Sindhi	BC	25 "	TS	CONS, GD	-
168.	do	,,	A168	do	do	BC	25 "	RS		Enterovirus
169.	do	,,	A169	0.551	do	HC	20 "	TS	GD	-
170.	do	,,	A170	do	do	HC	20 "	RS		Adenovirus
171.	do	,,	A171	0.552	do	BC	20 "	TS	DIA, DE	-
172.	do	,,	A172	do	do	BC	20 "	RS		-
173.	do	,,	A173	0.553	do	HC	15 "	TS	DIA, DE	Adenovirus
174.	do	,,	A174	do	do	HC	15 "	RS		-
175.	do	,,	A175	0.554	do	BC	8 "	TS	Mushy, DE	-
176.	do	,,	A176	do	do	BC	8 "	RS		-

Table 1 (contd)

S.No 1.	Date of collection. 2.	Place of collection 3.	Specimen number 4.	Animal Number 5.	Breed. 6.	Sex. 7.	Age. 8.	Kind of sample RS/TS/DS 9.	Condition of the animal 10.	Types of TC cytopathogenic agents 11.
177.	31.5.79	Central Cattle Breeding Farm, Alamadhi	A177	68/79	Buffalo	BC	1½ M	TS	GD	-
178.	do		A178	do	do	BC	1½ "	RS		-
179.	do		A179	75/79	do	BC	1 "	TS		-
180.	do		A180	do	do	BC	1 "	RS		-
181.	do	,,	A181	76/79	do	BC	20 D	RS	SOL,GD	-
182.	do	,,	A182	77/79	do	HC	20 "	TS	GD	-
183.	do	,,	A183	78/79	do	BC	8 "	TS	GD	-
184.	do	,,	A184	do	do	BC	8 "	RS		-
185.	do	,,	A185	81/79	do	HC	9 "	TS	GD	-
186.	do	,,	A186	do	do	HC	9 "	RS		-
187.	7.1.80	Research Station, Kattupakkam.	A187	MB95	do	BC	1 M	TS	Mushy, W	-
188.	do		A188	do	do	BC	1 "	RS		-
189.	do		A189	MB93	do	BC	1 "	TS	DIA,W	-
190.	do	,,	A190	do	do	BC	1 "	RS		-
191.	do	,,	A191	MB894	do	BC	1 "	TS	DIA,W	-
192.	do	,,	A192	do	do	BC	1 "	RS		-

Table 1 (contd)

S.No. 1.	Date of collection. 2.	Place of collection. 3.	Specimen number 4.	Animal No. 5.	Breed. 6.	Sex. 7.	Age. 8.	Kind of sample RS/TS/DS 9.	Condition of the animal 10.	Types of TC cytopathogenic agents 11.
193.	20.2.79	Central Cattle Breeding Farm, Alamadi	A193	10/79	Buffalo	HC	1 M	RS	DIA,DE	Enterovirus
194.	do		A194	11/79	do	HC	1 "	RS	SOL, GD	-
195.	do		A195	14/79	do	HC	20 D	RS	SOL, GD	-
196.	do		A196	15/79	do	HC	20 "	RS	SOL, GD	-
197.	do	,,	A197	18/79	do	HC	12 "	RS	SOL, GD	-
198.	do	,,	A198	19/79	do	BC	12 "	RS	GAD	-
199.	do	,,	A199	20/79	do	BC	10 "	RS	GD	-
200.	do	,,	A200	21/79	do	HC	10 "	RS	DE	-

MD: Model Dairy
LAC: Large Animal Clinic
BC: Bull Calf
HC: Heifer calf
M: Months
D: Days

RS: Rectal swab
TS: Throat swab
DIA: Diarrhoea
DE: Debility
SEMI: Semisolid faeces
GD: Good

RESP: Respiratory symptom
Mushy: Fatty stool
SOL: Solid faeces
- : No virus could be detected

Out of the 200 samples screened 31 (15.5%) were proved to be tissue culture positives with 14 (7%) for Enterovirus type of cytopathogenic effect (CPE), 12 (6%) for Adenotype CPE and 5 (2.5%) for Rotatype CPE (Table 1).

Farmwise incidence of viruses, season-wise incidence of viruses, age-wise incidence of viruses, breed and symptom-wise incidence were tabulated and the data were analysed statistically employing the Heterogeneity Chi-square using the formula $X^2 = \frac{1}{pq} (\mathcal{E} ap - n_1 p)$ to know the significance of incidence of the viruses on location, season and age (Ostle, 1966).

1) Farmwise distribution: Results of farm-wise incidence is presented in Table 2. Chi-square values on the occurrence of viruses in different farms is given in Table 3. The overall value of viral agents was 18.83 per cent which was found to be highly significant statistically ($P < 0.01$) (Table 3). The incidence was higher in District Livestock farms, Orathanad (29%), Pudukottai (23%) and Large Animal Clinic (LAC) of Madras Veterinary College (22%) than the other 3 places with 6 per cent and less (Table 3). The higher overall incidence of viral agents in livestock farms was probably due to an intensive calf rearing programme on the respective farms.

Table 2

Farm-wise Distribution of Viral Agents

S.No.	Name of the farm and place	Kind of sample		Result			Total number of tissue culture positive isolates
		Number of		Number of tissue culture positive isolates			
		Rectal swabs	Throat swabs	Entero virus	Adeno virus	Rota virus	
1.	District Livestock Farm, Orathanad, Thanjavur District.	24	24	6	6	2	14
2.	Government Dairy Farm, Madavaram.	21	20	-	-	2	2
3.	District Livestock Farm, Pudukottai, Pudukottai District.	11	18	3	6	-	9
4.	Central Cattle Breeding Farm (Buffalo) Alamadhi	21	11	1	-	1	2
5.	Large Animal Clinic, Madras Veterinary College, Madras.	11	7	4	-	-	4
6.	Others	11	11	-	-	-	-
	Total	109	91	14	12	5	31

Table 3

Statistical analysis of Farm-wise Distribution of Viral Agents

S.No.	Name of the farm (location)	Tissue culture			p = a/t1
		Positive for viral agent	Negative (a^1)	Total (t1)	
1.	Madras Veterinary College	4	14	18	0.22222
2.	District Livestock Farm, Pudukottai	9	30	39	0.23077
3.	Central Cattle Farm, Alamadi	2	30	32	0.06250
4.	District Livestock Farm, Orathanad	14	34	48	0.29167
5.	Government Dairy Farm, Madhavaram	2	39	41	0.04878
6.	Others	0	22	22	0.00000
		31 (n1)	169 (n2)	200 (t1) p =	0.15500

Chi-square value: X^2 = 18.83303 for 5 degree
statistically highly significant.

Among the occurrence of individual viruses, Enterovirus occurrence was more in Madras Veterinary College with 22 per cent and District Livestock Farm, Orathanad 14 per cent than the other farms with 7 per cent and less (Table 4) which was also statistically significant farmwise. But here it was observed that the incidence of Enterovirus was highest in L.A.C. of Madras Veterinary College since ailing animals showing symptoms of diarrhoea were brought in for treatment. Occurrence of Adenovirus was more in District Livestock Farm, Pudukottai with 15 per cent and Orathanad 12 per cent which are statistically significant and zero with the other farms (Table 5). Here again it was observed that the presence of Adenovirus was found to correlate with calf-rearing. Occurrence of Rotavirus was more or less equal in all the farms (Table 6) but were not statistically significant. Yet it could be observed that isolation of Rotavirus in tissue culture (TC) were not reported in India and that it was rather difficult to get TC isolates.

In the present study three types of tissue culture cyto-pathogenic agents, entero, adeno and rota in calf kidney cell cultures were demonstrated from the samples of ailing and apparently healthy animals from different farms. It was reported that samples from 378 cattle of below one year from different areas were screened using bovine kidney and testicular

Table 4

Statistical analysis of Farm-wise Distribution of Enterovirus incidence.

S.No.	Name of the farm (location)	Number of tissue culture		Total (tl)	$p = \frac{a}{a + a^1}$
		positive (a)	negative (a^1)		
1.	Madras Veterinary College	4	14	18	0.22222
2.	District Livestock Farm, Pudukottai	3	36	39	0.07692
3.	Central Cattle Breeding Farm, Alamadi	1	31	32	0.03125
4.	District Livestock Farm, Orathanad	6	42	48	0.14286
5.	Government Dairy Farm, Madhavaram	-	41	41	0.00000
6.	Others	-	22	22	0.00000
		14	186	200	0.07000

Chi-square value = X^2 = 15.79186 statistically highly significant

Table 5

Statistical analysis of Farm-wise incidence of Adenovirus

S.No.	Name of the farm (location)	Number of tissue culture: positive (a)	Number of tissue culture: negative (a^1)	Total (tl)	$p = \frac{a}{a + a^1}$
1.	Madras Veterinary College	0	18	18	0
2.	District Livestock Farm, Pudukottai	6	33	39	0.15385
3.	Central Cattle Breeding Farm, Alamadi	0	32	32	0
4.	District Livestock Farm, Orathanad.	6	42	48	0.12500
5.	Government Dairy Farm, Madhavaram	0	41	41	0
6.	Others	0	22	22	0
		12 (n)	188 (n^1)	200	p = 0.06

Chi-square value = χ^2 = 16.89894 statistically highly significant

Table 6

Statistical analysis of Farm-wise incidence of Rotavirus

S.No.	Name of the farm (location)	Number of tissue culture positive (a)	Number of tissue culture negative (a^1)	Total (tl)	$p = \frac{a}{a + a^1}$
1.	Madras Veterinary College, Madras	0	18	18	0
2.	District Livestock Farm, Pudukottai	0	39	39	0
3.	Central Cattle Breeding Farm (Buffalo) Alamadi	1	31	32	0.03125
4.	District Livestock Farm, Orathanad	2	46	48	0.04167
5.	Government Dairy Farm, Madhavaram	2	39	41	0.04878
6.	Others	0	22	22	0
		5	195	200	$\bar{p} =$ 0.025

Chi-square value = X^2 = 3.57 statistically not significant

culture and 2 entero and 22 respiratory cytopathogenic agents were isolated (Datt and Raghavan, 1969). Four cytopathogenic agents were isolated from rectal samples of 100 healthy calves in Kraslava district and identified as Enterovirus by serological test (Rumyantseva et al., 1972).

In the present study 31 cytopathogenic agents from 200 samples from calves from different farms in the State were isolated and the occurrence of these agents in different areas suggest their wide occurrence. The occurrence of Enterovirus, Adenovirus and Rotavirus observed in one and the same farm suggests higher frequency of incidence in calf-rearing programme of these farms.

ii) Season-wise distribution: The season-wise distribution of overall viral agents consisting of Enterovirus, Adenovirus and Rotavirus is presented in Table 7. Statistical analysis on the occurrence of these viruses during the different seasons (Jayarajan, 1968) is given in Table 8. The Chi-square value was found to be 9.9481, a statistically significant one.

The incidence as evidenced from the Table 8 was more in South-west monsoon with 21 per cent than in winter with 16 per cent and with no incidence observed during North-east monsoon and summer. The incidence of Enterovirus was more in South-west

Table 7

Seasonwise Distribution of Viral Agents

S.No.	Name of the season	Kind of sample		Result			
		Number of		Number of tissue culture positive			Total tissue culture positive
		Rectal swabs	Throat swabs	Entero virus CPE	Adeno virus CPE	Rota virus CPE	
1.	Summer (March, April, May)	13	13	-	-	-	-
2.	South-west monsoon June, July, August)	49	44	10	6	4	20
3.	North-east monsoon (September, October, November)	7	7	-	-	-	-
4.	Cold weather season (December, January, February)	40	27	4	6	1	11
	Total	109	91	14	12	5	31

Table 8

Statistical analysis of Seasonal Incidence of Viral Agents

S.No.	Name of the season	Number of tissue culture		Total (tl)	$p = \frac{a}{a + a^1}$
		positive (a)	negative (a^1)		
1.	Summer (March, April, May)	0	26	26	0
2.	South-west monsoon (June, July, August)	20	73	93	0.2151
3.	North-east monsoon (September, October, November)	0	14	14	0
4.	Cold weather season (December, January, February)	11	56	67	0.1642
	Total	31	169	200	$\bar{p}$ = 0.1550

Chi-square value = 9.9481 statistically significant

monsoon than in winter season (Table 9). For Adenovirus its occurrence was more in winter than in South-west monsoon (Table 10). The incidence of Rotavirus was more during South-west monsoon than in cold-weather season (Table 11). It was observed that the above variations among seasons for the individual viruses were not statistically significant (Tables 9, 10 & 11) though the incidence of overall viral agent was quite significant (Table 8).

It had been reported that infectious calf diarrhoea from which virus and bacteria isolated were observed to affect calves during all season but was more prevalent, during winter (Amstutz, 1965). The severity and complexity of disease like weak calf syndrome due to Adenovirus infection was found to be influenced by cold and wet weather (McClurkin and Coria, 1975) and it had been reported that incidence of Rotavirus in human beings was more in winter season but their reflection of increased survival rate of virus was not clear at this low temperature (McNulty, 1978).

In the present observation on the occurrence of viruses in South-west monsoon and cold weather seasons lend support to the above observation.

Table 9

Statistical analysis of Seasonal Incidence of Enterovirus

S.No.	Name of the season	Number of tissue culture positive (a)	Number of tissue culture negative (a^1)	Total (tl)	$p = \frac{a}{a + a^1}$
1.	Summer (March, April, May)	0	26	26	0
2.	South-west monsoon (June, July, August)	10	83	93	0.1075
3.	North-east monsoon (September, October, November)	0	14	14	0
4.	Cold weather season (December, January, February)	4	63	67	0.0597
	Total	14	186	200	$\bar{p}$ = 0.0700

Chi-square value = 5.1275 statistically not significant

Table 10

Statistical analysis of Seasonal Incidence of Adenovirus

S.No.	Name of the season	Number of tissue culture positive (a)	Number of tissue culture negative (a^1)	Total (tl)	$p = \frac{a}{a + a^1}$
1.	Summer (March, April, May)	0	26	26	0
2.	South-west monsoon (June, July, August)	6	87	93	0.0645
3.	North-east monsoon (September, October, November)	0	14	14	0
4.	Cold weather season (December, January, February)	6	61	67	0.0896
	Total	12	188	200	$\bar{p}$ = 0.06

Chi-square value = 3.6277 statistically not significant

Table 11

Statistical analysis of Seasonal Incidence of Rotavirus

S.No.	Name of the season	Number of tissue culture positive (a)	Number of tissue culture negative (a^1)	Total (tl)	$p = \frac{a}{a + a^1}$
1.	Summer (March, April, May)	0	26	26	0
2.	South-east monsoon (June, July, August)	4	89	93	0.0430
3.	North-east monsoon (September, October, November)	0	14	14	0
4.	Cold weather season (December, January, February)	1	66	67	0.0149
	Total	5	195	200	$\bar{p}$ = 0.0250

Chi-square value = 2.5369 statistically not significant

iii). Age-wise distribution: Occurrence of viruses that were tissue culture positives, in different age groups are given in Table 12. The grouping of age of the calves tested were done with a view to find out the effect of colostral antibody on virus isolation rate. Statistical analysis of the data revealed their occurrence in different age groups as significant one, with the heterogeneity value being 15.56103, occurring more in the 1-2 months age group with 50 per cent, followed by 30 per cent in 2 to 3 months group, with 26 per cent in 15 days to 1 month old calves and zero in 0 to 15 day old calves (Table 13).

As for individual viruses, Enterovirus occurred more in 1 to 2 months age group with 16 per cent followed by 15 days to 1 month calves with 15 per cent and 2 to 3 months group with 13 per cent (Table 14). Adenovirus occurred more in the 1 to 2 months age group following the similar pattern as Enterovirus (Table 15). Rotavirus occurred more in 1 to 2 months group with 12.5 per cent than in 2-3 months group with 6 per cent, with zero value in 0 to 1 month and above 3 months group (Table 16).

A critical review of the above findings, brings out the fact that colostral antibody which is likely to be active in the period 0 to 15 days of age is very important and essential

Table 12

Age-wise Distribution of Viral Agents

S.No.	Age	Enteric and respiratory affections	Apparently normal.	Result: Number of tissue culture positive			Total isolates
				Entero virus	Adeno virus	Rota virus	
1.	0-15 days	11	10	-	-	-	-
2.	15 days to 1 month	13	25	6	4	-	10
3.	1 - 2 months	9	15	4	5	3	12
4.	2 - 3 months	13	17	4	3	2	9
5.	Above 3 months	3	-	-	-	-	-
6.	Total	49	67	14	12	5	31

Table 13

Statistical analysis of Agewise Incidence of Viral Agents

S.No.	Age group	Number of tissue culture		Total (tl)	$p = \frac{a}{a + a^1}$
		positive (a)	negative (a^1)		
1.	0-15 days	0	21	21	0
2.	15 days to 1 month	10	28	38	0.26316
3.	1 - 2 months	12	12	24	0.5
4.	2 - 3 months	9	21	30	0.3
5.	3 months and above	0	3	3	0
		31 (n)	85 (n^1)	116	0.26724

Chi-square value = X^2 = 15.56103 statistically highly significant

Table 14

Statistical analysis of Age-wise Incidence of Enterovirus

S.No.	Age group	Number of tissue culture positive (a)	Number of tissue culture negative (a^1)	Total (tl)	$p = \frac{a}{a + a^1}$
1.	0-15 days	0	21	21	0
2.	15 days - 1 month	6	32	38	0.15789
3.	1 - 2 months	4	20	24	0.16667
4.	2-- 3 months	4	26	30	0.13333
5.	3 months and above	0	3	3	0
		14 (n)	102 (n^1)	116	0.12068

Chi-square value $X^2(4) = 4.31285$ statistically not significant

Table 15

Statistical analysis of Age-wise Incidence of Adenovirus

S.No.	Age group	Number of tissue culture		Total (tl)	$p = \frac{a}{a + a^1}$
		positive (a)	negative (a^1)		
1.	0 - 15 days	0	21	21	0
2.	15 days - 1 month	4	34	38	0.10526
3.	1 - 2 months	5	19	24	0.20833
4.	2 - 3 months	3	27	30	0.1
5.	3 months and above	0	3	3	0
		12	104	116	0.10345

Chi-square value = $X^2(4)$ = 5.62038 statistically not significant

Table 16

Statistical analysis of Age-wise Incidence of Rotavirus

S.No.	Age group	Number of tissue culture positive (a)	Number of tissue culture negative (a^1)	Total (tl)	$p = \frac{a}{a + a^1}$
1.	0 - 15 days	0	21	21	0
2.	15 days - 1 month	0	38	38	0
3.	1 - 2 months	3	21	24	0.125
4.	2 - 3 months	2	28	30	0.06667
5.	3 months and above	0	3	3	0
		5 (n)	111 (n^1)	116	0.04310

Chi-square value = X^2 (4) = 7.10087 statistically not significant

for the warding of the viral infections. As a matter of fact there were no virus isolations in the age group of 0-15 days (Table 12) and was found to increase as the colostral antibody wanes away in the higher age group of 15 days to one month and 1 to 2 months.

It had been reported that enterocytopathic agents were isolated in bovine kidneys from throat and rectal samples of 378 calves below 1 year (Datt and Raghavan, 1969). Two strains of Enterovirus were isolated in bovine embryo kidney cells from faecal samples of 15 dairy cows and two strains of Entero-virus from nasal swabs of 100 beef calves (Flammini and Allegri, 1969). Five Entero-virus were isolated in monkey kidney cells, bovine embryonic kidney and lung cells from 58 faecal samples of calves and yearling cattle (Durham and Burgess, 1977). In the present study, 14 enterocytopathogenic agents were isolated in calf kidney cells from 200 rectal and throat swabs of calves below 3 months age. The observation now made that Enterovirus occurring in different age group gathers support from the above authors suggesting that Enterovirus can occur in any age group when the colostral antibody wanes away. McNulty et al., (1976c) had also demonstrated colostrum fed to calves during the first three weeks of life had prevented the occurrence of Rotavirus and also diarrhoea.

The occurrence of Adenovirus type cytopathic agents in the present study occurring in 15 days to 1 month age group and above lends support of the findings of Mattson (1973) that Adenovirus affected rarely calves less than one week old.

Though Rotavirus affected characteristically young animals affecting newborn calves (Flewett et al., 1974; Albrey and Murphy, 1976), 3 days of age (Morin et al., 1974), 1-4 day old (Burges and Simpson, 1976), 5 to 7 days of age (Woode and Bridger, 1975), it had also been reported that Rotavirus affected both newborn and young animals (Holmes et al., 1974) and no age resistance had been observed (McNulty, 1978).

In the present study it had been observed that infection was seen in 1 to 2 months and 2 to 3 months age group which is in line with the findings of Holmes et al., (1974) and McNulty (1978). But in calves above 3 months of age there were no virus isolations probably because the animals had developed sufficient resistance due to earlier infection in life.

iv). species and symptomwise distribution: Distribution of the three cytopathogenic agents among breedwise and symptomwise are given in Table 17. The incidence was more in white cattle with 17.7 per cent than black cattle (buffaloes) with 11.3 per ce

Table 17

species and Symptom-wise Distribution

S.No.	Breed	Enteric and respiratory		Apparently healthy		Number of tissue culture positive					
						Enteric			Normal		
		Rectal swab	Throat swab	Rectal swab	Throat swab	Ev	Av	Rt	Ev	Av	Rt
1.	Pure bred and cross bred	28	26	37	33	3	6	-	8	2	3
2.	Buffalo	18	15	26	17	1	1	1	2	3	1
	Total	46	41	63	50	4	7	1	10	5	4

Ev: Enterovirus Av: Adenovirus Rt: Rotavirus

Among the white cattle the incidence in ailing animals was 16.6 per cent and apparently healthy animals 18.5 per cent. In black cattle it was 9 per cent and 13.9 per cent respectively.

The observation of Almeida et al., (1978) that host may be a factor to alter the clinical course of infection produced by enteropathic viruses supports the observations made in the present study and might be the reason that incidence was more in white cattle than black cattle.

Apart from isolation of enteropathogenic virus from the bovine kidney cell cultures from diarrhoeic cases (Krasnikov and Belokonov, 1970), from herds showing respiratory and enteric symptoms (Flammnini and Allegri, 1969; Raghavan and Datt, 1973) and from faecal samples of calves with diarrhoea (Durham and Burgess, 1977), viruses were isolated in bovine embryonic kidney cells from faecal samples of healthy calves also (Rumyantseva et al., 1972; Zebrowski et al., 1973).

Isolation of some strains of Adenovirus from apparently healthy animals (Mattson, 1973) and the observations about the symptomless infection of Rotavirus (McNulty, 1978; Almeida et al., 1978) correlates well with the present findings that viruses were isolated even in apparently healthy calves.

Symptomless infection could exist in naturally occurring avirulent strains of the viruses (McNulty, 1978) which explains the presence of virus isolations in healthy calves as well. Such a symptomless carriers of infection could prove to be a potential source of infection within the herd (Woode and Bridger, 1975), leading to development of sufficient resistance in animals beyond three months of age.

4. Summary

Higher overall incidence of viral agents in livestock farms was observed where large scale calf-rearing schemes are in progress. Adenoviruses, Enteroviruses and also Rotaviruses were found to be present. Incidence was found to be more in South-west monsoon and winter seasons. Calves of 1 to 2 months of age showed the highest isolation rates followed by the group 2 to 3 months. There was no virus isolates in the age group of 0-15 days indicating the importance and the activity of colostral antibodies. Symptomless carriers were also demonstrated by virtue of the fact that there were virus isolations in apparently healthy calves.

Chapter IV

CHARACTERISATION OF TISSUE CULTURE ISOLATES

1. Introduction

After broadly classifying the viral isolates into different categories based on their tissue culture cytopathogenicity one or two typical isolates in each group were taken for further characterisation with a view to identify the isolates. Techniques employed to characterise the virus isolates are described in this chapter and the results of the methods adopted for identification of them are also discussed.

2. Materials and Methods

i) Preparation of stock virus isolates: Selected isolates at the second passage level were inoculated in 0.2 ml aliquots into each of four bottles of calf kidney cell culture containing serum-free maintenance medium. When these bottles showed advanced cytopathic changes in the sheet they were harvested, frozen and thawed three times to release the viruses from the cells. The harvested fluids of representative isolates were pooled together and transferred to 30 ml sterile centrifuge tubes and centrifuged at 2000 rpm for 10 minutes. The supernatant fluid was collected and distributed into small aliquots of 5 ml each and were stored at -20°C for further study.

Of the three groups of TC cytopathic agents discussed in Chapter III representative isolates A042 from Adenovirus groups, A142 from Enterovirus group and A112 and A126 from Rotavirus group were chosen and stocks were prepared.

ii) Titration of the stock isolates: Titration of these viruses was done by making 10 fold serial dilution in serum-free maintenance medium (MM) using separate pipettes. 0.1 ml of the diluted virus suspension starting from 10^{-1} to 10^{-8} dilution was inoculated into each of four calf kidney (CK) culture tubes. Next, virus suspension was also inoculated into 4 CK tubes as control. The inoculated tubes were incubated at 37°C. All the inoculated tubes were examined daily for the evidence and progress of CPE and recorded according to the progressive nature of CPE as + to 4+. The highest dilution of the virus sample that gave + CPE was taken as the end dilution. The end titre of the virus was calculated as per Reed and Muench (1938) method.

iii) Studies on biophysical and biochemical characters of the isolates.

a) Determination of size: 5 ml of the stock virus of the each representative samples, namely A042, A142, A112 and A126 were taken out of the freezer and thawed at room temperature; 2 ml each of the stock virus was filtered through sterile

millipore filters of 100 mu and 50 mu by using a sterile syringe and piston. Each filtrate was made into 10 fold dilution with serum-free MM and titrated in CK cell cultures. The unfiltered samples were also titrated simultaneously in tissue culture tubes. A difference of more than 2 $\log_{10}$ dilution was taken as significant.

b) Ether sensitivity test: The stock viruses of representative samples were subjected to ether treatment. Equal volumes of the stock virus and diethyl ether (20% v/v) were taken in a sterile culture tube fitted with rubber cork and kept in an incubator at 37°C for 1 hour with periodical shaking at interval of 5 minutes. Untreated samples were also kept at 37°C for the same period in the incubator. Both the ether treated and untreated samples of the each isolate suepension was poured in sterile petridishes, partially covered in a sterile condition in a laminar flow cabin for evaporation of the ether. After this the samples were made into 10 fold serial dilution with serum-free MM starting from 10^{-1} to 10^{-8} and titrated in calf kidney cell culture as described earlier. Simultaneous titration of untreated samples were also carried out.

The end point of the virus titration in terms of $TCID_{50}$ of both the ether treated and the untreated samples were calculated

as per Reed and Muench formula (1938). A two $\log_{10}$ fall or more of the virus titre of the virus isolate indicated that the isolate was ether sensitive and if there was no change in the titre value, the isolate was considered to be ether resistant.

c) Cationic stabilisation: Stabilisation in cationic salts, viz. Magnesium sulphate (Mg So4) and Magnesium chloride ($Mg Cl_2$) solutions on heating at 50°C was done as per the method of Wallis and Melnick (1962).

The stock viruses were taken out of the freezer, thawed in room temperature and an aliquot was mixed with equal volumes of 2 Molar $Mg Cl_2$ and 2 Molar Mg So4 solutions. The 2 molar cationic solutions were sterilised by sintered glass filtration and the mixtures were poured into preheated sterile tubes and were subjected to 50°C in a circulating water bath for a period of 1 hour.

After heat treatment the viral aliquots were titrated in CK tubes. Simultaneous titration of the unheated aliquots was also performed and the titres were compared. A two $\log_{10}$ fall was taken as positive result for sensitivity to cations on heating.

d) Cytological and cytochemical studies.

i) Preparation and inoculation of coverslip cultures: CK cell cultures were prepared on flying coverslips (Masillamony, 1972). When the sheet was fully formed the coverslips were emptied in a sterile petridish aseptically. The stock virus isolates of each representative sample containing a multiplicity of infection of 1:30 was inoculated into the respective petridishes containing coverslips and incubated at 37°C for 2 hours for viral adsorption.

Then the coverslips were taken out using a separate spatula and dipped in petridishes containing MM to wash off the surface unadsorbed virus. Each coverslip was transferred to sterile tubes containing 1 ml of MM and incubated at 37°C.

The infected tubes of each sample were harvested at 8, 12, 16, 20, 24, 48, 72, 96 hours after infection. At each harvest 10 coverslips were renewed, 6 were fixed in Carnoy's fluid (absolute alcohol, chloroform and acetic acid 6:3:1), two for haematoxylin and eosin staining (H&E), two for enzyme digestion studies and 2 stored at 4°C for FA studies (Masillamony, 1972). Uninfected coverslips were also harvested to serve as control.

ii) Haematoxylin and Eosin staining: Two infected coverslips and one uninfected coverslip from each isolate were stained with H & E with the standard method (Dacie and Lewis, 1968).

iii) Giemsa staining: Two infected and one uninfected coverslip as control from each isolate were also stained with Giemsa with the standard staining procedure of Dacie and Lewis (1968).

iv) Acridine Orange staining: Two infected and one uninfected coverslips from each isolate, namely A042, A112, A126 and A142 fixed in Carnoy's fluid were treated with McIlvaine citrate-phosphate buffer (Cruickshank, 1972) of pH 4.0 for 8 minutes with one change of buffer at the 4th minute. They were stained with Acridine Orange fluorochrome - AO (BDH) 0.01 per cent solution for 8 minutes and washed in the buffer solution for 4 minutes and then examined under fluorescence microscope.

An Olympus fluorescence microscope with a mercury vapour lamp (HBO200) was used for observation. A combination of BG12 exiter filter and OG1 as barrier filter was used. Photomicrography were taken in Sakura colour negatives. Acridine Orange fluorescence stains RNA flame red, DNA yellowish green.

v) Enzyme digestion: In order to characterise the viral inclusion whether RNA or DNA, enzyme digestion was performed with ribonuclease (RNAse) and deoxy-ribonuclease (DNAse).

Deoxyribonuclease (DNAse) digestion was carried out as per the procedure of Rhim et al., (1962). Deoxyribonuclease II exbovine spleen (Koch-Light) was used as a source of DNAse and made into a solution containing 100 units of the enzyme per ml of 0.025 ml M Veronal buffer containing 0.003 M Magnesium sulphate. Infected and uninfected cells of A042, A112, A126 and A142 were exposed to enzyme digestion for 30 minutes at room temperature. As a control, one infected coverslip culture from each harvest was incubated with the enzyme diluent alone. Then the coverslips were stained with AO fluorochrome as described above.

Ribonuclease (RNAse) digestion was carried out using Ribonuclease from bovine pancreas (Sigma) at a concentration of 0.01 per cent in 0.003 M Magnesium chloride solution (Gomatos et al., 1962). Infected and uninfected cells of A042, A112, A126 and A142 were exposed to enzyme digestion for 1 hour at room temperature. Then they were stained with AO.

vi) Fluorescent antibody (FA) studies: The FA studies were carried out as per the method of Cherry et al., (1960). The

infected coverslip cultures of A042, A112, A126 and A142 of the representative isolates were air dried, fixed in acetone for 10 minutes, covered with immune serum prepared in rabbit against the respective isolates for 30 minutes, drained and finally covered with fluorescence-tagged anti-rabbit gamma globulin prepared in goats (Difco Laboratory, Detroit, U.S.A.). After 30 minutes they were washed in Coon's buffer and mounted in 10 per cent buffered glycerol (Cherry et al., 1960), uninfected cells incubated with immune serum and infected cells incubated with pre-immunised serum served as controls in these tests. As an additional check on stain specificity, the infected cells were directly treated with anti-rabbit globulin. The results were interpreted according to the nature of fluorescence either cytoplasmic or nuclear.

vii) Study of haemagglutinating activity (HA) of the virus isolates: Certain viruses possess the property of agglutinating erythrocytes of different species, some having narrow range like Enterovirus and others having broad range. This property is useful in differentiating viruses at group level and subgroup level.

HA titrations of the virus isolates A042, A112, A126 and A142 were carried out at 37°C by using 2-fold dilution starting from 1:5 to 5120. The RBC used were from human - O,

monkey, bovine, sheep, goat, rabbit, rat, guinea pig and mouse. The method of HA titration was done by using the microtitre technique of Sever (1962).

viii) Serum neutralisation studies.

A. Type specific antisera: Attempts were made to get type specific sera from various sources for Bovine Adenovirus type 1, 3 and 5, Bovine Enterovirus and Rotavirus. Bovine Adenovirus type 5 sera was kindly donated by Dr. R.Raghavan, Bangalore Veterinary College, Hebbal. Bovine Adenovirus type 1, 3 and 5 were donated by Dr. C.T.Perry, Ministry of Agriculture, Fisheries and Food, Central Veterinary Labotarory, New Haw, Weybridge, Surrey, whereas Bovine Enterovirus sera could not be obtained from any sources. Rotavirus serum was also not available but Dr. T.J.John, Director, Enterovirus Laboratory, Christian Medical College was kind enough to test the isolates in counter immunoelectrophoresis against SA11 antiserum.

The Bovine Adenovirus serotype 1 (Dr. Perry), type 3 (Dr. Perry), Bovine Adenovirus serotype 5 (Dr. Perry and Dr. Raghavan) were used in the serum neutralisation tests against A042 isolate.

B. Test procedure: A constant virus of 100 $TCID_{50}$ in 0.1 ml was used for each tube containing CK cells. Double fold

dilution of the serum samples was made upto 1 in 640 in MM. Equal amount of (0.5 ml) the diluted serum samples and the reconstituted constant virus suspension were taken in small test tubes and closed with sterile rubber corks. These tubes containing serum:virus mixture were incubated for 1 hour at 37°C and inoculated into each of the tubes containing 0.8 ml of the CK cells suspension. For each dilution of serum 4 tubes were used. Along with the inoculation of the serum:virus mixture, a check titration of the virus suspension used, was also conducted simultaneously. All the tubes for serum neutralisation were examined daily for inhibition of CPE. Tubes for check titration were also examined daily to confirm the appearance of CPE. The highest dilution of the serum capable of neutralising the cytopathogenic effect (CPE) of 100 $TCID_{50}$ of the virus was taken as the end titre.

Two days after the appearance of characteristic CPE in the control tubes, final reading in the other tubes were made and serum neutralising titre calculated as per Reed and Muench (1938) method and expressed as number of SN_{50} units/inoculum volume (Hoskins, 1967).

ix) Counter immunoelectrophoresis: The two tissue culture isolates A112 and A126 were sent to Christian Medical College, Vellore for counter immunoelectrophoresis (CIEP) against SA11

antiserum which is closely related to calf Rotavirus - Nebraska Calf Diarrhoea Virus.

The procedure of CIEP is described in detail in Chapter V. The test was repeated at Madras Veterinary College using antisera prepared in rabbits against the isolate A126.

3. Results and Discussion

The results of the tests for determination of size by millipore filtration (Table 18) for ether sensitivity (Table 19), Cationic stabilisation (Table 20) and for the presence of haemagglutinins (Table 21) of the four isolates, viz. A042, A112, A126 and A142 are presented and discussed for identification of the viral isolates. In addition to the above, the type of formation of inclusion body in cell culture, their staining features in Giemsa, and Haematoxylin and Eosin under conventional microscopy and in Acridine Orange fluorochrome with or without nuclease digestion under fluorescent microscopy were taken into consideration while identifying the isolates.

The results of serum neutralisation test against type specific sera of Adenovirus type 1, 3 and 5 are presented in Table 22.

Table 18

Size Determination of the Virus Isolate

Virus isolate	Neat	Titre of virus after millipore filtration		Size range
		50 mu	100 mu	
A042	10^6	$10^{2.7}$	10^6	50 mu - 100 mu
A112	$10^{7.5}$	$10^{3.5}$	$10^{6.5}$	50 mu - 100 mu
A126	$10^{6.5}$	10^3	$10^{6.5}$	50 mu - 100 mu
A142	$10^{6.5}$	10^6	10^6	>50 mu

Table 19

Ether Sensitivity Test

Virus isolate	Titre of virus TCID50/0.1ml		Interpretation
	Neat	Ether treated	
A042	$10^{5.5}$	$10^{5.5}$	Ether resistant
A112	10^{7}	$10^{6.5}$	Ether resistant
A126	$10^{6.3}$	10^{7}	Ether resistant
A142	$10^{7.5}$	$10^{7.3}$	Ether resistant

Table 20

Test for Cationic Stabilisation

Virus isolate	Neat	Titration after heating at 50°C for one hour with		Result of stabilisation after heating at 50°C for one hour with	
		1M-$MgCl_2$	1M-$MgSO_4$	1M-$MgCl_2$	1M-$MgSO_4$
A042	$10^{5.3}$	10^{2}	10^{3}	negative	negative
A112	$10^{5.3}$	$10^{2.7}$	$10^{4.5}$	negative	positive
A126	10^{6}	10^{3}	$10^{4.5}$	negative	positive
A142	$10^{7.5}$	$10^{7.5}$	$10^{7.3}$	positive	positive

Table 21

Test for Haemagglutination Activity of the Virus Isolates

Source of erythrocytes (RBC)	Virus Isolate			
	A042	A112	A126	A142
Bovine	40*	0	0	0
Sheep	0	0	0	0
Goat	0	0	0	0
Rat	0	0	0	0
Mouse	0	0	0	0
Monkey	0	0	0	0
Human-O	0	0	0	0
Rabbit	0	0	0	0
Guinea pig	0	ND	ND	0
Cell culture (control)	0	0	0	0

ND = Not done

* = Reciprocal of the highest dilution showing HA.

Table 22

Serum titre against 100 $TCID_{50}$ of A042

Serum	Neutralisation titre
Adenovirus type 1	10
type 3	10
type 5	80

1) Adenovirus group

A. Isolate No. A042

a) Biophysical and biochemical features: A042 was found to be having a size ranging from 50 mu to 100 mu (Table 18), resistant to diethyl ether (Table 19) and was not stabilised by Cations 1 molar Magnesium chloride and 1 molar Magnesium sulphate on heating at 50°C for one hour (Table 20).

b) Cytological and cytochemical features: A042 was found to produce delayed cytopathogenic effect (CPE) in the first passage level which started appearing quickly and rapidly in CK cell culture at the second passage. By the 8th hour of injection with a higher multiplicity of the virus unstained preparations showed the presence of rounded refractile cells in the midst of normal sheet which became more prominent by 16 hours having a tendency to clump like cluster of grapes by 24 hours post-infection (Figure 1).

Flying coverslip preparations showing grapelike clusters of Adenovirus infection when stained with Haematoxylin and Eosin showed the presence of basophilic nuclear inclusions. The chromatins of the nucleii were pushed to the periphery and basophilic masses of viral inclusions with halo around were seen in the centre of the nucleii (Figure 2). When stained with Giemsa these nuclear inclusions remained basophilic indicating

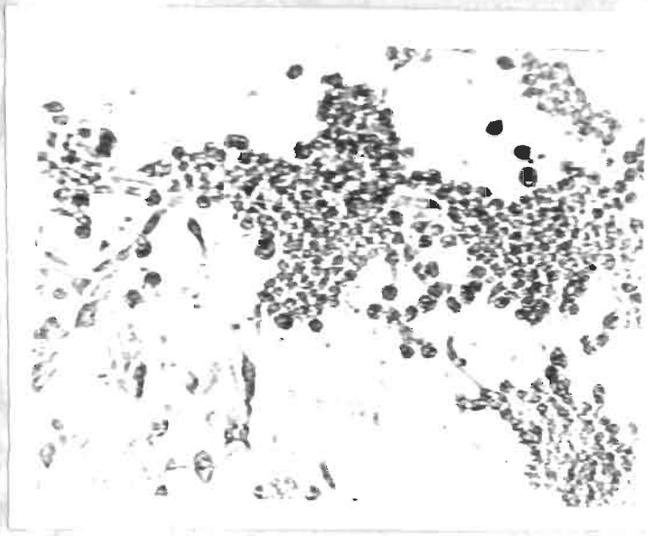

Figure 1: A042. The advanced CPE of CK cell sheet showing the rounded refractile cells clumped like clusters of grapes. Unstained preparation - 200X

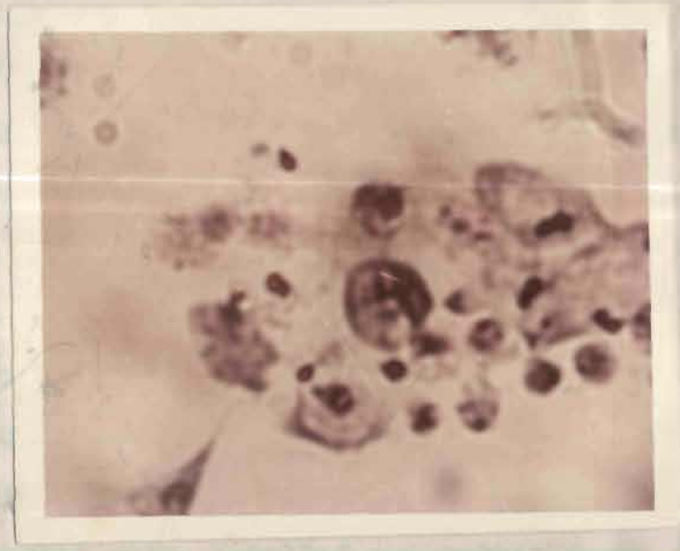

Figure 2: Adenovirus A042 showing extensive nuclear changes containing basophilic inclusions with halo around them pushing the nuclear chromatin to the periphery. H & E. 1000X

that they are DNA materials (Figure 3). These grapelike clusters on staining with Acridine Orange fluorochrome (AO) showed the presence of nuclei and chromatin inclusions pushed to the periphery and large masses intranuclear greenish yellow (Figure 4).

Cytochemical studies with RNAse digestion followed by AO staining and viewing under fluorescence microscope did not remove the intranuclear yellowish green viral inclusions whereas the cytoplasmic RNA got digested (Figure 5). A similar preparation with DNAse digestion did remove the yellowish green intranuclear DNA viral inclusions as well as the cellular DNA but not the flame red cytoplasmic RNA (Figure 6). These intranuclear inclusions gave a positive fluorescence at the nuclear site against type specific Adenovirus antisera prepared in rabbit and coupled with labelled antirabbit gamma globulin (Figure 7). These cytological and cytochemical changes accompanying the infection of A042 did reveal that the virus isolate is a double stranded DNA virus capable of producing specific virus neoprotein at the nuclear site of the cell, which has a tendency to cluster like a bundle of grapes.

c) Serological feature: A042 was found to possess a low titre of HA activity against bovine cells but not with other RBC (Table #1). The results of serum neutralisation against 100 $TCID_{50}$ of Adenovirus type 1, 3 and 5 showed that the virus

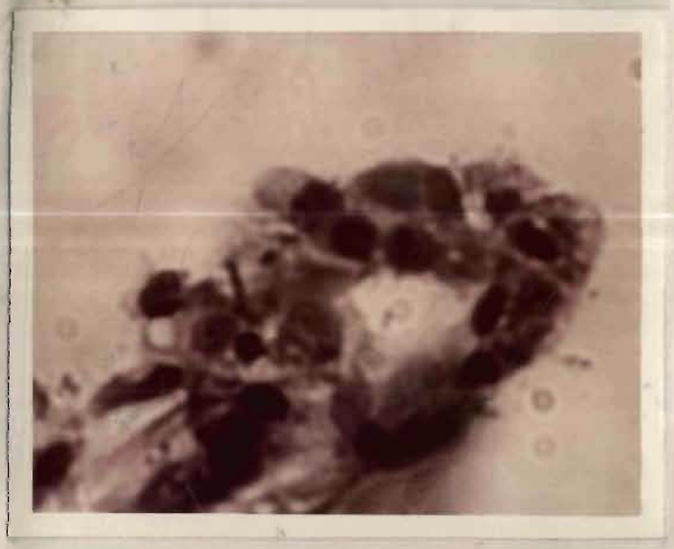

Figure 3: Adenovirus isolate A042 - A grapelike cluster of adenovirus infected cells showing nuclear inclusions basophilic in nature. Giemsa 1000X

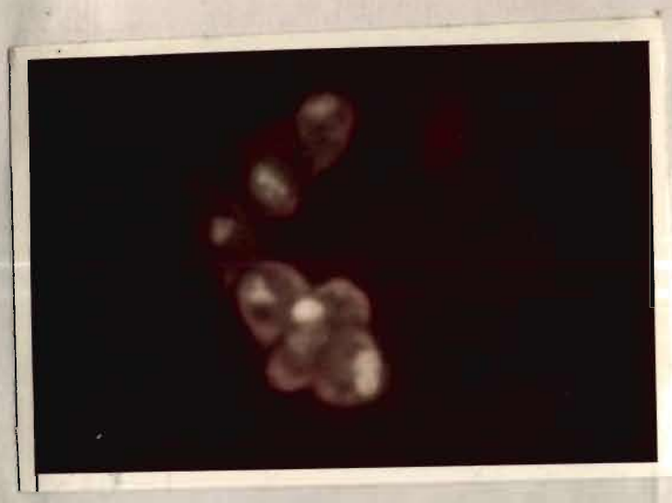

Figure 4: Adenovirus AC42 - Grapelike cluster of cells. Nucleoli are pushed to outer edge of the nucleus and greenish nuclear inclusions seen.

AO staining 600X

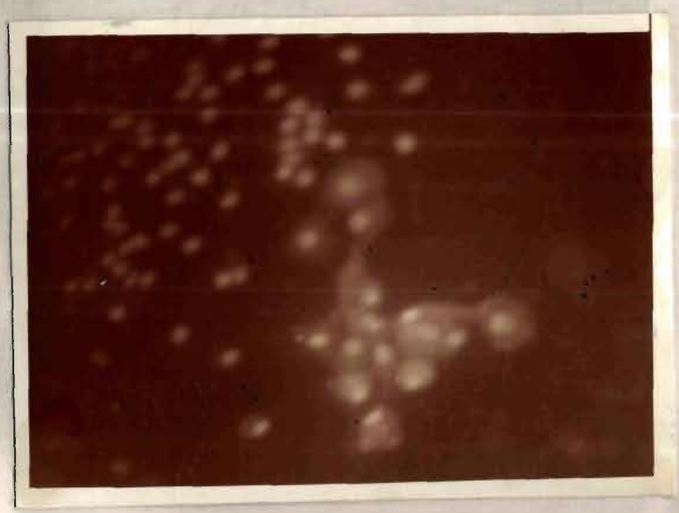

Figure 5: Adenovirus A042 - RNAse digestion. The infected cell cluster retains the greenish nuclear inclusions and the nuclear material whereas the red cytoplasmic RNA got digested out due to the action of RNAse. AO staining 600X

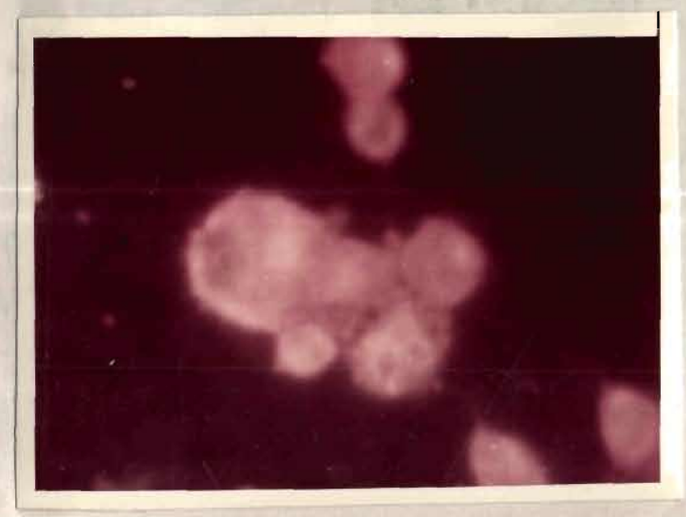

Figure 6: Adenovirus A042 - DNAse digestion. The infected adenovirus cell clusters show the absence of greenish yellow nuclear inclusions and the nucleii material due to the digestion of DNAse. Cells show punched out appearance at the nuclear sites whereas flame red cytoplasmic RNA is not digested out.

AO 1000X

Figure 7: CK cell aggregates showing positive fluorescence of the nucleii indicating the presence of adeno viral protein in the nucleus. IFA staining against specific antiserum prepared in rabbits coupled with labelled anti-rabbit gamma globulin - 1000X

was neutralised by prototype Adenovirus type 5 antiserum (Table 22).

Hence it could be concluded that A042 isolate was Adenovirus type 5.

ii) Rotavirus group

A. Isolate No. A112.

a) Biophysical and biochemical features: A112 was found to be having a size ranging from 50 mu to 100 mu (Table 18) resistant to ether (Table 19) and was not stabilised by 1 molar Magnesium chloride whereas stabilised by 1 molar Magnesium sulphate on heating to 50°C for one hour (Table 20) which is a characteristic feature of Rotavirus (McNulty, 1978; Estes et al., 1979).

b) Cytological and cytochemical features: The cytopathogenic effect following the infection of A112 was found to appear only by 7th or 8th post-infection day at the first passage. The sheet of CK cells presented a moth-eaten appearance and became granular. The appearance of CPE got advanced to the 2nd or 3rd post-infection day as the virus got concentrated in the subsequent passages.

At a higher multiplicity of infection, the virus produced granular, moth-eaten type of CPE with extensive vacuolation and cytoplasmic processes making cells to flutter in the fluid

and get stuck to the surface of the glass by these processes (Figure 8).

Flying coverslip preparations when stained with haematoxylin and eosin showed the presence of eosinophilic cytoplasmic inclusions with extensive pyknotic nucleii and vacuolations at the perinuclear area (Figure 9)10).At higher magnification it was seen that the nuclear chromatin was pushed to one side and the cytoplasm showed abundant eosinophilic masses of viral inclusions which did not coalesce to form large inclusions as seen in cells infected with reovirus (McNulty, 1978). These cytoplasmic inclusions fluoresced specifically with FA staining.

These cytoplasmic inclusions presented a metachromatic staining reaction with Giemsa showing basophilic inclusions (Figure 11). Higher magnification showed the presence of small vacuoles in the cytoplasm with particulate basophilic viral inclusions (Figure 12). Under oil immersion these basophilic inclusions were seen as spherical particulate bodies strewn in the cisternae of the endoplasmic reticulum (Figure 13) giving picture of reticulum-like inclusions which fail to condense at the perinuclear area unlike Reovirus (McNulty, 1978).

With AO staining the inclusions were stained yellowish green (Figures 14 and 15) which got digested out with RNAse

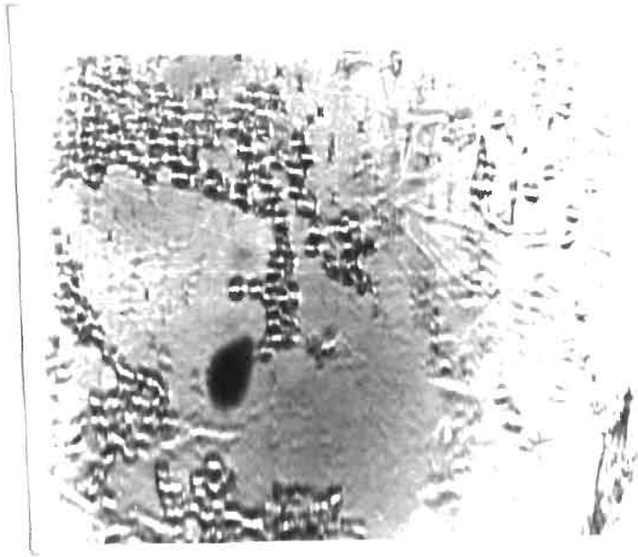

Figure 8: A112 - Advanced CPE of CK cell sheet showing granular (Rotavirus type) cells like moth- eaten appearance with some cells hanging on. Unstained preparation. 200X

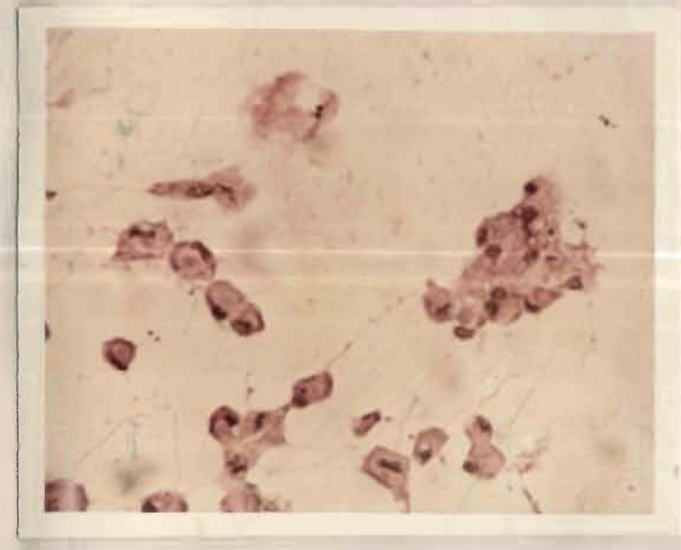

Figure 9: Rotavirus A112. Advanced CPE of CK cells showing cytoplasmic processes, pyknotic nucleii and cytoplasmic inclusions stained red with H & E. - 400X

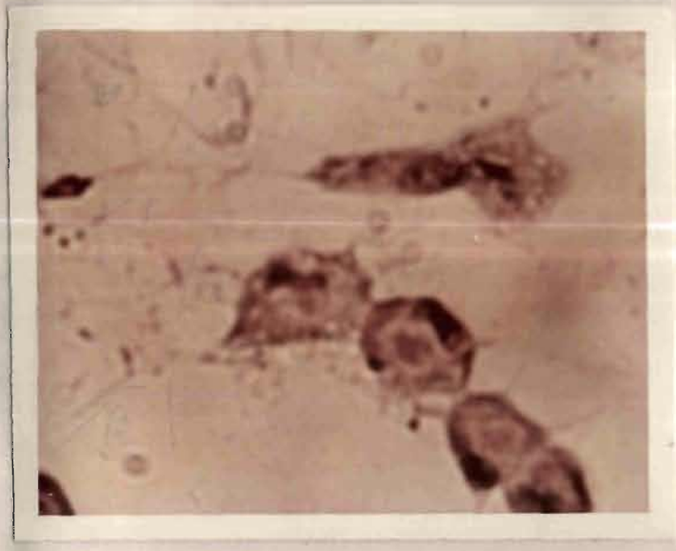

Figure 10: Rotavirus A112 - Advanced CPE of CK cells showing eosinophilic cytoplasmic inclusions with pyknotic nucleii and vacuolation at perinuclear area. H & E 1000X

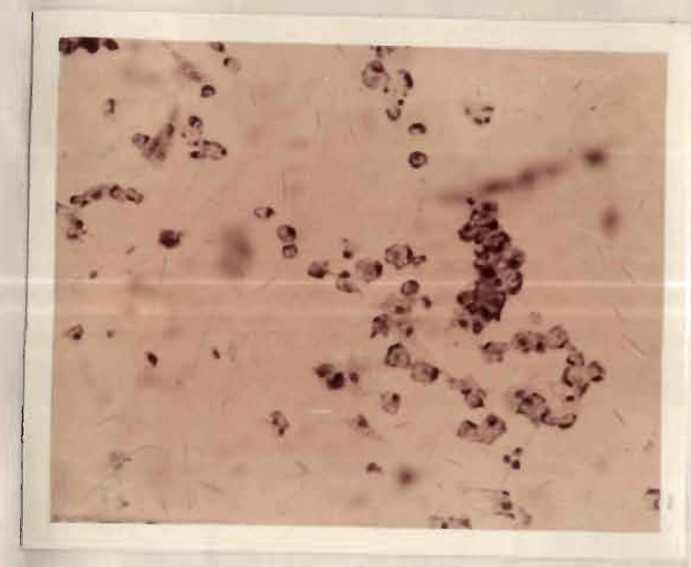

Figure 11: Rotavirus A112. Advanced CPE - CKO cells stained with Giemsa showing blue cytoplasmic inclusions - nucleus are pushed to one side. The cytoplasm shows extensive vacuolation and are filled with inclusions - 100X

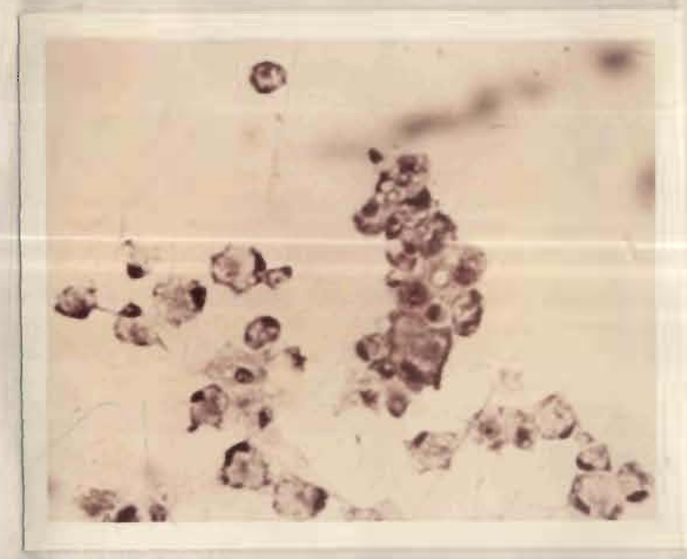

Figure 12: Rotavirus A112. Infected calf kidney cells showing basophilic cytoplasmic inclusions staining blue with Giemsa stain. Cytoplasm of the infected cells show extensive vacuoles.
Giemsa - 400X

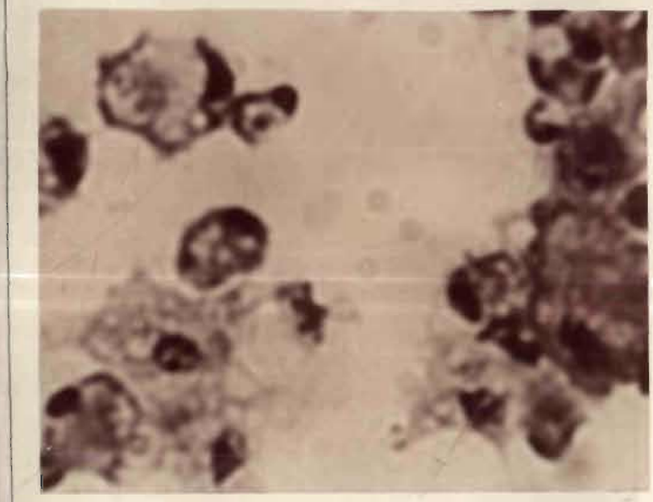

Figure 13: Rotavirus A112. Advanced CPE of the CK cells showing pyknotic nucleus, numerous cytoplasmic vacuoles which coalesce at the perinuclear area pushing the nucleus to one side with characteristic basophilic cytoplasmic inclusions in cisternae of cytoplasm with Giemsa staining - 1000X

Figure 14: Rotavirus A112. A pair of cells seen in the centre of the picture one without any change and the other showing greenish material filling the entire cytoplasm in which the nucleus is pushed to one side. AO staining 600X

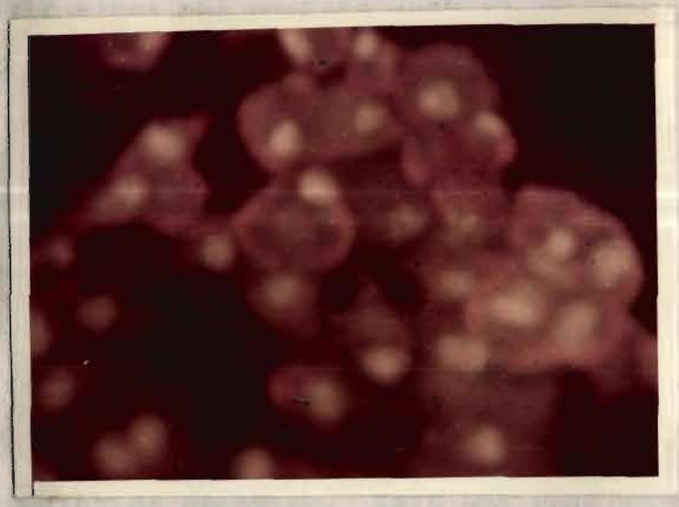

Figure 15: Rotavirus A112. Yellowish green viral inclusions are seen in the cytoplasm. Vacuolation of cytoplasm also seen.
AO staining 400X

digestion (Figure 16) leaving the nuclear DNA intact. DNAse digestion of these virus bearing cells showed the absence of nuclear DNA fluorescence with undisturbed greenish viral inclusion (Figure 17), indicating that the enzyme DNAse did not digest the Rotavirus inclusion staining greenish yellow in AO but RNAse did since the nucleic acid of the Rotavirus happened to be a double stranded RNA virus.

c) Serological features: Rotavirus sera could not be obtained from other sources. So the antigen A112 was tested against antiserum prepared against A126 and was found to produce specific precipitation band in CIEP (Figure 18). Further antigen A112 and A126 in turn were tested against SA11 at Christian Medical College, Vellore by CIEP and found to produce specific precipitation reaction. SA11 and calf Retavirus are reported to be identical with Nebraska calf diarrhoea virus.

B. Isolate No. A126.

a) Biophysical and biochemical features: A126 was found to be having a size ranging from 50 mu to 100 mu (Table 18), resistant to ether (Table 19) and was not stabilised by 1 molar Mg Cl_2 whereas stabilised by 1 molar Mg So4 on heating to 50°C for one hour (Table 20) which is a characteristic feature of Rotavirus (McNulty, 1978).

Figure 16: A112. RNAse digestion - A Rotavirus infected cluster of cells shows the absence of cytoplasmic inclusions digested by the action of ribonuclease. AO staining 600 X

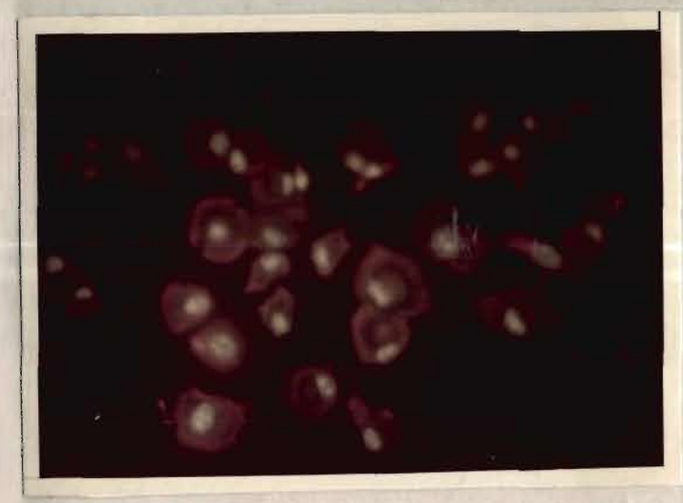

Figure 17: A112 isolate - calf kidney cells infected with DNAse digestion - The nuclear DNA got digested leaving behind the yellow nucleii as yellow spots. Some undigested nucleii are also seen whereas red stained RNA cytoplasm and yellowish green semi-lunars double stranded RNA inclusions remained unaffected. Suggestive of double stranded RNA viruses, viz. Rotaviruses - AO staining 400 X

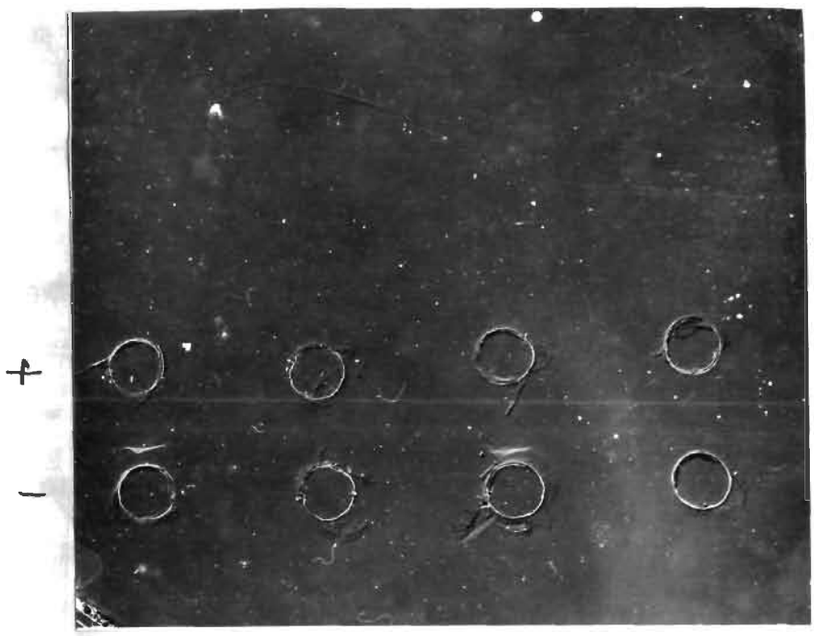

Figure 18: Well one represents A126, a tissue culture isolate showing specific precipitating line against its own antiserum prepared in rabbits and the antiserum A126 cross-reacted with another tissue culture isolate A112 in well No.3. No specific precipitating line was observed with antiserum A126 against A142 (entero type) in well No. 2 and control cell culture fluid in well No. 4 as evidenced from counter immunoelectrophoresis.

b) Cytological and cytochemical features: As seen in A112 the cytopathogenic effect was noted only during the second week of observation by which time the sheet presented a moth-eaten appearance and becams granular. In subsequent passages the characteristic features of Rotavirus CPE started appearing by the 2nd or 3rd day after infection.

Unstained preparation by the 4th day after infection showed the presence of long cytoplasmic processes with detachment of cells fluttering in the fluid (Figure 19).

Flying coverslip preparations when stained with haematoxylin and eosin showed the presence of eosinophilic cytoplasmic inclusions with extensive pyknotic nucleii and vacuolations at the perinuclear area (Figure 20) and cytoplasmic processes are quite evident in the colour plate (Figure 21). A similar preparation when stained with Giemsa showed the presence of metachromatic reaction of basophilic inclusions (Figure 22). Advanced CPE is characterised by the presence of large vacuoles of the cytoplasm basophilic inclusions in the reticulum and displaced pyknotic nucleii (Figure 23).

The inclusions fluoresced yellowish green with AO staining (Figure 24) and also with FA staining around the dense yellowish nucleus in the perinuclear area. In some cells the entire cytoplasm is filled with viroplasm showing a marginal amount of

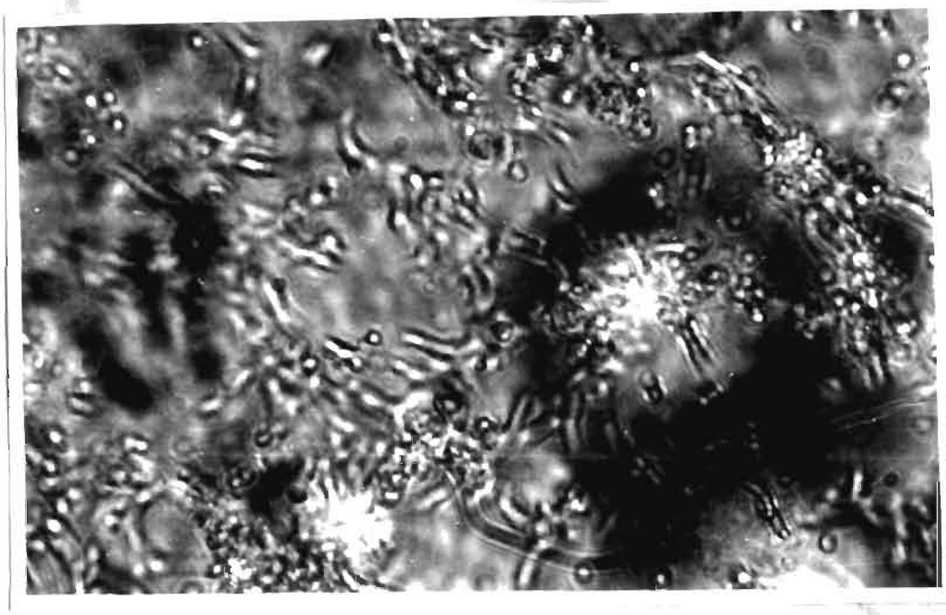

Figure 19: A126. Advanced stage of CPE seen on the 4th day after infection showing detachment of cells with cytoplasmic long processes sticking to the glass surface. Sheet showing granularity

- unstained 200 X

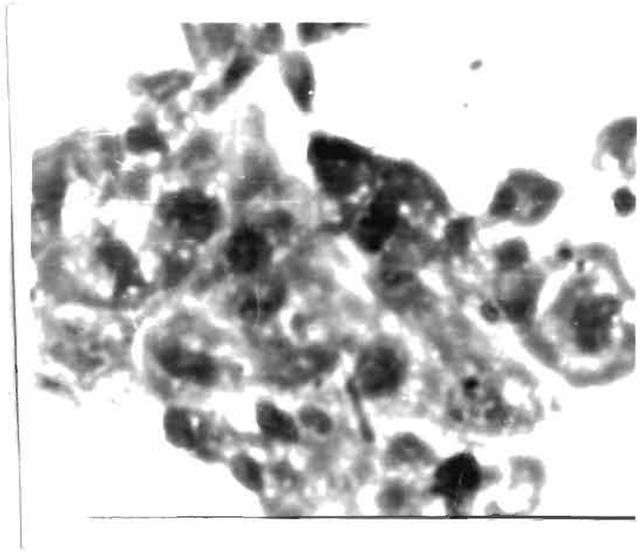

Figure 20: A126 (Rotavirus) CK Cells showing cytoplasmic inclusions pushing the pyknotic nucleus to one side. Cells show extensive vacuolation and cytoplasmic inclusions which do not coalesce at the perinuclear area.

H & E - 600 X

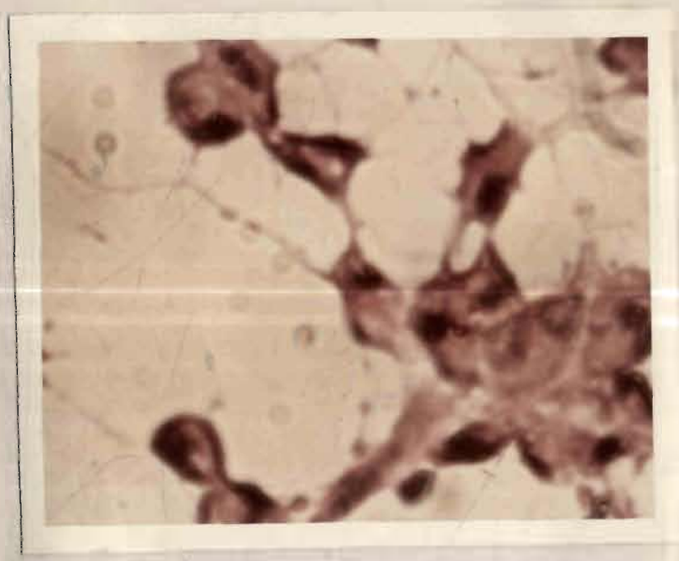

Figure 21: Rotavirus Isolate No.A126 - Advanced CPE of the CK cells showing pyknotic nucleus, vacuolation of the cytoplasm with eosinophilic inclusions displacing the nucleii to the sides. The infected cells show long processes by which they remain stuck to the glass - H & E 600 X

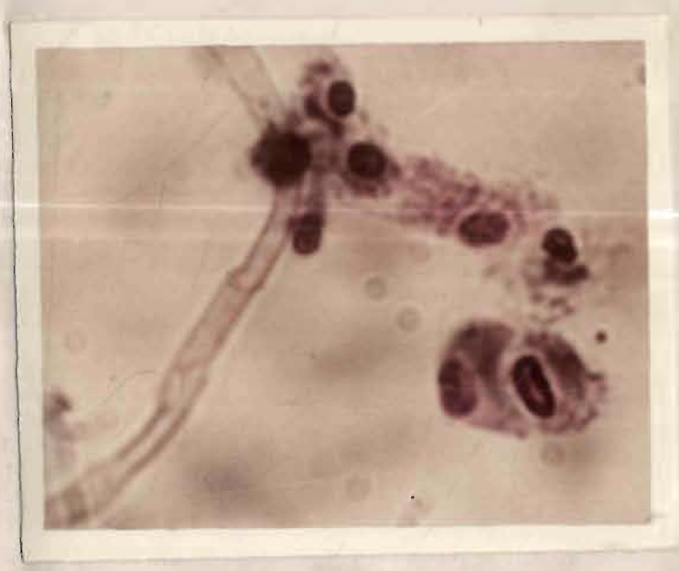

Figure 22: A126. CK cells infected with Rotavirus showing pyknotic nucleii and basophilic inclusions in the perinuclear area
Giemsa 1000 X

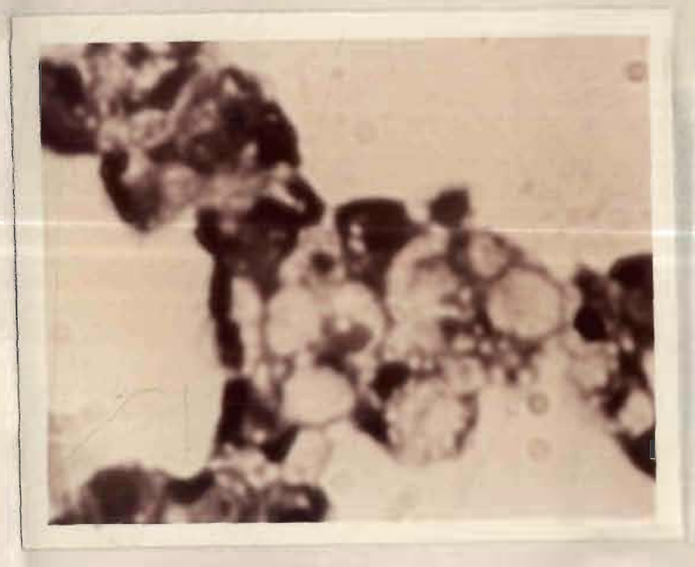

Figure 23: Rotavirus (Isolate A126) - Advanced CPE showing large coalesced perinuclear vacuolation and basophilic cytoplasmic inclusions. Nucleii are condensced, pyknotic and displaced to one side - Giemsa 1000 X

Figure 24: Calf kidney cells infected with A126 isolate showing the presence of yellowish green cytoplasmic inclusions around the dense yellowish nucleus in the perinuclear area - a characteristic feature of Rotavirus infection

AO staining - 1000 X

flame red cytoplasmic RNA (Figure 25). The DNAse digestion in such infected cells removed only the nuclear DNA and the nucleolar matter remained yellowish with the viral nucleic acid undigested at the inclusion sites (Figure 26), whereas RNAse digestion removed the viral nucleic acid.

c) Serological features: A126 produced specific arc with SA11 antiserum in CIEP when tested along with other faecal samples at Christian Medical College, Vellore (Figure 30). It also produced similar arc against its own antiserum prepared in rabbits (Figure 18). The antiserum of A126 prepared in rabbits cross-reacted with A112 antigen in CIEP (Figure 18) whereas the antiserum of A126 did not react with another RNA virus A142 and also against control cell culture fluid (Figure 18).

Hence it could be concluded that A126 and A112 were two tissue culture cytopathogenic agents which are Rotavirus isolation in calf diarrhoea

iii) Enterovirus group.

A. Isolate No. A142.

a) Biophysical and biochemical features: A142 was found to be having a size below 50 mu (Table 18) ether resistant (Table 19) and got stabilised with 1 molar Magnesium sulphate and 1 molar Magnesium chloride on heating to 50°C for one hour (Table 20)

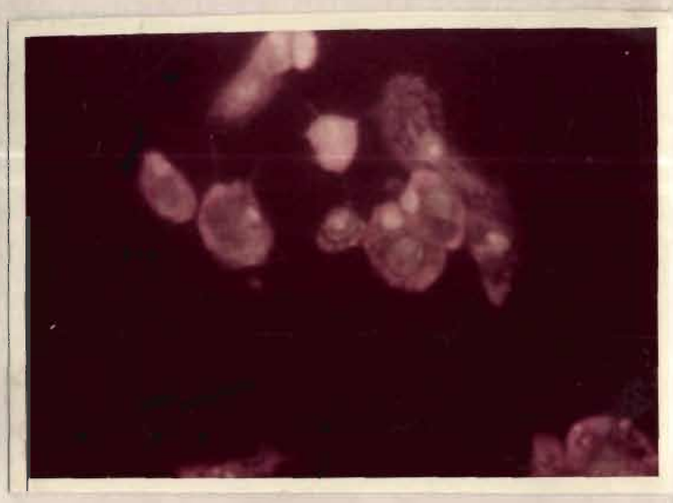

Figure 25: Rotavirus (A126). Cells show the presence of yellowish green inclusion in the cytoplasm and large vacuolations around the inclusions. AO staining 600 X

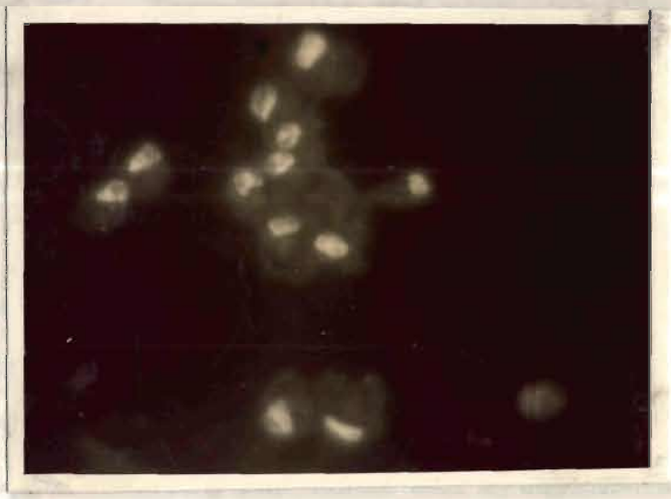

Figure 26: Rotavirus - DNAse digestion (Isolate A126). Rotavirus infected cells retained the greenish inclusions in the cytoplasm whereas the nucleii lost their greenish nuclear component due to DNAse digestion. The bright yellow nucleoli of the nucleii remained intact.

AO staining - 1000X

which is a characteristic feature of Enteroviruses (Wallis and Melnick, 1962).

b) Cytological and cytochemical features: Unstained preparations of CK cell sheets with A142 showed small refractile rounded cells occurring individually and were strewn throughout the sheet (Figure 27). Haematoxylin and eosin staining did not reveal any inclusions. Acridine Orange staining showed characteristic retraction of cell margins, with high cytoplasmic RNA activity staining flame red in colour and distortion of nucleii leading to contortion and lysis of cell (Figure 28). FA staining showed positive cytoplasmic fluorescence. Such a feature was described as the characteristic CPE of Enteroviruses (Wenner and Behbehani, 1968). DNAse digestion removed only the nuclear DNA material leaving the viral and cytoplasmic RNA intact (Figure 29).

c) Serological features: Haemagglutination test using red cells of bovine, sheep, goat, human O, bonnet monkey, rabbit, rat and guinea pigs did not reveal the presence of any haemagglutinins either at 4° or 37°C (Table 21).

CIEP test did not produce any specific precipitation arc against A126 antiserum prepared in rabbits (Figure 18).

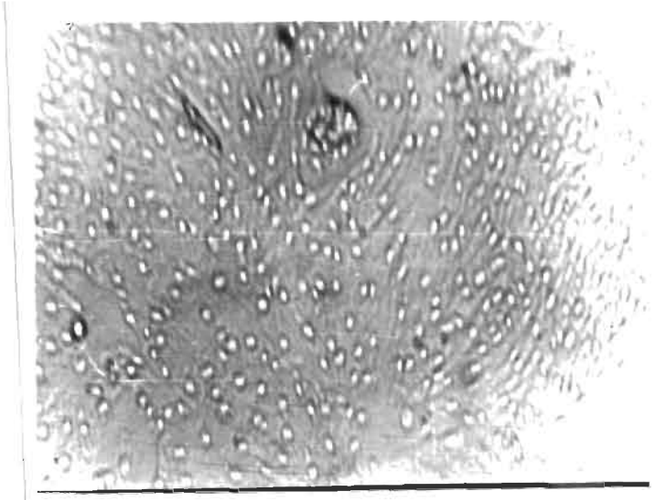

Figure 27: A142 (Enterovirus type). Advanced CPE of the CK cell showing refractile rounded cells occurring individually and are strewn throughout the sheet. Unstained preparation in CKC 200X

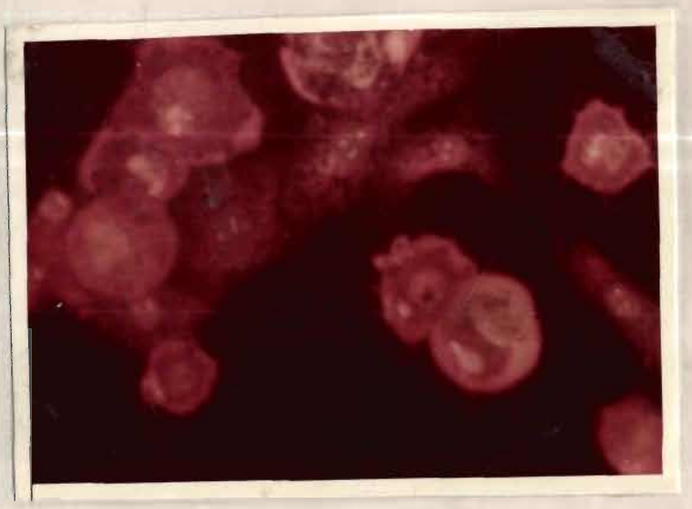

Figure 28: RNA virus (A142) Rounded cells showing flame red cytoplasmic staining indicating intense synthesis of viral RNA and nuclear distortion. AO staining 600 X

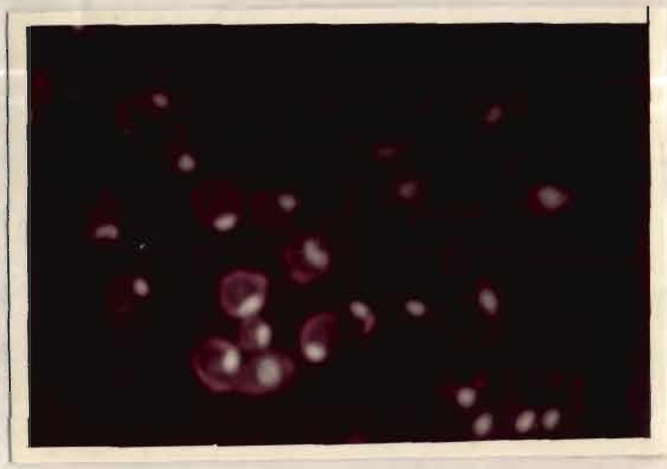

Figure 29: Entero (A142) - DNAse digestion.
The infected cells showed the absence of greenish yellow fluorescence of nucleii material due to the digestion of DNA whereas the RNA viral component remained undigested.
AO staining 400X

Serum neutralisation test could not be done against any prototype Enterovirus antiserum since no such prototype sera could be obtained.

Based on the cytopathogenic effect and other biophysical and biochemical properties A142 could be identified as Bovine Enterovirus.

4. Summary

The four cytopathogenic agents A042, A112, A126 and A142 were subjected to various biophysical, biochemical, cytological and cytochemical studies with a view to identify them individually.

It was found that A042 proved to be Adenovirus Type 5, A112 and A126 were found to be Rotaviruses and A142 was Bovine Enterovirus.

Chapter V

COLLECTION OF SAMPLE AND PROCESSING FOR COUNTER-IMMUNOELECTROPHORESIS

1. Introduction

Counter immunoelectrophoresis (CIEP) is one of the rapid methods employed to detect the virus and virus antigen using hyperimmune serum which in an electric field forms clear precipitating line in agarose gel. CIEP was found to be four times sensitive than Agar gel immunodiffusion (AGID) regardless of which the antigen was either faecal or tissue antigen as observed by Mohammed et al., (1978) in detecting bovine Rotavirus. CIEP was successfully employed in detecting Rotavirus from stools of children with gastro-enteritis (Jesudoss et al., 1978).

In this chapter detection of Rotavirus using CIEP from dung samples of calves is presented.

2. Materials and Methods

i) Collection and processing: Dung was collected from 44 calves showing diarrhoea, debility and apparently healthy condition from different places and were transported in liquid nitrogen at -196°C and stored in the laboratory at -20°C for further processing.

ii) CIEP antigen.

a) Faecal antigen: A twenty per cent dung suspension of each sample was made in Phosphate Buffer Solution (PBS) for centrifugation. The centrifuge tubes were prerinsed with nutrient broth containing 0.05 per cent bovine albumin to avoid sticking of viral protein matter to the sides. High speed centrifugation at 10,000 rpm for 30 minutes was done at Christian Medical College, Vellore and at Biochemistry Department, A.C. College of Technology campus, Adyar, Madras. The supernatant obtained was used as faecal antigen in CIEP.

b) Tissue culture antigen for identification in CIEP: Along with the dung samples, fluids from the two tissue culture positive isolates A112 and A126 whose CPE was suggestive of rota type were also screened in CIEP. The stock virus was used in these tests.

iii) Antiserum for CIEP: SA11 antiserum was used against the samples supplied by Enterovirus Laboratory, Christian Medical College Hospital, Vellore.

iv) Procedure for counter immunoelectrophoresis: CIEP was performed at the ICMR centres for advance research in virology, Christian Medical College Hospital, Vellore as per the procedure described by Jesudoss et al., (1978) and was also

repeated at Madras Veterinary College, Madras 7, by using 2 per cent agarose in Tris-barbitone buffer pH 8.6. Wells were cut with a distance of 3 mm between the two wells. The fluid of the each sample was put in the negative terminal and the SA11 antiserum in the positive terminal. The slide was then placed in a electrophoretic tank containing the buffer. The slide was connected with a filter paper between the terminals. Current was applied with 150 volts for 30 minutes. After electrophoresis, the slides were washed overnight in cold saline (0.15M) and for one hour in distilled water. They were dried at room temperature and stained with Coomassie blue for fifteen minutes. The slides were thereafter destained with destaining solution consisting of ethanol (96%) 4 parts, glacial acetic acid 1 part and distilled water 4 parts. The slides were observed for the presence of precipitating lines against each sample. In all the tests SA11 MK3 Rotavirus antigen was used as control.

A similar procedure was repeated at our laboratory using antiserum prepared in rabbit inoculated with A126 antigen (which was positive against SA11 serum) against A112, A142 and normal cell culture fluid as control.

3. Results and Discussion

1) Demonstration of faecal Rotavirus by CIEP: Out of 44 dung samples (25 from diarrhoeic and 19 from apparently healthy calves) screened in CIEP for the presence of Rotavirus against SA11 antiserum, 37 samples were found to be positive accounting for 84.1 per cent (Figures 30 and 31).

Among the 25 diarrhoeic and debility samples screened, 23 samples were found to be positive accounting for 92 per cent (Table 23) which closely resembled the findings of Bridger and Woode (1975) who reported the presence of Rotavirus in 88 per cent of the diarrhoeic sample when examined by immunofluorescence and electron microscope. Other workers had also reported about the presence of Rotavirus in 53.8 per cent of the faecal samples in one herd and 72.3 per cent in another herd by employing electron microscope and virus isolation methods (Frey et al., 1979).

Among the 19 apparently healthy samples screened, 14 samples were found to be positive (Figure 31) for Rotavirus accounting for 73.7 per cent (Table 24). In District Livestock Farm, Orathanad, it was seen that apparently healthy calves were completely free from Rotavirus, whereas Government Dairy Farm, Madhavaram did show the presence of Rotavirus whether or not they were diarrhoeic or healthy. Probably in this farm

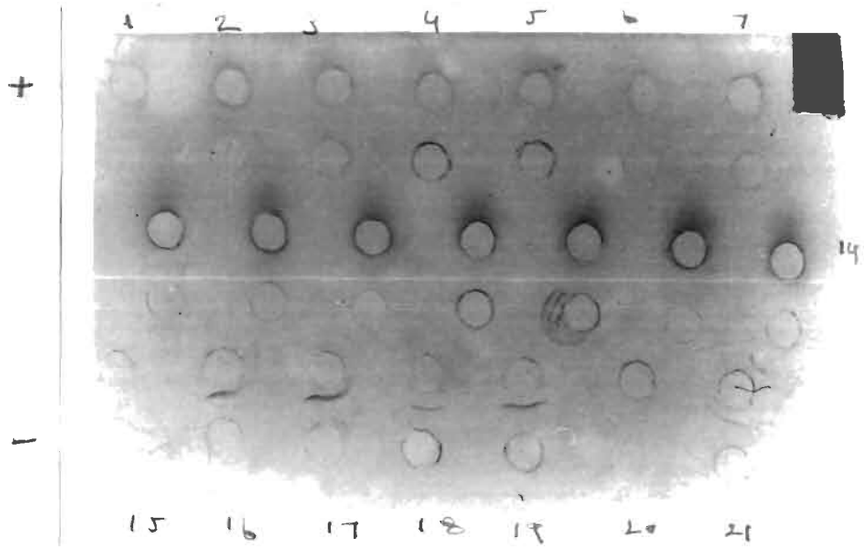

Figure 30: Wells 16 and 17 represents tissue culture isolates A112 and A126 respectively showing sharp precipitating line against SA11 antiserum. Wells 18, 19, 20 and 21 represents dung samples of 0.553, 0.554, Loyola College sample and Aliken (private dairy) sample respectively all diarrhoeic samples showing clear precipitating lines against SA11 antiserum - as seen in counter immunoelectrophoresis.

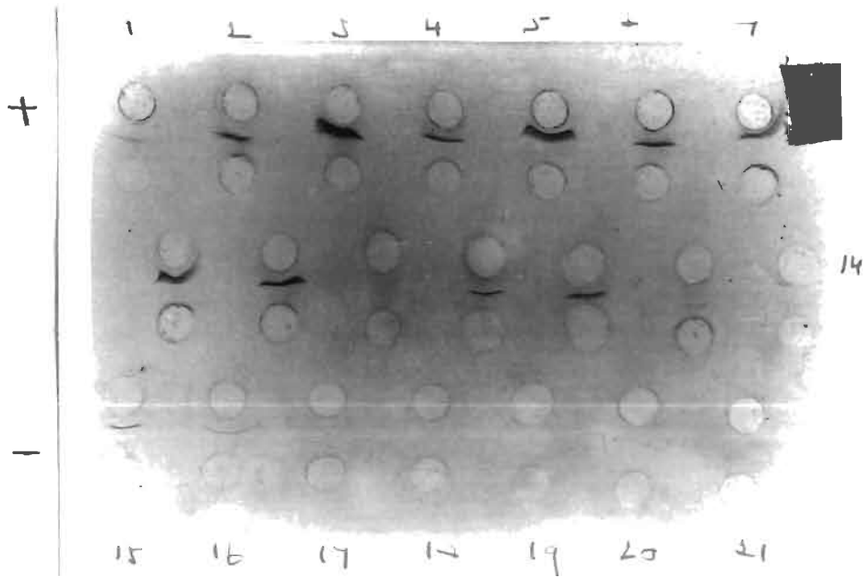

Figure 31: Well No.1 represents SA11 tissue culture fluid rotavirus antigen and shows sharp precipitating lines against SA11 antiserum. Well No. 2 representing E652 diarrhoeic sample showing precipitating line. Well Nos. 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16 represent dung samples from apparently healthy calves of K123, K151, K163, P745, P814, P818, P928, X405, X407, X408, X413, X414 and X419 respectively showing specific precipitating lines against SA11 antiserum.

Well Nos. 17, 18, 19, 20 and 21 represents dung samples also from apparently healthy calves of 0.519, 0.529, 0.535, 0.539, 0.540 and 0.544 respectively showing no specific lines indicating negative against SA11 serum as seen in counter immune electrophoresis (CIEP).

Table 23

Diarrhoeic dung samples screened in counter immunoelectrophoresis

S.No.	Date of collection.	Place of collection	Animal number	Breed	Symptoms if any of the animal		General condition	Whether positive or negative against SA11 antiserum
					Enteric	Respiratory		
1.	29.6.79	District Livestock Farm, Orathanad.	0.542	Jersey cross	DIA	-	DE	+
2.	do		0.553	Sindhi	DIA	-	DE	+
3.	do	,,	0.554	do	Mushy	-	DE	+
4.	do	,,	0.546	Buffalo	SEMI	-	GD	+
5.	do	,,	0.519	Jersey cross	-	-	DE	+
6.	29.7.79	Government Dairy Farm, Madhavaram.	K115	Cross breed	DIA	-	DE	+
7.	do		N119	do	Mushy	-	DE	-
8.	do	,,	N200	do	Mushy	-	DE	+
9.	do	,,	N228	do	Mushy	-	DE	+
10.	do	,,	N279	do	Mushy	-	DE	+
11.	do	,,	B803	Buffalo	Mushy	-	-	+
12.	31.8.79	,,	K143	Cross bred	DIA	-	GD	+
13.	do	,,	K145	do	DIA	-	DE	+
14.	do	,,	K153	do	SEMI	-	DE	+
15.	do	,,	K894	do	DIA	-	W	+

Table 25 (contd)

S.No.	Date of collection	Place of collection	Animal number	Breed	Symptoms if any of the animal		General condition	Whether positive or negative against SA11 antiserum
					Enteric	Respiratory		
16.	27.10.79	Government Dairy Farm, Madhavaram	K172	Cross bred	Mushy	-	DE	+
17.	26.11.79		N280	do	DIA	-	DE	+
18.	do	,,	E652	do	DIA	-	DE	+
19.	do	,,	R55	do	DIA	-	DE	+
20.	do	,,	B320	do	SEMI	-	W	+
21.	1.12.79	Private dairy unit, Kilpauk, Madras	-	do	DIA	-	GD	+
22.	10.12.79	Loyala College Madras	-	do	DIA	-	GD	+
23.	7.1.80	Livestock Research Station, Kattupakkam	MB93	Buffalo	Mushy	RESP	DE	+
24.	do		MB94	do	DIA	-	W	+
25.	do		MB95	do	Mushy	-	W	+

DIA: Diarrhoea
DE: Debility
Mushy: Fatty stool
RESP: Respiratory symptoms
SEMI: Semisolid

GD: Good
W: Weak
- Negative
+ Positive

Table 24

Dung sample from apparently healthy calves screened in CLRP

S.No.	Date of collection.	Place of collection	Animal Number	Breed	Symptoms if any of the animal		General condition	Whether positive or negative against SA11 antiserum
					Enteric	Respiratory		
26.	29.6.79	District Livestock Farm, Orathanad.	0.529	Jersey cross	-	-	GD	-
27.	do		0.535	do	-	-	GD	-
28.	do	,,	0.539	Sindhi	-	-	GD	-
29.	do	,,	0.540	do	-	-	GD	-
30.	do	,,	0.544	do	-	-	GD	-
31.	29.7.79	Government Dairy Farm Madhavaram	K123	Cross bred	-	-	GD	+
32.	31.8.79		K151	do	-	-	GD	+
33.	27.10.79	,,	K163	do	-	-	GD	+
34.	26.11.79	,,	P745	do	-	-	GD	+
35.	do	,,	P814	do	-	-	GD	+
36.	do	,,	P818	do	-	-	GD	+
37.	do	,,	P928	do	-	-	W	+
38.	do	,,	E652	do	-	-	W	+

Table 24 (contd)

S.No.	Date of collection.	Place of collection	Animal number	Breed	Symptoms if any of the animal		General condition	Whether positive or negative against SA11 antiserum
					Enteric	Respiratory		
39.	27.9.79	Agricultural College, Coimbatore	X405	Cross bred	-	-	GD	+
40.	do		X407	do	-	-	GD	+
41.	do	,,	X408	do	-	-	GD	+
42.	do	,,	X413	do	-	-	GD	+
43.	do	,,	X414	do	-	-	GD	+
44.	do	,,	X419	do	-	-	GD	+

DE: Debility
GD: Good
W: Weak
DIA Diarrhoea
\+ Positive
\- Negative

the infection of Rotavirus had run right through the neonates. This finding is in line with the reports of Albrey and Murphy (1976) that Rotavirus was found to be excreted in 52 per cent of the neonates without any accompanying diarrhoea and the findings of Woode and Bridger (1975) who had observed the excretion of virus from clinically normal calves. It is probable that severe disease of the small intestine of young animals producing malabsorption syndrome and thus loss of essential nutrients would have permanent effect on the general metabolism leading to growth retardation (Woode and Bridger, 1975). Hence Rotavirus could be a major factor in the causation of diarrhoea as well causing recurrent infection within the herd by virtue of harbouring in the clinically normal calves. The demonstration of Rotavirus in calves in India had not been reported so far.

ii) Identification of Rotavirus isolate in CIEP: The two tissue culture positive isolates, namely A112, A126 were also found to be positive in CIEP against SA11 antiserum indicating that the isolates were Rotavirus (Figure 30). The serum raised in rabbits against A126 cross-reacted with A112 but did not react with A142, a different isolate and with normal cell culture fluids. The SA11 MKP3 and P6 Rota antigen which were used as control also gave positive results in all the tests done (Figure 31).

The characters described for A112 and A126 (Chapter IV) in addition to the occurrence of distinct precipitation are in CIEP suggested that the isolates were Rotavirus.

4. Summary

Counter immuno-electrophoresis (CIEP) had been employed to demonstrate the presence of faecal Rotavirus in the dung samples of calves showing diarrhoeic symptoms as well as those which were apparently healthy. There was a slightly higher percentage (92%) of Rotavirus isolation in CIEP in the dung of diarrhoeic calves than in apparently healthy calves having a percentage of 73.7. In all, 84.1 per cent of dung samples examined showed the presence of Rotavirus.

The two virus isolates (A112 and A126) were identified as Rotavirus giving a positive specific arc against SA11 antiserum in CIEP. Isolates A112 and A126 showed similar arcs against their own type specific antiserum and were also found to be cross-reactive with each other.

Chapter VI

COLLECTION OF SAMPLE AND PROCESSING FOR ELECTRON MICROSCOPIC STUDY

1. Introduction

Viruses were among the first biological entities to be examined in electron microscope when it first became a practical instrument for biological research in the late 1930's (Habel and Salzman, 1969). The vast improvements in the instrument and the development of various preparations, techniques have greatly widened the scope of electron microscopy in virology (Habel and Salzman, 1969). For example those viruses which did not grow in routine cell cultures and the small virus like Small Round virus (SRV), Astro virus and Calicivirus which were found along with other virus like Adenovirus, Rotavirus could be seen and detected only by means of the electron microscope and the identification of different viruses with electron microscope depends on the recognition of characteristic morphological features (Madeley, 1979).

2. Materials and Methods

a) Collection and processing: Dung samples from 59 calves 30 diarrhoeic and 29 non-diarrhoeic, were collected in sterile

tubes from different farms and were transported in liquid nitrogen at -196°C to the laboratory where they were stored at -20°C for further processing.

The 30 diarrhoeic samples were processed at Christian Medical College Hospital, Vellore following the method of Flewett (1973) with little modifications. 10 ml of 20 per cent dung suspension was made in phosphate buffer solution (PBS) in a centrifuge tube which was prerinsed with nutrient broth with 0.05 percent bovine albumin. Preliminary centrifugation of the suspension was spun in a refrigerated centrifuge (angle type) at 10,000 g for 30 minutes. The supernatant obtained, was again centrifuged at 25,000 g for 120 minutes in a refrigerator ultracentrifuge. The pellet was then resuspended in 0.5 ml to 1 ml sterile distilled water and homogenised.

The 29 non-diarrhoeic samples were first centrifuged at 5000 rpm in a refrigeration centrifuge in our laboratory for one hour and the supernatant was centrifuged at 50000 g for 1 hour in a MSE refrigeration centrifuge attached to the Biochemistry department of University of Madras at Adyar campus. The pellet [redacted] was then resuspended in 0.5 ml to 1 ml sterile distilled water for staining and screening under electron microscope (EM).

b) Staining the sample for EM examination: A drop of the suspension was placed on a carbon-formvar electron microscope grids and left for 10 minutes for sufficient adsorption of viral agents, the excess fluid was drained by blotting. A drop of 3 per cent ammonium molybdate solution was then placed on the grid as suggested by Grist et al., (1979) and left for 10 minutes, the excess stain was drained and dried in a dessicator. The grids were then examined under EM, 100 Jem C, Joel Japan.

Photomicrographs were taken by using electron sensitive Kodak fine grain plates.

3. Results and Discussion

Electron microscopic examinations of the dung samples from neonatal calves had revealed a host of viruses. These viruses could be classified into three groups as was done by Madeley (1979) in human stools. Group I included those viruses that can be recovered from stools by cell culture like Adenovirus, Parainfluenza virus, etc. Group II includes those viruses that cannot be grown routinely in cell cultures, and group III comprised the bacteriophage and other viruses which are parasitic on the bacterial flora of the gut. The viruses observed in faecal samples under electron microscopic study are presented in Table 27.

Group I viruses: Adenovirus, Parainfluenza virus, Paramyxovirus and Enteroviruses were observed under the electron microscope in the faecal samples (Table 27). These are the viruses that could be grown in the routine tissue culture and are demonstrable under electron microscope.

Adenoviruses with icosahedral bright profile of 60-80 nm (closed arrows - Figure 32) were observed in the dung sample. Further in the same field of observation numerous small regularly spherical satellite viruses (open arrows - Figure 32) and bacteriophages (dotted arrows - Figure 32) which are spherical bodies with filamentous tail were observed.

Parainfluenza virus with lipoprotein envelope filled with tubular nucleocapsid were also observed (Figure 33). Strands of such tubular capsids of size 18 nm were seen extruding out of the viral envelope (closed arrow - Figure 33) like springs and were also seen free in the area (open arrow - Figure 33).

A partially disrupted Paramyxovirus ( ) showing loosely packed tubular structures which are helical nucleocapsid and Enteroviruses of size 30 nm were also observed in the faecal samples under electron microscope.

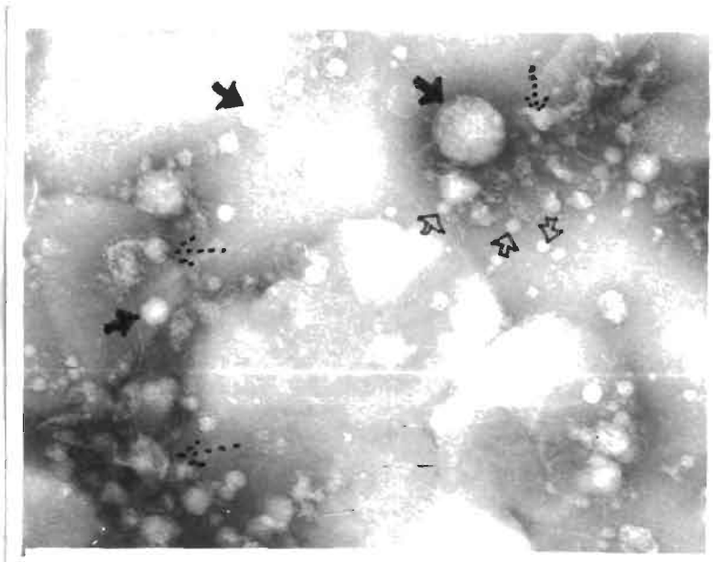

Figure 32: Electron micrograph of a dung sample showing one large Adenovirus with a bright icosohedral profile of 60-80 nm (closed arrow) and numerous small regularly spherical statellite viruses (open arrows) and other bacteriophage like bodies (dotted arrows) with spherical objects attached with filamentous tail. Direct microscopic magnification 50,000X. Photographic magnification 1,50,000X.

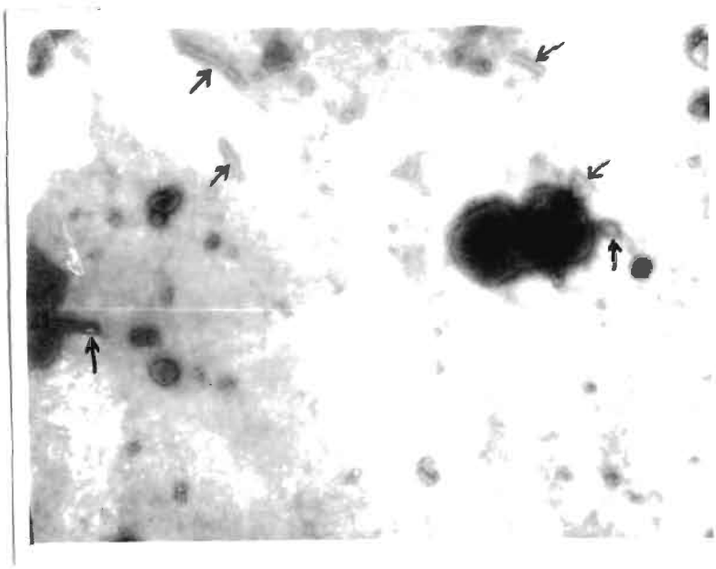

Figure 33: Parainfluenza virus showing lipoprotein envelope filled with numerous linear tubular strands of nucleocapsid. Strands of such capsids are seen projecting out of the envelope and broken bits of strands are seen free in the field. Direct microscopic magnification 26,000X. Photographic magnification 78,000X

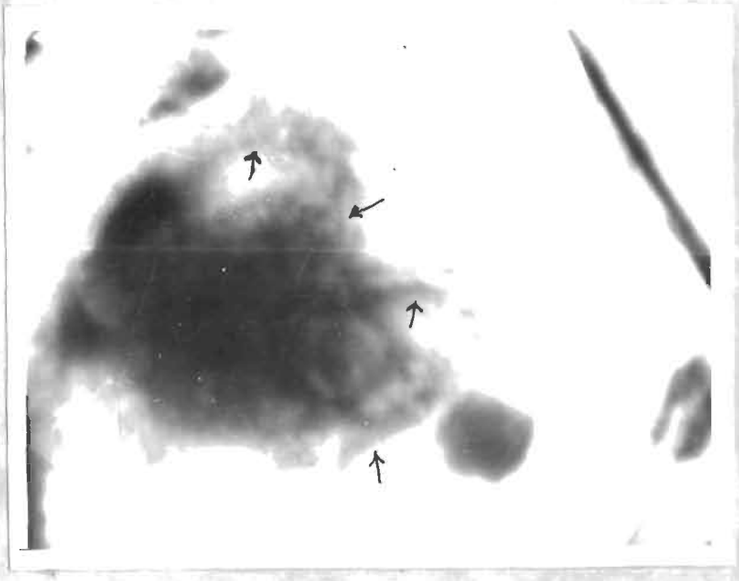

Figure 34: Paramyxovirus: partially disrupted paramyxovirus showing tubular structures of the helical nucleocapsid. Direct microscopic magnification 20,000X.
Photographic magnification 60,000X

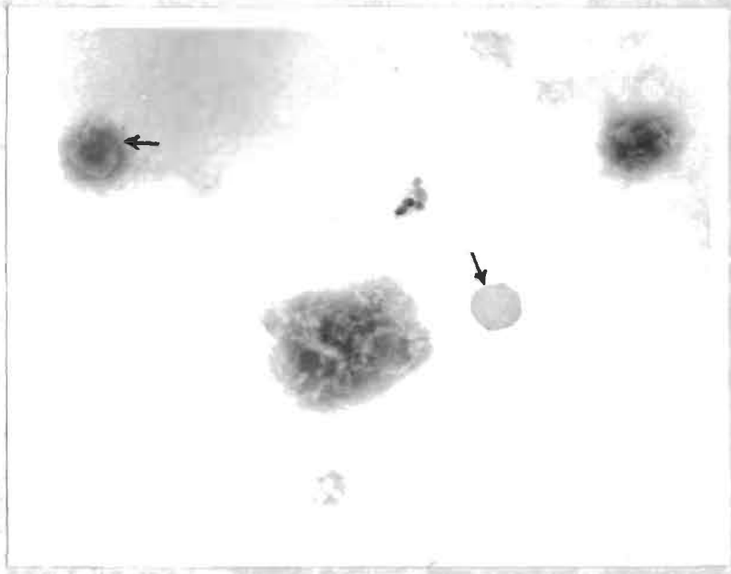

Figure 35: Enterovirus (closed arrows) particles of size 30 nm with icosohedral in shape.
Direct microscopic magnification 50,000X
Photographic magnification 2,25,000X

Group II viruses: Rotaviruses, Coronavirus and Small Round viruses were the other animal viruses (Table 27) which could be demonstrated in the dung samples. These viruses could not be grown in the routine cell culture technique (Madeley, 1979). Prominent among these is Rotavirus which showed the characteristic feature of electron dense central hub with radiating capsomeres with an outer shell (Figure 34). In many samples small round viruses of size 30 nm with a central electron dense viral genome were seen (Figure 35). In one sample membrane bound structures were found packed with numerous irregular pleomorphic coronavirus (?) without any corona jewels (Figure 36).

Group III viruses: In addition to the above animal viruses in the dung sample, many other bacterial viruses and phages with or without tail were seen in the faecal samples (Table 27). Figure 32 shows the presence of tailed bacteriophages (dotted arrow). These bacteriophages are normal inhabitants of the intestine which are not implicated in any disease (Madeley, 1979) and hence will not be discussed further.

The results of diarrhoeic faecal samples examined under electron microscope are presented in Table 25 and the non-diarrhoeic samples in Table 26.

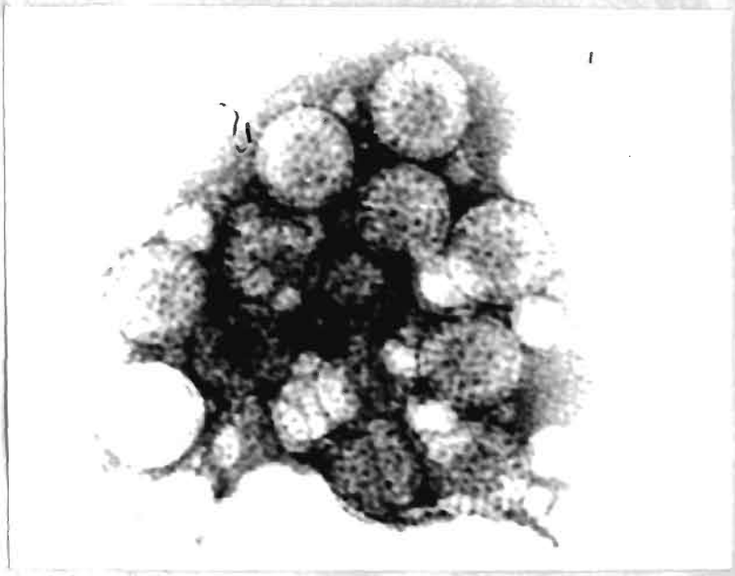

Figure 34: Rotavirus: Characteristic feature of electron dense central hub with radiating capsomeres with outer additional shell consisting of capsomeres giving an appearance of a sharply defined rim attached short spokes upon a wide hub.

Direct microscopic magnification 20,000 X
Photographic magnification 60,000 X

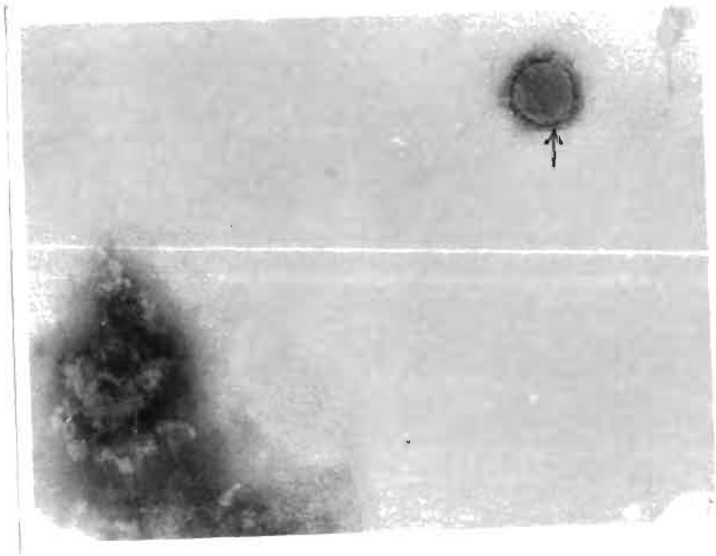

Figure 35: Small round viruses (SRV). A high magnification of small round virus with a spherical contour of size 30 nm filled with electron dense viral genome.
Direct magnification 66,000X
Photographic magnification 2,30,000X

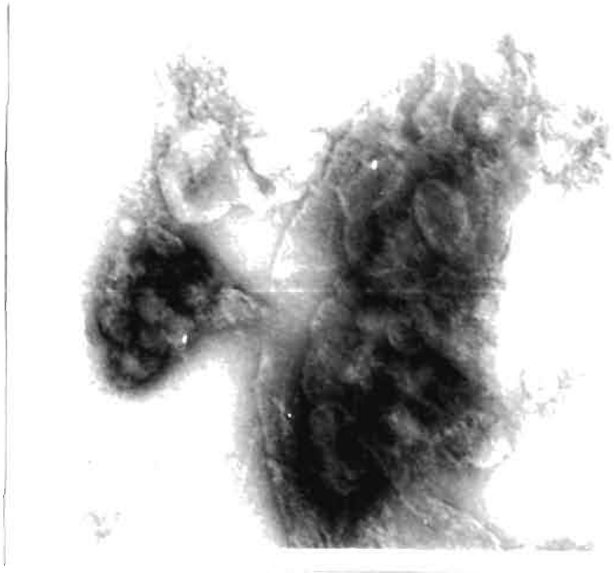

Figure 36:

"Other agents" seen under EM.

Direct microscopic magnification 50,000X

Photographic magnification 2,00,000X

Table 25

Diarrhoeic Dung samples examined under electron microscope

S.No.	Date of collection.	Place of collection	Animal number	Breed	Symptoms if any of the animal		General condition	Results of electron microscopy
					Enteric	Respiratory	GD/DE/W	
1.	29.6.79	District Livestock Farm, Orathanad.	O.542	Jersey cross	DIA	-	DE	Rotavirus
2.	do		O.546	Buffalo	SEMI	-	GD	Rotavirus
3.	do	,,	O.553	Sindhi	DIA	-	DE	Rotavirus; Paramyxovirus & SRV
4.	do	,,	O.544	do	Mushy	-	DE	Rotavirus; Enterovirus
5.	29.6.79	Government Dairy Farm Madhavaram	K115	Cross bred	DIA	-	DE	Rotavirus & SRV
6.	do		M119	do	Mushy	-	DE	Rotavirus
7.	do	,,	M200	do	Mushy	-	DE	Rotavirus & SRV
8.	do	,,	M228	do	Mushy	-	DE	Rotavirus & SRV
9.	do	,,	M279	do	Mushy	-	DE	SRV & Enterovirus
10.	do	,,	B803	Buffalo	Mushy	-	DE	SRV
11.	31.8.79	,,	K143	Crossbred	SEMI	-	GD	Rotavirus & SRV
12.	do	,,	K145	do	DIA	-	DE	Rotavirus & SRV
13.	do	,,	X834	do	SEMI	-	GD	SRV
14.	27.10.79	,,	K153	do	DIA	-	DE	SRV
15.	do	,,	K172	do	Mushy	-	DE	Rotavirus & SRV
16	26.11.79	,,	R55	do	DIA	-	DE	Rotavirus & SRV
17.	do	,,	M280	do	DIA	-	DE	Rotavirus & SRV

Table 25 (contd)

S.No.	Date of collection	Place of collection	Animal number	Breed	Symptoms if any of the animal		General condition	Results of electron microscopy
					Enteric	Respiratory	GD/DE/W	
18.	26.11.79	Govt. Dairy Farm, Madhavaram	E652	Crossbred	DIA	-	DE	Rotavirus & SRV
19.	do		P820	do	SEMI	-	W	Rotavirus
20.	1.12.79	Private dairy unit, Madras.	-	do	DIA	-	GD	Enterovirus
21.	10.12.79	Loyola College Madras.	-	do	DIA	-	GD	Coronavirus(?) and SRV
22.	7.1.80	Livestock Research Station, Kattupakkam	MB93	Buffalo	Mushy	RESP	DE	Rotavirus; Para-influensa & SRV
23.	do		MB94	do	DIA	-	W	Adenovirus & SRV
24.	do	,,	MB95	do	Mushy	-	W	Rotavirus and Adenovirus
25.	7.4.80	Private dairy unit Madhavaram	1198	Crossbred	DIA	-	W	SRV
26.	do		DM429	do	DIA	-	DE	SRV
27.	do	,,	T975	do	DIA	-	DE	SRV
28.	do	,,	GNS	do	DIA	-	DE	SRV
29.	do	,,	R55	do	DIA	-	DE	SRV
30.	do	,,	K198	do	DIA	-	DE	SRV

DE: Debility
W: Weak
GD: Good
RESP: Respiratory symptoms

Mushy: Fatty stool
SEMI: Semisolid
DIA: Diarrhoea
SRV: Small Round Virus

Table 26

Nondiarrhoeic dung samples examined under electron microscopy

S.No.	Date of collection.	Place of collection	Animal number	Breed	Symptoms if any of the animal		General condition	Results of electron microscopy
					Enteric	Respiratory	GD/DE/W	
31.	29.6.79	District Livestock Farm, Orathanad	0.515	Sindhi	-	-	GD	-
32.	do		0.516	Buffalo	-	-	GD	-
33.	do	,,	0.519	Jersey cross	-	-	DE	-
34.	do	,,	0.521	Buffalo	SEMI	-	GD	Enterovirus;SRV
35.	do	,,	0.529	Jersey cross	-	-	GD	-
36	do	,,	0.531	do	CONS	-	DE	SRV
37.	do	,,	0.535	do	-	-	GD	SRV
38.	do	,,	0.538	Sindhi	-	-	GD	SRV
39.	do	,,	0.539	do	-	-	GD	-
40.	do	,,	0.540	do	-	-	GD	-
41.	do	,,	0.543	Buffalo	-	-	GD	-
42.	do	,,	0.544	Sindhi	-	-	GD	-
43.	do	,,	0.547	do	-	-	GD	-
44.	do	,,	0.548	Buffalo	-	-	GD	-
45.	do	,,	0.549	Jersey cross	-	-	GD	SRV
46.	do	,,	0.551	Sindhi	-	-	GD	-

Table 26 (contd)

S.No.	Date of collection	Place of collection	Animal number	Breed	Symptoms if any of the animal		General condition	Results of electron microscopy
					Enteric	Respiratory	GD/DE/W	
47.	27.10.79	Govt.Dairy Farm, Madhavaram	K123	Crossbred	-	-	GD	Enterovirus & SRV
48.	do		K151	do	-	-	GD	-
49.	do	,,	K163	do	-	-	GD	SRV
50.	do	,,	P745	do	-	-	GD	-
51.	27.10.79	,,	P814	do	-	-	GD	SRV
52.	do	,,	P818	do	-	-	GD	SRV
53.	do	,,	P928	do	-	-	GD	Parainfluenza
54.	27.10.79	Agriculture College, Coimbatore	X405	do	-	-	GD	-
55.	do		X407	do	-	-	GD	SRV
56.	do	,,	X408	do	-	-	GD	-
57.	do	,,	X413	do	-	-	GD	-
58.	do	,,	X414	do	-	-	GD	SRV
59.	do.	,,	X419	do	-	-	GD	-

GD: Good
DE: Debility
W: Weak

SEMI: Semisolid dung
CONS: Constipated
SRV: Small Round Virus

Table 27

Viruses found in dung samples of calves under electron microscope

Group	No. of samples
1. Viruses that can be grown and passaged	
a) Enterovirus	5
b) Adenovirus	2
c) Parainfluenza	2
2. Viruses detected by electron microscope	
a) Rotavirus	17
b) Small Round Virus	34
c) Other agents	1
3. Bacteriophages	
a) Tailed phages	multiple

Of the 30 samples from diarrhoeic calves screened, all the samples showed the presence of some virus or other, out of which 17 samples showed the presence of Rotavirus either alone or in combination with other viruses like Small Round Virus (SRV), Paramyxovirus, Parainfluenzavirus and Adenovirus, whereas SRV was seen in 23 samples either alone or in combination with others. SRV alone was seen in 9 samples and Rotavirus alone was seen in 4 samples. Three calves with diarrhoea showed the presence of Enteroviruses. Paramyxovirus along with Rotavirus and SRV was seen in one calf which was showing diarrhoea and debility (Table 25). One calf showed the presence of Other agents along with SRV. The Coronavirus did not show the presence of the peplomer of corona jewel. Parainfluenza Virus was seen in a debilitated calf that showed respiratory symptoms along with mushy dung. This calf showed the presence of Parainfluenza, Rota and SRV.

Of the 29 samples from non-diarrhoeic calves, 11 showed the presence of SRV, one showed Parainfluenza virus and two Enterovirus in combination with SRV (Table 26).

Small Round Virus of size 20-30 nm was more or less ubiquitous in diarrhoeic as well as in non-diarrhoeic dung sample. Enteroviral excretion with no evidence of illness is often found in young children (Bell et al., 1961;

Patterson and Bell, 1963) and this has to be borne in mind when considering the role of other viruses found in the gut (Madeley, 1979). But in case of the presence of Rota Viruses it should be borne in mind that morphologically similar viruses had caused diarrhoea in gnotobiotic animals (Middleton et al., 1975; Mebus et al., 1977; Snodgrass et al., 1977).

4. Summary

Thirty dung samples from diarrhoeic calves and 29 non-diarrhoeic calves were subjected to high speed centrifugation to pellet the viruses in them and were examined under electron microscope. Electron microscopy revealed the presence of Rotavirus, Small Round Viruses, Adenoviruses, Enteroviruses, Paramyxovirus, Parainfluenza Virus and Other agents Rotaviruses were seen associated with diarrhoeic samples and Adenovirus was found in a weak diarrhoeic calf. A calf with respiratory symptoms and diarrhoea showed a mixed infection of Parainfluenza, Rota Virus and SRV.

Chapter VII

COMPOSITE PICTURE OF THE STUDY OF RESPIRATORY AND ENTEROPATHOGENIC VIRUSES IN CATTLE

A study on the respiratory and enteropathogenic viruses in cattle especially calves were undertaken in the hope that our knowledge about the occurrence of viral agents in neonatal calves and their disease potential could be extended. Such a knowledge about the role of virus infection in neonatal calves would go a long way in the progressive livestock breeding and calf rearing programmes in India in preserving the cross-bred calves from morbidity and mortality due to viral infections.

Apparently healthy and diarrhoeic calves of age generally less than 3 months were selected for the study since older calves are likely to develop resistance against these infections. In order to obtain a widespread picture of the incidence of viruses in calves various livestock farms which were involved in cross-bred calf rearing projects, were chosen and were studied during the different seasons of one calendar year.

For the isolation of respiratory and enteropathogenic viruses in calves, throat swabs and rectal swabs were collected since these viruses were predominantly seen in them. Care was

taken to employ serum-free maintenance medium as a vehicle for the rectal and throat swabs to avoid preneutralisation of the viruses due to the presence of specific antibodies in the serum and the samples were straightaway quenched in liquid Nitrogen at -196°C and then transported to the laboratory wherein the samples were inoculated into calf kidney cell culture without undue delay in the storage at -20°C.

The routine procedure of calf kidney cell culture with a slight modification of avoiding two times washing of trypsinised kidney cells with Hanks' BSS and addition of whole serum to completely inhibit the action of trypsin was carefully standardised with a view not to avoid the presence of trypsin absolutely. Probably this procedure had helped in the present study to isolate Rotaviruses which needed traces of trypsin at 5 ug/ml for isolation (Babiuk et al., 1977). In the present study the calf kidney cell culture was found to be useful in giving a good number of isolates of cytopathogenic agents which were classified basdd on the nature of CPE. Accordingly the isolates were identified as belonging to Adenovirus group, Enterovirus group and Rotavirus group. For the preparation of stock virus isolate, for the titration of the stock and to study the biophysical, biochemical, cytological, cytochemical and serological characters of the representative isolates of

these above groups, calf kidney cell culture was employed and was found to be useful.

Livestock farms where large scale calf rearing schemes were in progress did present a higher overall incidence of viral agents in young calves. Adenoviruses, Enteroviruses and Rotaviruses were found to be present in every farm and was more in South-West monsoon and Winter seasons. Madeley (1979) had found that children may be found to excrete Enteroviruses and other viral agents where there was overcrowding, poor sanitation and hygiene. It is possible that in Tamil Nadu, inclement monsoon weather would give rise to a poor sanitary and hygienic conditions in the farm, resulting in more incidence of virus infection.

An analysis of age susceptibility to virus incidence had brought to light that calves of 1 to 2 months of age were worst affected followed by the age group of 2 to 3 months and 15 days to 1 month with no incidence in 0-15 days. Hence it could be said that colostral antibody is very essential in affording resistance to these bouts of virus infection and that the colostral antibodies may wane away completely and natural infection from apparently healthy calves in the herd takes the upper hand. Since apparently healthy calves could act as a carrier and become a source of infection for those calves which had less amount of colostrum. Holmes et al., (1974) and McNulty (1978) had

reported similar findings in their study of the role of colostral antibodies in the viral infections of newborn calves.

Statistical analysis of the type of viruses isolated had revealed that Enteroviruses were more commonly isolated followed by Adenovirus and then Rotavirus, which could well be anticipated when primary calf kidney cell culture alone was used for isolation studies. But the presence of Rotavirus in calves in India had not been reported so far and the present study had revealed the occurrence of Rotavirus in almost every farm in which the study was undertaken. Hence further investigation is warranted in finding out the aetiopathogenic significance of Rotavirus in calves.

Only one or two representative isolates of each group of tissue culture cytopathogenic agents had been studied in detail with a view to identify them. It was found that one isolate from Adenovirus group was identified as Adenovirus Type 5 which is responsible for weak calf syndrome, two from Rotavirus group was taken and identified as Rotavirus and one from Enterovirus group as Enterovirus. Other samples could not be identified for want of specific sera and time.

While identifying these virus isolates, ether sensitivity test, size determination by millipore filtration and EM studies, cationic stabilisation to heat at 50°C, cytological and

cytochemical studies to find out the property of the type of viral inclusions and the nucleic acid contained in them, serum neutralisation test and counter immuno-electrophoresis were employed.

Of these techniques, counter immuno-electrophoresis and electron microscopic screening of dung samples were further employed to investigate the presence of viral agents in some samples collected from diarrhoeic and non-diarrhoeic calves.

Counter immunoelectrophoresis was found to be easily operated since it did not involve any costly instruments like electron microscope. Further it was quick and quite specific in their identification. As a matter of fact just with 1/2 hour of actual electrical run and within another 12-24 hours results could be made available in typing the faecal or tissue samples upto the specific seretype if only the type specific sera is available. It was reported that counter immuno-electrophoresis was four times more sensitive than agar gel immunodiffusion regardless of which the antigen was either faecal or tissue antigen (Mohammed et al., 1978). Further, McNulty (1978) while reviewing the various techniques of identifying the presence of Rotavirus in faecal sample reported that opinions on the sensitivity of counter immunoelectrophoresis differ and that electron microscopic examination of faecal sample

would be better than CIEP (Middleton et al., 1976) and Grauballe et al., (1977) found CIEP was more sensitive than electron microscopy.

In the present study it was found that CIEP was really useful in bringing out the presence of 92 per cent Rotavirus isolation in the dung of diarrhoeic calves and 73.7 per cent of the same in non-diarrhoeic calves. Whereas electron microscopic study of faecal samples revealed the presence of Rotavirus in diarrhoeic stools rather than in non-diarrhoeic stools. This is probably due to the fact that for electron microscopic study one may require at least 10^6 to 10^7 viral particles per 0.1 ml for easy demonstration of the agent (Grist et al., 1979). Such a concentration is possible only when there is active infection. But CIEP requires the presence of type-specific antiserum and to get type-specific antiserum one must be in a position to isolate the specific viral agent and prepare a stock in vitro. Madeley (1979) had observed many more viruses under electron microscope which could not be routinely isolated in cell cultures.

Hence electron microscopy was attempted in the present study to screen the incidence of respiratory and enteropathogenic viruses in calves. With a little amount of expertise

this method could also be adopted as a routine diagnostic method if the equipment is made available.

In the present study, the incidence of respiratory and enteropathogenic viruses of calves was studied by using the conventional tissue culture technique and identified the isolates by other conventional virological techniques as well as the sophisticated modern techniques like counter immuno-electrophoresis and electron microscopy.

Incidence of Adenovirus, Rotavirus and Enteroviruses were established in calves under 3 months old in every Livestock farm of the State wherein calf rearing programmes are adopted in large scale. In the present study the conventional tissue culture was quite useful in isolating Rotavirus, Adenovirus Type 5 and Enterovirus from the faecal samples of calves. This is the first report of the presence of Rotavirus in calf diarrhoea in India (John, 1980). Much work has to be undertaken in this direction to establish the aetiopathology of Rotavirus.

Besides the conventional tissue culture technique, Rotavirus was found to be present by CIEP and also by electron microscopy. The results of these sophisticated techniques confirmed the primary isolation of Rotavirus in calf diarrhoea and the incidence of other viruses in calves.

Further work is warranted in finding out the pathogenic potential of these viruses like Rotavirus, Adenovirus and Enterovirus in calves. Such a study would help in developing a polyvalent vaccine if need be in India as well against calf diarrhoea and other related syndrome as already in vogue in the western countries, thus preventing the heavy economic loss to the farmers who rear crossbred calves.

Chapter VIII

SUMMARY

Attempts to isolate viruses from throat and rectal swabs and faecal samples from diarrhoeic and non-diarrhoeic calves of age generally less than 3 months were undertaken in order to gain more insight into the aspects of viral aetiology of calf morbidity and mortality.

The conventional tissue culture technique and other modern virological techniques like counter immunoelectrophoresis and electron microscopy were employed to diagnose viruses from calves.

Two hundred samples of rectal swabs and throat swabs were screened for the presence of tissue culture cytopathogenic agents. Higher overall incidence of viral agents in livestock farms was observed where large scale calf-rearing schemes are in progress. Adenoviruses, Enteroviruses and Rotaviruses were found to be present. Incidence was found to be more in South-West Monsoon and Winter seasons. Calves 1 to 2 months of age showed the heaviest isolation rates followed by the group 2 to 3 months. There were no virus isolation in the age group 0-15 days demonstrating the importance and the activity of

colostral antibody. Symptomless carriers were also demonstrated by virtue of the fact that there were virus isolations in apparently healthy calves.

Various biophysical, biochemical, cytological, cytochemical, serological and counter immunoelectrophoretic (CIEP) studies were employed to characterise one or two of the representative isolates belonging to Adenovirus, Enterovirus and Rotavirus groups. Such a characterisation revealed the identity of Adenovirus Type 5, Rotavirus related to SA11 and bovine Enterovirus in calves studied. The present Rotavirus in calves is recorded for the first time in India.

Since Rotavirus isolations were obtained in the routine virus isolation studies and were easily identified as Rotavirus in CIEP, 44 faecal samples from calves with or without diarrhoea were screened for the presence of Rotavirus in CIEP. It was found that 92 per cent of diarrhoeic calves, 73.7 per cent of non-diarrhoeic calves and an overall per cent of 84.1 of dung samples examined, showed the presence of Rotavirus.

Direct examination of faecal viruses in calves were also done under electron microscope. 30 diarrhoeic and 29 non-diarrhoeic faecal samples were subjected to high speed centrifugation to pellet viruses present in the dung of calves. These pellets were stained and examined under electron

microscope. Electron microscopy revealed the presence of Rotavirus, Small Round Viruses, Adenoviruses, Enteroviruses, Paramyxovirus, Parainfluenzavirus, Corona-like viruses and bacteriophages. Rotaviruses were seen associated with diarrhoeic samples and Adenovirus was found in a weak diarrhoeic calf. A calf with respiratory symptoms and diarrhoea showed a mixed infection of Parainfluenza virus, Rotavirus and Small Round Virus.

The pathogenic potential of these viruses like Rotavirus, Adenovirus Type 5 and Enteroviruses in calves have to be studied in greater detail to understand their aetiologic and pathogenic significance by experimental infection so that a polyvalent vaccine if need be, could be evolved to protect against the natural infection of these viruses in calves. Such a study is warranted to prevent the heavy economic loss encountered by livestock breeders.

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