DETECTION OF ROTAVIRUS IN THE FAECES OF DIARRHOEIC CALVES BY REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION AND SILVER STAINING

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DECLARATION

I hereby declare that the thesis entitled **"DETECTION OF ROTAVIRUS IN THE FAECES OF DIARRHOEIC CALVES BY REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION AND SILVER STAINING"** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that the thesis entitled **"DETECTION OF ROTAVIRUS IN THE FAECES OF DIARRHOEIC CALVES BY REVERSE TRANSCRIPTASE – POLYMERASE CHAIN REACTION AND SILVER STAINING"** is a record of research work done independently by **Dr. Ambily. R.,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, associateship or fellowship to her.

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INTRODUCTION

1. INTRODUCTION

India is an agrarian country, where livestock sector forms the back bone of national economy. Cattle and buffaloes have been a part of rural society from ancient times. Milk, meat and manure contribute to the major source of their income. The infectious and noninfectious diseases affecting cattle and buffaloes have been a cause of great concern to farmers and veterinarians all over the world. Neonatal diarrhoea is one of the most important disease syndromes affecting calves, which causes substantial economic losses due to increased mortality, treatment costs and reduced growth rates. The etiology of calf diarrhoea is complex, often involving a multitude of infectious agents and a range of nutritional and environmental factors. The role of rotavirus as a cause of diarrhoea in calves was first demonstrated by Mebus *et al.* in 1968 at Nebraska Agricultural Experiment Station. The name given was Nebraska Calf Scours Virus, later called as Rotavirus (Flewett *et al.*, 1974). It is now recognized as the single most important viral agent responsible for gastroenteritis in the young ones of animals and children.

The virus belongs to Family *Reoviridae*. It consists of double stranded RNA with 11 segments having molecular weights in the range of 2.2×10^6 to 0.2×10^6 dalton (Todd and McNulty, 1977). Rotaviral diarrhoea is most common in calves between one to three weeks of age and this is related to rapid decline in specific colostral antibody (Radostits *et al.*, 2000). The clinical signs include profuse watery diarrhoea and dehydration which is similar to bacterial and dietary gastroenteritis, bovine viral diarrhoea and corona virus infection. Hence, an accurate laboratory diagnosis is essential for clinical and epidemiological management of the disease. Virus isolation (VI) in cell culture and electron microscopy (EM), although highly specific, are too time consuming and expensive. The conventional serological tests like Agar Gel Immuno Diffusion

(AGID), Counter Immuno Electrophoresis (CIE), Complement Fixation Test (CFT) and Haemagglutination Inhibition (HI) are less sensitive. Hence, the search for alternative methods for rapid diagnosis of bovine rotavirus (BRV) infection have been focused on nucleic acid based technique like RNA – Polyacrylamide gel electrophoresis (RNA-PAGE) and Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR).

The minimum amount of particles required to be detected in RT-PCR is 10^4 per ml and hence this is found to be more sensitive than Enzyme Linked Immuno Sorbent Assay (ELISA) which requires 10^6 particles per ml (Argüelles *et al.*, 2000).

Rotaviruses are assigned to various serogroups – A through G. Group A rotavirus in recognized as the common cause of neonatal diarrheoa in bovines. The serogroups are further classified into serotypes based on the specificity of outer capsid proteins VP7 (G types) and VP4 (P types). The characteristics of the virus at molecular levels and strain variation can be studied using advanced molecular techniques. Several works were carried out in this aspect in different parts of India. However, incidence of bovine rotavirus in Kerala has not been studied.

Keeping these in view, the present study was undertaken with the following objectives.

1. Detection of BRV in faecal samples of diarrhoeic calves by RNA-PAGE, RT-PCR and AGID

2. Attempts for isolation of BRV using cell lines (Madin Darby Bovine Kidney)

3. To study the influence of age on the occurrence of BRV infection.

4. To study the protein profile of BRV by SDS-PAGE.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 ROTAVIRUS

Rotavirus is the major etiological agent of acute gastroenteritis in a wide variety of species throughout the world with its greatest frequency and severity within first two weeks of life (McNulty, 1978).

2.2 HISTORICAL BACKGROUND

The virus was first isolated from faecal filtrates of diarrhoeic calves in 1968 at the Nebraska Agricultural Experiment Station (Mebus *et al.*, 1969).

The agent was originally called as Nebraska Calf Scours Virus (Mebus *et al.*, 1971).

It was later named as Reo-like virus because of its resemblance to the members of Family *Reoviridae* (Welch, 1971).

The term 'Rota virus' was first proposed by Flewett *et al.* (1974) due to the 'cart-wheel' like appearance of the virus under electron microscope. They found that the virus differed morphologically from both *Reo* and *Orbiviruses.*

Rotaviruses were named as 'duo virus' because electron micrographs depicted a double shelled structure and also they were frequently detected in duodenum (Mebus, 1975).

According to Woode *et al.* (1976), the virus causing Epizootic Diarrhoea of Infant Mice (EDIM) and the enteritis virus from pigs and foals were morphologically similar as well as antigenically related to human and bovine rotavirus.

2.3 INCIDENCE

2.3.1 Global

Subsequent to identification of BRV in1969, the disease was reported from various places in United States such as California, South Dakota and Illinois (White *et al.*, 1970).

There were reports of the disease from Australia (Turner *et al.*, 1973), Great Britain (Woode *et al.*, 1974), Scotland (Herring *et al.*, 1982), Japan (Fukai *et al.*, 1998), Sweden (de verdier Klingenberg *et al.*, 1999), Italy (Falcone *et al.*, 1999), Brazil (de Brito *et al.*, 2000), Turkey (Cabalar *et al.*, 2001) and Nigeria (Adah *et al.*, 2001).

2.3.2 India

An epidemic of human rotavirus diarrhoea occurred in Manipur, in 1979 (Gupta *et al.*, 1981).

Singh *et al.* (1993) studied the prevalence of rotavirus in cow calves and buffalo calves during 1987 in Haryana by RNA -PAGE and rotavirus could be detected in 34.5 per cent of diarrhoeic cow calves and 24 per cent of buffalo calves.

Electropherotyping of bovine rotavirus using RNA-PAGE was done by Gulati *et al.* (1995) and found that different electropherotypes had been prevailing in organized dairy farms in Haryana.

The prevalence of bovine rotavirus infection in organized dairy farms at Ambala and Meerut during 1995-'96 was studied by Grover *et al.* (1997) and observed that rotavirus was associated with 25 per cent of diarrhoeic cases.

Wani *et al.* (2004) conducted RT- PCR based detection of bovine rotavirus infections in Kashmir.

2.3.3 Kerala

An outbreak of rotaviral diarrhoea was reported in children less than five years in Palghat district of Kerala (Gupta *et al.*, 1995)

Arun *et al.* (2006) demonstrated rotavirus in diarrhoeic faeces of piglets by RNA-PAGE.

2.4 ETIOLOGY

2.4.1 Taxonomy

According to Estes and Cohen (1989), rotaviruses were assigned to seven serogroups –A through G – with group members sharing distinctive common antigens.

Bovine Rotavirus belongs to the Genus Rotavirus of Family *Reoviridae* (Murphy *et al.*, 1999).

Rotavirus serogroups were further classified into serotypes based on specificity of the outer capsid proteins VP7 (G types) and VP4 (P types). Currently, on the basis of antigenic and genetic characterizations, 15 G serotypes and 23 P types have been identified (Hoshino *et al.*, 2002).

2.4.2 Properties of the Virus

2.4.2.1 Morphology

Flewett *et al.* (1974) observed that rotavirus particles had a diameter of 60 to 66 nm consisting of a centre of 36 to 38 nm diameter bounded by a membrane from which short cylindrical capsomeres radiated outwards. Attached to the ends, an additional outer layer of capsomeres was there which gave the appearance of a wide hub. Rotaviruses are nonenveloped viruses with icosahedral symmetry and double layered capsid.

The genome is double stranded RNA consisting of 11 segments ranging in molecular weight from 2.2 x 10^6 to 0.2 x 10^6dalton (Todd and Mc Nulty., 1977).

Estes and Cohen (1989) reported that the virion consists of three concentric layers of proteins – two independent neutralizing antigens on the outer capsid namely Viral Protein 7 (VP7) encoded by 7, 8 and 9 segments and VP4 encoded by segment 4.

According to Mattion *et al.* (1994), the segments of rotavirus coded for six structural (VP1, VP2, VP3, VP4, VP6 and VP7) and five nonstructural proteins (NSP1 to NSP5). The structural proteins were located in the core (VP1, VP2, and VP3), inner shell (VP6) and outer shell (VP4 and VP7).

The inner layer of the virus, made of VP2, enclosed the genomic RNA and two minor proteins, VP1 and VP3, with which it formed the viral core (Berois *et al.*, 2003).

2.4.2.2 Physico Chemical Properties

Kasza *et al.* (1970) conducted heat stability test and found that porcine rotavirus was stable at 56°C for 120 min.

The double shelled calf rotavirus particles were degraded to single shelled stage when treated with β-galactosidase. There was no alteration in morphology when treated with proteases (Rodger *et al.*, 1977).

According to Gulati *et al.* (1995), bovine rotavirus could resist sonication, freezing and thawing. The virus was resistant to ether and chloroform. The virus could resist a temperature of 50°C for more than one hour. But, it was thermo labile when heated at 50°C in presence of magnesium chloride.

The survival of the bovine rotavirus in air and on surfaces was directly influenced by the level of relative humidity. They survived well in aerosol state

and medium range of relative humidity and air could act as one of the vehicles for virus dissemination (Radostits *et al.*, 2000).

2.4.2.3 Antigenic Properties

Antigenic similarities among human and calf rotavirus and Epizootic Diarrhoea of Infant Mice (EDIM) had been demonstrated by immuno electron microscopy where as no cross reaction between *Rota* and *Reo* or *Orbi* virus had been found (Flewett *et al.*, 1974).

According to Hoshino *et al.* (1983), animal and human rotaviruses showed some differences in their antigenic properties. Both VP4 and VP7 proteins of rotaviruses were involved in virus neutralization

Woode *et al.* (1974) studied the antigenic relationship among some bovine rotaviruses by cross protection studies in gnotobiotic calves and found that cross protection occurred only between rotaviruses of same serotype. Even a minor serotype difference is sufficient to show lack of protection.

Kapikian *et al.* (1987) reported that the strains of rotaviruses derived from animals and human showed antigenic similarity.

2.5 PATHOGENESIS

Holmes *et al.* (1976) suggested that the enzyme lactase present in the brush border of the intestinal epithelial cells interacted with the glycosylated surface polypeptide of calf rotavirus causing removal of the outer shell of the capsid. This hypothesis was consistent with the observation that rotaviruses could infect only gut epithelial cells. Infant animals, with high lactase concentration were more susceptible to infection than adults.

Lesions occurred within 24 h after experimental infection; villous epithelial cells of small intestine were infected and become detached. The cells returned to normal within seven days after recovery (Radostits *et al.*, 2000).

2.6 CLINICAL SIGNS

The clinical signs of rotavirus infection varied from inapparent infections to severe profuse watery diarrhoea with yellow coloured faeces. The temperature might be elevated or subnormal. The nose might be reddened and crusted. There could be excess salivation. Varying degrees of dehydration with accompanying electrolyte imbalances were evident prior to death (Mebus *et al.*, 1971).

The mortality was found to be highest in young animals which had received insufficient colostrum and those which were subjected to severe weather conditions. Explosive outbreaks occurred and up to 50 per cent of calves from five to fourteen days of age developed the disease (Radostits *et al.*, 2000).

2.7 EPIDEMIOLOGY

2.7.1 Natural Host Range

Rotavirus was first detected in calves (Mebus *et al.*, 1969). It was later found to infect a wider host range including piglets (Flewett *et al.*, 1974), lambs (Snodgrass *et al.*, 1976) and avian species (Woode *et al.*, 1976), and foals (Radostits *et al.*,2000).

According to Flewett and Woode (1978), rotaviruses were the major etiological agents of acute viral gastroenteritis in human infants and several species of domestic livestock.

Hoshino *et al.* (1981) detected feline rotavirus from diarrhoeic cats by electron microscopy.

Rotavirus of bovine origin had been isolated from faeces of dogs (Sharma and Adlakha, 1994).

2.7.2 Factors Influencing Infection

2.7.2.1 Age

Acres and Babuik (1978) observed that the onset of rotaviral diarrhoea occurred at around three to ten days of age or earlier.

Tzipori *et al.* (1981) reported that calves were susceptible to rotavirus infection only during their first week of life and clinical signs were observed only during this period.

The high rotaviral antibody prevalence among cattle and the onset of diarrhoea between three to ten days suggested widespread distribution of rotavirus, carrier state and age susceptibility. Animals confined to sheds and barns had a high incidence of the disease (Sharma and Adlakha, 1994).

Sato *et al.* (1997) isolated bovine rotavirus G8P6 [1] from adult cattle with diarrhoea.

Calves were most susceptible to rotavirus diarrhoea between one to three weeks of age and susceptibility was related in part to rapid decline in specific colostral antibody to rotavirus (Radostits *et al.*, 2000).

Nath *et al.* (2004) studied rotavirus infection in piglets and found that piglets of zero to two months of age were actively infected.

According to Wani *et al.* (2007), prevalence of rotavirus was more in one month old calves (44.23 per cent) followed by three week old calves (37.50 per cent).

2.7.2.2 Season

According to Singh *et al.* (1993), a high prevalence of calf rotavirus could be noticed during winter months (January to February). Rotavirus infection in buffalo calves was more prevalent during July to October, coinciding with

calving period of buffaloes. Relative humidity appeared to be the determining factor in survival and transmission of rotavirus. There was no effect for low temperature.

Gulati *et al.* (1995) noticed a high prevalence of rotavirus infection in calves in Hissar during winter months.

Grover *et al.* (1997) studied the prevalence of bovine rotavirus infection in organized dairy farms at Ambala and Meerut. They found that there were more incidences during July to August followed by December to February.

2.8 DIAGNOSIS OF ROTAVIRUS INFECTION

Rotavirus induced diarrhoea must be differentially diagnosed from dietary gastroenteritis and bacterial diarrhoea. But an accurate diagnosis was not easy (Acres and Babuik, 1978).

According to Moon *et al.* (1978), the clinical signs of rotaviral diarrhoea were similar to parvo virus and corona virus infections, bovine viral diarrhoea and infectious bovine rhinotracheitis indicating the essentiality of differential diagnosis.

Benfield *et al.* (1984) opined that the clinical signs of diarrhoea were of little value in the diagnosis of rotaviral enteritis because bacterial, parasitic and protozoan pathogens caused similar clinical signs. Hence, an accurate laboratory diagnosis of rotaviral diarrhoea was essential for the clinical and epidemiologic management of the disease.

The laboratory diagnostic methods for rotaviral diarrhoea were based predominantly on the detection of the virus, viral antigen or viral nucleic acid in faecal samples (de verdier Klingenberg and Esfandiari, 1996).

The faecal samples should be collected from affected animals as soon as possible after the onset of diarrhoea and submitted to the laboratory in chilled state for the detection of virus (Radostits *et al.*, 2000).

According to Cabalar *et al.* (2001), the faecal samples should be collected directly from the affected animal and stored at –30°C until it is processed.

2.8.1 Detection of Virus

2.8.1.1 Electron Microscopy

Turner *et al.* (1973) demonstrated virus particles in the intestinal contents of calves with diarrhoea by EM. These particles had cubic symmetry and the virions had an extra capsid.

Flewett *et al.* (1974) studied the morphology of human and animal rotavirus by EM and found that there was no difference in size or structure between the two. In electron micrographs, these viruses differed in appearance from *Reo viruses* which did not possess the well defined circular outline of calf and human gastroenteritis virus. Immuno electron microscopy (IEM) revealed that the calf antibody reacted with the inner but not the outer capsid layer of human virus; where as the human antibody reacted with both the layers.

Electron microscopy of negatively stained faecal extracts could be used to demonstrate viral particles having 'cart wheel' like appearance (Flewett *et al.*, 1974a and Albrey and Murphy, 1976).

Rotavirus infected intestinal epithelial cells *in vivo* and calf kidney cells *in vitro* were examined by EM of the negatively stained smears. Rotavirus particles with single and double capsid could be detected (Chasey, 1977).

According to Mohammed *et al.* (1978), electron microscopy was less sensitive when compared to serological tests like AGID, CIE, HI and ELISA.

Benfield *et al.* (1984) compared the sensitivity of EM with various serological tests and found that it was as sensitive as ELISA in detecting bovine rotavirus where as it was more sensitive than ELISA, Fluorescent Antibody Technique (FAT) and VI in detecting porcine rotavirus.

Ellis and Daniels (1988) compared electron microscopy with commercial Enzyme Immuno Assay (Rotazyme) for the detection of rotavirus and observed that the latter was at least three times more sensitive.

According to Al-Yousif *et al.* (2000), Transmission EM could detect up to 10^5 virus particles/g of faeces. But it was expensive and required skilled personnel.

2.8.1.2 Virus Isolation/ Propagation

2.8.1.2a Primary Cell Culture

Kaşza *et al.* (1970) propagated porcine reovirus in canine thyroid adeno carcinoma cultures.

According to Mebus *et al.* (1971), primary foetal bovine kidney, lung, thyroid and choroid plexus cells from eight to nine month old foetus, primary baby hamster kidney and embryonic bovine trachea could be used for the propagation of neonatal calf diarrhoea virus.

Primary foetal bovine kidney cells, primary lamb kidney cells, embryonic bovine trachea (EBT) and bovine embryonic skin could be used to adapt and propagate rotavirus (Fernelius *et al.*, 1972).

Bovine embryonic kidney cell cultures were the most satisfactory for propagation of rotavirus (Welch and Twiehaus, 1972).

Bridger *et al.* (1975) isolated bovine rotavirus from faecal filtrates in primary calf kidney cells prepared from three to seven day old calves.

According to Benfield *et al.* (1984), virus isolation was the least sensitive technique for the detection of rotavirus.

2.8.1.2b Continuous Cell Line

Mebus *et al.* (1971) used established cell lines of Baby Hamster Kidney (BHK-21), African green monkey kidney (Vero), Monkey kidney (LLC-MK_2) and human cervical carcinoma cells (He La cells) for propagation of neonatal calf diarrhoea virus.

Bovine rotavirus could be grown in Madin Darby Bovine Kidney cells (MDBK), LLC-MK_2 cells and He La cells. Madin Darby Bovine Kidney cells produced ten fold more virions than Microbiological Associates (MA-104), LLC-MK_2 and He La cells. The infectivity of BRV could be increased by trypsin treatment up to three fold (Clarke *et al.*, 1979).

The isolation of rotavirus could be done in African green monkey kidney cell line, BSC-1 and there was an enhanced production of infectious virions in BSC-1 cell cultures in presence of trypsin, cortisol, retinoic acid and Vitamin B12 (Begin, 1980).

Urquidi *et al.* (1981) found that BRV multiplied efficiently in LLC-MK_2 cells, a continuous cell line of rhesus monkey kidney with low concentration of trypsin in virus inoculum for infectivity. They observed that the polypeptide pattern of uninfected cells was modified by rotavirus infection.

Hoshino *et al.* (1981) observed reproducible clear cut plaques in infected MA-104 cells by feline rota virus in presence of trypsin.

An established foetal rhesus monkey kidney cell line, MA-104 could be used for the isolation and passage of bovine rotavirus (Fukai *et al.*, 1998).

2.8.1.2c Cytopathic effect (CPE)

Fernelius *et al.* (1972) noticed that rotavirus infected pig kidney cells showed swelling and rounding. The cytopathic effect of infected L cells consisted of leathery appearance of cells which had a tendency to remain attached to glass surface. The infected embryonic bovine tracheal cells became densely granulated or pigmented.

The cytoplasmic changes detected after inoculation with rotavirus were cytoplasmic vacuoles, eosinophilic inclusions, degeneration of cells and detachment from monolayer (Welch and Twiehaus, 1972).

Albrey and Murphy (1976) attempted to propagate human rotavirus in trypsinised foetal bovine intestinal cells and demonstrated intracytoplasmic fluorescence in infected cells. There was no evidence of CPE in any stage.

Calf kidney cells and pig kidney cells were equally susceptible to infection with calf and pig rotaviruses. Cytopathic effect was observed only with first passage of field virus and not in subsequent passages. Granular intracytoplasmic fluorescence could be observed in both cells (Woode *et al.*, 1976).

Chasey (1977) isolated bovine rotavirus in calf kidney cells and observed discrete foci of cytopathic effect in the infected monolayer. The cytoplasm of the infected cells contained large number of virus like particles, associated with dense unenveloped inclusions and rough endoplasmic reticulum.

Hoshino *et al.* (1981) observed reproducible clear cut plaques in infected MA-104 cells by feline rotavirus in presence of trypsin. The cytopathic effect produced was flagging of cells one day post infection.

According to Minakshi *et al.* (2004), avian rotavirus could be propagated in MA-104 cells and CPE was characterized by cell rounding, cell detachment and lytic foci.

Bora *et al.* (2007) isolated porcine rotavirus in MA-104 cell line and found that no observable cytopathic effect was exhibited up to eight passages. Two samples caused alteration in cellular configuration and elongation of cells after fourth passage and cell monolayer was completely detached after four to five days post infection.

2.8.2 Serological Methods

2.8.2.1 Immuno Fluorescent Assay

Flewett *et al.* (1974) reported that Immuno Fluorescent Assay was more convenient in detecting rotavirus because diagnosis could be made in few hours without the use of an EM. They observed that human and calf sera possessing neutralizing antibody fluoresced with calf rotavirus in calf kidney cell cultures suggesting that human and calf rotaviruses are related, probably possessing a serologically similar internal capsid protein.

According to Bridger et al. (1975), immuno fluorescent assay could be used to detect rotavirus antigen in faecal smears or in intestinal sections.

Calf kidney cell cultures inoculated with lamb rotavirus showed specific intracytoplasmic fluorescence with calf rotavirus antiserum and not with foetal calf serum (Snodgrass *et al.*, 1976).

According to Mohammed *et al.* (1978), immunofluorescence test is more sensitive in detecting BRV than EM and VI.

Clarke *et al.* (1979) used fluorescein conjugated anti-bovine rotavirus rabbit Immunoglobulin G for staining the monolayer infected with calf rotavirus. It was found that the titre in cell culture extracts were 2×10^8 Fluorescent cell forming units (FCFU) per millilitre after 72 h of infection.

According to Benfield *et al.* (1984), FAT could demonstrate rotavirus antigen in the villous epithelial cells of affected animals. It was found to be valid for up to six hours after the onset of diarrhoea in calves.

Al-Yousif *et al.* (2000) showed that immunofluorescence assay had a higher sensitivity than indirect ELISA and was found to be a specific and rapid method to study viral pathogenesis.

2.8.2.2 Enzyme Linked Immuno Sorbent Assay (ELISA)

According to Herring *et al.* (1982), the sensitivities of PAGE, EM and ELISA were approximately equal in detecting BRV in faecal samples.

Enzyme Linked Immuno Sorbent Assay was considered as a sensitive test for the detection of bovine rotavirus. It was as sensitive as EM and more sensitive than FAT and VI (Benfield *et al.*, 1984).

Besser *et al.* (1988) reported that ELISA was more sensitive than FAT in detecting calf rota virus.

Snodgrass *et al.* (1990) used a monoclonal antibody (MAb) based ELISA for G typing of bovine rotavirus and found that 66 per cent of the isolates were G6 and only 7.4 per cent were G10.

Enzyme Linked Immuno Sorbent Assay failed to detect viral antigens in faeces which also contain antibody. The test could provide results within 24 h after collection of samples (Radostits *et al.*, 2000).

Al-Yousif *et al.* (2000) first produced and characterized MAbs against VP7 of bovine rotavirus and were used in antigen capture ELISA.

Adah *et al.* (2001) employed ELISA with a group A common monoclonal antibody directed to VP6 for the detection of BRV.

Bora *et al.* (2007) reported that ELISA could detect rotavirus antigen in slightly higher numbers (29.35 per cent) than that of PAGE (24.84 per cent).

Wani et *al.* (2007) reported that ELISA could detect 10^6 rotavirus particles per millilitre of faeces and PAGE could detect 10^{11} particles indicating that the former was more sensitive.

2.8.2.3 Radio Immuno Assay (RIA)

Radio Immuno Assay (RIA) was found to be thousand times more sensitive than Counter Immuno Electrophoresis (CIE), but only ten fold better than Haemagglutination Inhibition (HI) in detecting rotavirus antibody (Mohammed *et al.*, 1978).

2.8.2.4 Counter Immuno Electrophoresis

Counter Immuno Electrophoresis was a more sensitive method than VI, EM and AGID. For routine detection of rotaviral antigen in faecal samples, it appeared to be the best test due to its simplicity and speed (Mohammed *et al.*, 1978).

The CIE test utilized reagents that are both stable and non radioactive. It was ideal for handling large number of specimens. The advantage of CIE was that the test could be read with naked eye (Radostits *et al.*, 2000).

2.8.2.5 Agar gel Immunodiffusion

Mohammed *et al.* (1978) detected rotavirus in the faecal samples by AGID using faecal concentrated viral antigen and faecal soluble antigen. The procedure was more sensitive in detecting the latter. Agar Gel Immunodiffusion was found to be the least sensitive test when compared with CIE, RIA and HI.

2.8.2.6 Immuno chromatography

De verdier Klingenberg and Esfandiari (1996) conducted a one step procedure, immunochromatography for the detection of group A rotavirus in bovine, porcine and equine faeces and found that the test was 89 per cent sensitive and 99 per cent specific as compared to ELISA.

Al-Yousif *et al.* (2002) developed immunochromatography for the detection of rotavirus in bovine faecal samples. It was found to be 75 per cent sensitive and 91 per cent specific compared to Latex Agglutination Test (LAT).

2.8.3 Molecular Methods

2.8.3.1 Extraction of viral RNA

Clarke and McCrae (1982) described a method based on end labeling of total faecal nucleic acid followed by CF 11 cellulose purification of the double stranded RNA.

The double stranded RNA could be extracted by using phenol chloroform method in which the faecal sample was mixed with an equal volume of sodium acetate buffer and phenol: chloroform: isoamyl alcohol in the ratio 25:24:1 (Herring *et al.*, 1982).

The isolation of total RNA can be done using TRI reagent which is a complete and ready to use reagent combining phenol and guanidine thiocyanate in a monophase solution to facilitate the immediate and most effective inhibition of RNase activity (Manufacturer's Protocol of Molecular Research Centre, 1995).

Fukai *et al.* (1998) extracted the RNA from stool suspension using TRIzol reagent.

Falcone *et al.* (1999) extracted the double stranded RNA from cell culture fluids and faecal samples using RNeasy mini kit. In this method, the sample was

added to lysis buffer followed by addition of two volume of ethanol and then applied to RNeasy mini spin column.

Rotavirus double stranded RNA could be extracted from faecal samples by a guanidine and silica method (Gentsch *et al.*, 2002).

2.8.3.2 Poly Acrylamide Gel Electrophoresis (PAGE)

Poly acrylamide Gel Electrophoresis is a simple, rapid and economical method for the ultra sensitive detection of the double stranded segmented genome of rotavirus. It is also used for identifying the electropherotypes (Clarke and McCrae, 1982).

Herring *et al.* (1982) employed RNA-PAGE for rapid and sensitive detection of the segmented genome of rotavirus using five per cent polyacrylamide slab gels. Electrophoresis was performed at room temperature for 16 h at 20 mA and 70 volt .The bands were visualized by silver staining.

According to Singh and Pandey (1988), genome electropherotyping was a common method for taxonomic and epidemiological studies. They found that the electropherotype of rotavirus positive samples from buffalo calves had RNA migration pattern similar to that of cow calf strains.

Pande and Pandey (1998) compared the sensitivity of PAGE and nucleic acid hybridization in detecting bovine rotavirus and observed that the latter was 1000 times more sensitive.

De Brito *et al* (2000) carried out the electrophoretic characterization of Brazilian bovine rotavirus and observed that all samples belonged to group A rotavirus. This study showed two electropherotypes differing in the migration patterns of segments five and ten.

Adah *et al.* (2001) conducted RNA-PAGE in 10 per cent acrylamide gels of two millimetres thick for 16 h at 20 mA at room temperature for the detection of human rotavirus RNA from diarrhoeic stools.

Minakshi *et al.* (2001) detected bovine group A rotavirus by RNA-PAGE and found that all isolates had long RNA genome pattern which suggested that different G and P genotypes in combination might have similar long migration patterns.

The avian rotaviruses could be distinguished from bovine rotaviruses on the basis of the RNA profile. The eleven double stranded RNA genomic segments were arranged in a 5:1:3:2 pattern typical of avian rotaviruses. The bovine rotaviruses exhibited a 4:2:3:2 pattern (Minakshi *et al.*, 2004).

Wani *et al.* (2005) reported that RNA-PAGE was less sensitive when compared to RT-PCR and ELISA in detecting bovine rotavirus. Faecal samples from nine diarrhoeic calves were screened for the presence of rotavirus. Three samples negative by sandwich ELISA and PAGE were proved to be negative by RT-PCR. Two samples positive by ELISA, but negative by RNA-PAGE were found positive by RT-PCR as well.

2.8.3.3 Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR)

Reverse Transcriptase – Polymerase Chain Reaction based assay was used for the detection of bovine rotavirus by Snodgrass *et al.* (1990). It was found that the most prominent serotypes were G10 and G6.

Gentsch *et al.* (1992) identified group A rotavirus gene 4 types by RT-PCR. The use of rotavirus specific primers in an RT- PCR had allowed not only the sensitive detection but also typing for both G and P types when the type specifying sequence diversity was exploited.

Gouvea *et al.* (1994) developed a PCR typing assay to identify rotavirus P types (VP4 specificity) of bovine and porcine strains and observed that the PCR

results agreed with previous characterizations by monoclonal antibodies, sequence analysis and hybridization assays except for a single strain of porcine rotavirus (Gottfried) which showed a distinct P type.

According to Hussein *et al.* (1996), RT-PCR provided a very sensitive method for G typing of bovine rotavirus field isolates, when compared with monoclonal antibody based ELISA. Reverse Transcriptase – Polymerase Chain Reaction could efficiently detect mixed infection with G6 and G10 serotypes.

Pande and Pandey (1998) applied RT-PCR for detection of bovine group A rotavirus for the first time in India.

Fukai *et al.* (1998) employed RT-PCR based assays to study the distribution of G serotypes and P genotypes of bovine group A rotavirus in Japan and found that G6P5 was the most common serotype in Japan.

G typing of rotavirus strains in Swedish cattle herds using RT-PCR enabled to study the molecular epidemiology of the virus. G10 was found to be the most common serotype followed by G6 (De Verdier Klingenberg *et al.*, 1999).

The VP7 type of human rotavirus in South America was examined by multiplex RT-PCR with a cocktail of primers and observed that G8 strains predominated (Steele *et al.*, 1999).

A nested RT-PCR assay was employed by Gulati *et al.* (1999) for studying the relative frequencies of G and P types among rotaviruses from Indian diarrhoeic cow and buffalo calves and observed that over 81 per cent of bovine rotaviruses were G10 P8[11] strains.

The minimum amount of rotavirus particles required to be detected in RT-PCR was found to be 10^4 per ml and hence this method was more sensitive than ELISA and RNA-PAGE which required 10^6 and 10^{11} particles per ml respectively (Arguelles *et al.*, 2000).

De Brito *et al.* (2000) conducted semi nested RT-PCR assay for G and P genotyping of Brazilian bovine rotavirus strains and found that 40 per cent of samples were G10 P(11) and the others showed evidence of mixture of strains G6 G10 and P(5) P(11). The presence of various combinations of G and P serotypes among field isolates of BRV indicated the possibility of genetic reassortments frequently occurring between viral strains with genes encoding different G and P serotypes.

Minakshi *et al.* (2001) employed VP4 gene specific RT-PCR and observed that the lowest detection limit of RT-PCR was 20 femtogram or 20 virus particles per millilitre of faeces.

According to Adah *et al.* (2001), strains with serotype G8 had VP4 genotype P [1] or remained untypeable. The stool specimen from which strain G8 P [1] was isolated did not reveal any nucleic acid upon PAGE analysis.

Many G1 and P [8] strains from Libya and Kenya could be typed using alternative typing primers, since type specific products could not be obtained by conventional primers. Strains that could not be typed even by using alternative primers were considered nontypeable (Cunliffe *et al.*, 2001).

El-Attar *et al.* (2002) reported the first genomic characterization of a bovine G3 rotavirus, by RT PCR using VP7 specific primers followed by nucleotide sequencing.

Martella *et al.* (2003) reported the detection and molecular characterization of rotavirus strain isolated from faeces of a diarrhoeic buffalo calf in Italy and was found to be G6 P [3].

According to Wani *et al.* (2004), of the ten selected samples, only six were identified by RT-PCR while four failed to produce an amplified viral band despite being known to contain sufficient rotaviral particles by RNA-PAGE and sandwich ELISA. This could be due to some nonspecific inhibitors of PCR.

Distefano *et al.* (2005) introduced a novel rotavirus VP7 typing assay using a one step RT-PCR protocol and product sequencing. A 365 bp RT-PCR product was generated from VP7 gene segment using a pair of novel degenerate primers and sequencing of these products was performed using truncated versions of original primers. Unlike other RT-PCR methods, this assay did not rely on gel patterns to determine VP7 types.

The sensitivity of ELISA and RT-PCR was found to be equal for detection of bovine rotavirus in faecal samples (Wani *et al.*, 2005).

Minakshi *et al.* (2007) detected BRV from faecal samples by RT-PCR using VP4 gene specific primers (Con 3 and Con 2) and a product of 876 bp was generated.

2.9 PROTEIN PROFILE OF BOVINE ROTAVIRUS

The polypeptides of human and calf rotaviruses (infantile gastroenteritis virus and neonatal calf diarrhoea virus) had been fractionated in polyacrylamide gels with a continuous buffer system and five polypeptides were obtained, four in the inner shell and fifth comprising the outer shell (Newman *et al.*, 1975).

Rodger *et al.* (1975) fractionated the polypeptides of human and calf rotavirus in polyacrylamide gels with discontinuous buffer system and noticed that the two viruses had very similar polypeptide components.

Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis of the surface glycoproteins of calf rotavirus was carried out by Rodger *et al.* (1977). It was found that calf rotavirus consisted of nine polypeptides having molecular weights in the range of 14.5 to 131 kDa.

Thouless (1979) reported that the molecular weight of rotavirus polypeptides in faecal samples and infected LLC-$MK_{2\ cells}$ were identical with the expection of nonstructural polypeptide 2 (NS2). The difference was that it moved

0.5 mm less far in the infected LLC-MK2 cells than in the faecal samples. Molecular weights were in the range of 24.6 kDa to 135.5 kDa.

The structural protein analysis of diarrhoeic calves in India was done using SDS-PAGE by Gulati *et al* (1997). Five protein bands of sizes 116 to 120 kDa (VP1), 95 kDa (VP2), 90 kDa (VP3/VP4), 44 kDa (VP6) and 34 kDa (VP7) were detected.

Group A rotavirus consisted of 11 polypeptides ranging in molecular weights from 21.7 kDa to 125 kDa (Desselberger, 1998).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

Molecular grade chemicals procured from Genei, Bangalore and analytical grade chemicals purchased from Sisco Research Laboratories Private Limited (SRL) and Hi-Media Laboratories Private Limited, Mumbai were used, wherever the source is not mentioned. Glassware of Borosil brand and plastic ware of Tarsons brand were used in this study.

3.1 COLLECTION AND PROCESSING OF FAECAL SAMPLES

Faecal samples were collected from diarrhoeic calves of different age groups from veterinary hospitals and farms attached to Kerala Agricultural University, various organized dairy farms and some individual private farms in and around Thrissur. Collection of samples was made using dry sterile cotton swabs and was immersed in sterile phosphate buffered saline (PBS, pH 7.2). Freshly voided faeces were also collected and transferred immediately to the laboratory in an ice box and preserved at -20°C until use.

3.2 DETECTION OF VIRAL NUCLEIC ACID

3.2.1 Extraction of RNA

3.2.1.1 *Materials*

3.2.1.1a TRI Reagent obtained from Molecular Research Institute, Inc., Cincinnati was used.

3.2.1.1b Chloroform (AR)

3.2.1.1c Isopropanol (AR)

3.2.1.1d Ethanol (75 per cent)

Ethanol 75 ml

DNase RNase free distilled water to make 100 ml

Stored at 4°C until use

3.2.1.1e Diethyl Pyrocarbonate treated water (GIBCO, USA)

Diethyl Pyrocarbonate (DEPC) 0.1 ml

Triple glass distilled water 99.9 ml

Stirred for six hours using a magnetic stirrer and sterilized by autoclaving.

3.2.1.2 Method

The RNA was extracted from the diarrhoeic faecal samples as per the Manufacturer's protocol, Molecular Research Centre Inc., Cincinnati (1995) with minor modifications. All the micro tips and eppendorf tubes used were washed with DEPC treated water to make it RNase free.

To 0.2 ml of faecal sample taken in a sterile RNase free tube, one millilitre of TRI reagent was added and homogenized. Following homogenization, the insoluble material from the homogenate was removed by centrifugation at 10,000x g for 10 min at 4°C. The clear supernatant was transferred to a fresh tube and 0.2 ml of chloroform was added, followed by vigorous shaking for 15 seconds. The resulting mixture was kept at room temperature for 10 min and centrifuged at 10,000x g for 15 min at 4°C in a refrigerated centrifuge. The upper aqueous phase containing RNA was transferred to an RNase free tube. To this, 0.5 ml of isopropanol was added for precipitation of RNA and kept at room temperature for five minutes followed by centrifugation at 10,000x g for eight minutes at 4°C. The supernatant was discarded

and the RNA pellet was washed with 75 per cent ethanol by vortexing. It was then centrifuged at 7500x g for five minutes at 4°C. The ethanol was removed and air dried the RNA pellet for five minutes. The pellet was dissolved in 20 µl sterile DEPC treated water.

3.2.2 Ribonucleic Acid – Poly Acrylamide Gel Electrophoresis (RNA-PAGE)

The rotavirus genome was detected in faecal samples by RNA-PAGE following the method of Herring *et al.* (1982) with minor modifications.

3.2.2.1 Materials

3.2.2.1a Separation gel buffer (1.5 M Tris Hydrochloride)

Tris base	18.17 g

Dissolved in about 80 ml distilled water. The pH was adjusted to 8.8 with 10N HCl, and made up the volume to 100 ml.

3.2.2.1b Stacking gel buffer (0.5 M Tris hydrochloride)

Tris base	6.06 g

Dissolved in about 80 ml distilled water. The pH was adjusted to 6.8 with 10N HCl and made up the volume to 100 ml.

3.2.2.1c Acrylamide-Bis acrylamide Solution

Acrylamide	30 g
N, N Methylene bis acrylamide	0.8 g
Distilled water	100 ml

The solution was filtered through Whatman No.1 filter paper and stored at 4°C.

3.2.2.1d Tris-Glycine Reservoir Buffer (1X)

Tris base	3.0 g
Glycine	14.4 g
Distilled water to make	1000 ml

3.2.2.1e RNA-PAGE sample buffer (2X)

Stacking gel buffer	5.0 ml
Glycerol	3.0 ml
SDS (10 per cent)	200 µL
Bromophenol Blue (0.05 per cent)	400 µl
Distilled water	11.4 ml

3.2.2.1f Ammonium per sulphate (10 per cent)

Ammonium per sulphate	100 mg
Distilled water	1 ml

3.2.2.1g Separation gel (eight per cent)

Acrylamide-Bisacrylamide solution	6.7 ml
Separation gel buffer	6.3 ml
Ammonium per sulphate	250 µl

TEMED	15 µl
Distilled water	11.5 ml

3.2.2.1h Stacking gel (five per cent)

Acrylamide-Bisacrylamide solution	1.0 ml
Stacking gel buffer	0.75 ml
Ammonium per sulphate	60 µl
TEMED	6 µL
Distilled water	4 ml

3.2.2.1i Fixer solution

Ethyl alcohol (10 per cent)	20 ml
Acetic acid (Five per cent)	10 ml
Distilled water	170 ml

3.2.2.1j Silver Nitrate Solution (0.011M)

Silver nitrate	0.37 g
Distilled water	200 ml

3.2.2.1k Developer solution

Sodium hydroxide pellets	7.5 g
Formaldehyde (38 per cent)	2 ml

Distilled water	250 ml

3.2.2.1l Stopping solution

Glacial acetic acid	10 ml
Distilled water	190 ml

3.2.2.1m Positive sample

Bovine group A rotavirus RuBV128 kindly provided by Dr. T. N. Naik, Deputy Director and Head, National Centre for Cholera and Enteric Diseases, Kolkata was used as positive sample in the whole study.

3.2.2.2 Method

The gels were prepared in 16 x 20 cm glass plates supplied with the vertical electrophoresis apparatus with one millimetre thick spacer.

Eight per cent separation gel was prepared and poured in between the glass plates. Over this, three ml of distilled water was added to get uniform surface and allowed to polymerize for 20 min.

After polymerization, water was removed and a comb was inserted between the two glass plates. Then five per cent stacking gel was added and allowed to polymerize for 20 minutes. The comb was removed after complete polymerization. The wells were washed with Tris-Glycine reservoir buffer to remove unpolymerized particles. Each well was loaded with 20 µl of RNA sample along with equal quantity of RNA-PAGE sample buffer (2 X). One of the wells was loaded with the positive sample. The glass plates containing gel was then transferred to the vertical slab gel electrophoresis system (Hoefer, USA). The upper and lower buffer tanks were filled

with 1X Tris-glycine reservoir buffer and electrophoresis was carried out at 100 V and 16 mA for 20 h.

3.2.2.3 Silver staining

When the electrophoresis was over, glass slab containing the gel was dismantled and the gel was separated out. The stacking gel was discarded and the remaining gel was visualized after silver staining (Herring *et al.*, 1982).

The gel was placed in fixative solution for one hour at room temperature with intermittent gentle shaking. It was then taken to dark room and placed in silver nitrate solution for one hour with gentle shaking. The staining solution was drained off and washed with two changes of distilled water to remove traces of silver nitrate and then developer solution was added to develop the stained RNA bands. The tray was rocked gently till the brown coloured bands were clearly visible in a yellow background. The developer was removed and replaced with stopping solution to stop the reaction and the gel was photographed.

3.2.3 Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR)

3.2.3.1 Materials

3.2.3.1a MMuLV Reverse transcriptase 20U/µl

3.2.3.1b 10X Reverse Transcriptase Buffer

3.2.3.1c dNTP mix (100 mM)

3.2.3.1d 10X PCR buffer

3.2.3.1e Magnesium chloride 25 mM

3.2.3.1f Taq polymerase (3U/µl)

3.2.3.1g Primers

Consensus primers (Gouvea *et al.*, 1994) for an 877 bp nucleotide sequence of the VP4 gene of bovine rotavirus were used. The desalted primers were synthesized and supplied by M/s Imperial Biomedics, USA

Primer 1 (Forward)

Con 3 (11-32) 5"-TGGCTTCGCCATTTTATAGACA-3'

Primer 2 (Reverse)

Con 2 (868-887) 5"-ATTCGGACCATTTATAACC-3'

3.2.3.1h Water for PCR

To 99.9 ml of triple glass distilled water, 0.1 ml of DEPC was added, stirred for six hours and sterilized at 121°C for 15 min.

3.2.3.1i DNA molecular weight marker – pBR322 DNA/AluI Digest containing double stranded DNA segments of 63/57/49, 100/90, 226, 257, 281, 403, 521, 659/656 and 908 bp.

3.2.3.2 Method

The RT-PCR reaction was carried out as per the method of Gouvea *et al.* (1994) with some modifications.

3.2.3.2.1 Reconstitution of primers

The tubes containing lyophilized primers were centrifuged at 10,000x g for five minutes in a refrigerated centrifuge. To the tube containing forward primer (Con 3), 321 µl of nuclease free water was added and mixed well. From this, 10 µl was taken and 90 µl nuclease free water was added. To the tube containing reverse

primer (Con 2), 389 µl water was added and mixed well and this was diluted to one in ten as described above. This constituted 10 picomole solution, which was used in PCR.

3.2.3.2.2 Reverse Transcription

The primer mix was prepared by adding the following components on ice for 25µl volume in a clean DEPC treated tube.

Viral ds RNA	2.5 µl
Forward primer	1.0 µl (10 pm)
Reverse primer	1.0 µl (10 pm)
DEPC treated water	16.0 µl

The contents were mixed by vortexing. The tube was then placed in thermal cycler at 99°C for five minutes, and snap chilled on ice. The following reagents were added on ice.

10 X RT buffer	2.5 µl
100 mM dNTPs	1.0 µl
MMuLV-RT 20 U/µl	1 µl

The reaction mixture was incubated at 25°C for 10 min. The reverse transcription was carried out at 42°C for 60 min in thermal cycler (Eppendorf Master Cycler Gradient TM, Germany). This was followed by heat inactivation of MMuLV-RT at 90°C for five minutes. The strand of cDNA thus synthesized was used as template for PCR.

3.2.3.2.3 Polymerase Chain Reaction

The reaction mixture (25µl) for PCR was prepared as follows:

cDNA	5.0 µl
Forward primer Con 3	1µl
Reverse Primer Con 2	1µl
DEPC treated water	13 µl

The mixture was heated in thermocycler at 99°C for five minutes and snap chilled on ice. This was followed by the addition of the following reagents on ice.

10X PCR buffer	2.5 µl
25 mM $MgCl_2$	1.5 µl
100 mM dNTPs	0.5 µl
Taq polymerase (3U/µl)	0.5 µl

The reagents were mixed gently by vortexing. The amplification was carried out with an initial denaturation at 94°C for five minutes, followed by the sequence of 30 cycles as follows: 94°C for one minute, 50°C for two minutes and 72°C for two minutes. This was followed by final extension at 72°C for 10 min.

Programme of amplification

One cycle	Denaturation	94°C	Five minutes
30 cycles	Denaturation	94°C	One minute
	Annealing	50°C	Two minutes
	Extension	72°C	Two minutes
One cycle	Extension	72°C	10 minutes

3.2.3.2.4 Submarine Agarose Gel Electrophoresis

3.2.3.2.4.1 Materials

3.2.3.2.4.1a Tris-Acetate EDTA (TAE) buffer (50X), pH 8.0

Tris base	48.4 g
Glacial acetic acid	11.42 ml
0.5 M EDTA pH 8.0	20 ml
Distilled water to	1000 ml

Autoclaved and stored at room temperature

3.2.3.2.4.1b Agarose Gel (1.5 per cent)

Agarose low EEO (Genei)	1.5 g
TAE buffer	100 ml

3.2.3.2.4.1c Gel loading buffer (6X)

Bromophenol blue	0.25 g
Xylene cyanol	0.25 g
Sucrose	40 g
Distilled water	100 ml

Stored at 4°C

3.2.3.2.4.1d Ethidium Bromide

Ethidium Bromide	100 mg
Distilled water	10 ml

Stored at 4°C in amber coloured bottles

3.2.3.2.4.2 Method

The PCR product was detected by electrophoresis in 1.5 per cent agarose gel in TAE buffer (1X). Agarose was dissolved in TAE buffer by heating. When the mixture cooled to around 50°C, ethidium bromide was added to a final concentration of 0.5µg/ml. The clean, dry gel platform edges were sealed with adhesive tape and the comb was kept in proper position before pouring agarose. Once the gel was set, the comb and adhesive tape were removed gently and the tray containing the gel was placed in the buffer tank. Buffer (TAE 1X) was poured until the gel was completely covered.

The PCR product (five microlitre) was mixed with one microlitre of 6X gel loading buffer and the samples were loaded in the wells. Electrophoresis was carried out at 5 V/cm until the dye migrated the full length of the gel.

The gel was visualized under Ultraviolet transilluminator (Hoefer, USA) and the results were documented in a gel documentation system (Bio-Rad Laboratories, USA).

3.3 DETECTION OF VIRAL ANTIGEN

3.3.1 Preparation of Anti-Bovine Rotavirus (Anti-BRV) hyper immune serum

Anti-BRV hyper immune serum was raised in rabbits as per the method described by Mohammed *et al.* (1978).

3.3.1.1 Materials

3.3.1.1a Freund's complete and incomplete adjuvant

3.3.1.1b Experimental animals

Two healthy rabbits of New Zealand white breed, aged six months and weighing 2.5 kg, procured from Small Animal Breeding Station, KAU were used.

3.3.1.2 Method

Faecal antigen consisted of a virus preparation obtained from diluted faeces (1/5) after two cycles of centrifugation at 3000x g for 20 min to remove the majority of organic matter. The supernatant was clarified by filtration through 0.45 µm Millipore filter. The filtered antigen was then further centrifuged at 1, 00,000x g for three hours in a Servo Combi Plus No.80 rotor. The pelleted antigen was resuspended in PBS pH 7.2.

Each rabbit was injected intramuscularly with one milliliter of antigen emulsified in equal quantity of Freund's adjuvant. Totally two injections were given at an interval of two weeks. Freund's complete adjuvant (FCA) was used for the first injection and Freund's incomplete adjuvant (FICA) was used for subsequent

injections. The rabbits were given a booster dose with FICA on day 21 and test bled from ear vein one week after the last injection and serum samples were tested by AGID for the presence of antibody.

3.3.2 Agar Gel Immunodiffusion (AGID) Test

3.3.2.1 Materials

3.3.2.1a Gel for AGID

Agarose	0.8 g
Sodium chloride	0.85 g
Sodium azide	0.01 g
Distilled water to	100 ml

To dissolve the agarose in saline, the solution was boiled for five minutes.

3.3.2.1b Agar coated slides

Clèan glass slides were coated by smearing 0.5 per cent melted agar in distilled water and drÿing in air by keeping the slides horizontally over glass rods.

3.3.2.2 Method

Agar gel precipitation test was done as per the method of Mohammed *et al.* (1978) with minor modifications. Three millilitres of melted agarose was poured onto precoated glass slides and allowed to set. One central well and five peripheral wells, each with five millimetre diameter were punched out on the solidified agar over the slide. Central well was charged with 20 µl of test rabbit serum. One of the peripheral wells was loaded with 20 µl of the positive BRV control. The test antigens were loaded in the peripheral wells. One of the wells loaded with faecal antigen from a healthy calf was kept as negative control. The slides were incubated in a moist chamber at 37°C for about 24 to 48 h, to allow development of precipitin lines. The

gels were washed in saline overnight and then in distilled water for two hours, dried in a 37°C incubator, and stained with 0.17 amido black.

3.4 ISOLATION OF BOVINE ROTAVIRUS

Madin Darby Bovine Kidney (MDBK) cell line obtained from National Centre for Cell Sciences (NCCS), Pune, was employed for the purpose.

3.4.1 Subculturing and Maintenance of Cell Line

3.4.1.1 Materials

3.4.1.1a Eagle's minimum essential medium (MEM)

Dehydrated MEM was reconstituted as per manufacturer's instruction and filtered using membrane filter (0.2 μm).

3.4.1.1b Sodium bicarbonate (7.5 per cent)

Prepared in triple distilled water and sterilized by filtration using membrane filter (0.2μm).

3.4.1.1c Calcium Magnesium Free - Phosphate Buffered Saline (CMF-PBS 0.15M, pH 7.2)

Dehydrated CMF-PBS was reconstituted as per manufacture's instruction and sterilized by autoclaving at 15 lbs pressure at 121°C for 15 min.

3.4.1.1d Trypsin-Versene Glucose (TVG) solution (working solution)

Trypsin (1:250)	0.25 g
EDTA	0.02 g
Glucose	0.05 g
CMF-PBS (0.15 M, pH 7.2)	100 ml

Sterilized by filtration through membrane filter (0.2 µm), distributed in small aliquots and stored at -20°C and prewarmed before use.

3.4.1.1e Neonatal calf serum: collected aseptically from colostrum deprived male calves maintained at University Livestock Farm, Mannuthy.

3.4.1.1f Cell culture growth medium: Eagle's MEM containing 10 per cent foetal calf serum and 1 mM sodium pyruvate was prepared and pH was adjusted to 7.2 with 7.5 per cent sodium bicarbonate.

3.4.1.1g Cell culture maintenance medium: Differed from growth medium in having two per cent foetal calf serum.

3.4.1.1h Antibiotics

Benzyl penicillin	10 lac units
Streptomycin sulphate	1 g
Sterile triple distilled water	40 ml

Sterilized by filtration through membrane filter (0.2 µm), distributed in aliquots and stored at -20°C. It was used both in growth and maintenance media at a final concentration of 100 IU of penicillin and 100 µg of streptomycin per milliliter of the medium.

3.4.1.1i Tissue culture bottle of 50 ml capacity

3.4.1.2 Method

The maintenance medium was poured off from tissue culture bottle containing confluent monolayer of healthy growing cells. The cell sheet was washed twice with CMF-PBS. Two millilitres of prewarmed TVG solution was added to

bottle containing monolayer and then shaken gently for one minute. Then, the TVG solution was discarded and the bottle was incubated at 37°C for five minutes. When the cells started dislodging from the monolayer, added a small quantity of freshly prepared growth medium and the cells were detached from the surface by mechanical disruption using sterile pipette attached with bulb. A split ratio of 1:3 was employed for seeding into new tissue culture bottles and the cells were also seeded into test tubes containing coverslips. The tissue culture bottles and test tubes containing cover slip were enriched with growth medium at the rate of eight millilitres and two millilitres respectively. The bottles and tubes were incubated at 37°C. They were observed daily for the formation of monolayer. When monolayer was formed (usually within three to four days), it was used for further infection with BRV.

3.4.2 Inoculation of Cell Lines

3.4.2.1 Materials

3.4.2.1a Fresh monolayer of MDBK cells in tissue culture bottles and cover slips

3.4.2.1b Faecal samples processed for inoculation

3.4.2.1c Trypsin (1:250)

Faecal sample which were found positive for BRV by RNA-PAGE was filtered through Millipore syringe filter (0.2 μm) and incubated at 37°C for one hour and was used as inoculum for infecting cell line after treating with 10 μg trypsin per millilitre of inoculum .

3.4.2.2 Method

Tissue culture bottle with fresh monolayer was selected. The growth medium was poured off and washed with CMF-PBS. The monolayer was inoculated with 0.5

millilitre of inoculum and incubated at 37°C for one hour to facilitate adsorption of the virus. The inoculum was removed and the monolayer was washed with CMF-PBS. Then eight millilitres of maintenance medium was added to the tissue culture bottle containing fresh monolayer. Control culture bottles were prepared simultaneously in which CMF-PBS was used as inoculum instead of clinical samples.

All the tissue culture bottles were incubated at 37°C and were examined at 24h interval for a period of five to six days, under an inverted microscope for evidence of any cytopathic effect (CPE).

For infecting coverslip cultures in the test tubes, 0.2 ml of inoculum and two millilitres of maintenance medium were used. Control tubes were also treated in the same manner in which CMF-PBS was used as inoculum. Infected coverslip cultures were collected at 24 h interval for a period of five days, for studying the CPE by May-Grunwald Giemsa staining. The control cover slips were also stained and studied.

3.4.3 Passaging of Virus

3.4.3.1 Materials

3.4.3.1a Inoculated monolayer in tissue culture bottles

3.4.3.1b Fresh monolayer of MDBK cells in tissue culture bottles and cover slips

3.4.3.2 Method

Five days post inoculation, the inoculated monolayer were freeze thawed three times and centrifuged at 2000x g for 15 min in a cooling centrifuge (Remi C-24), to sediment cell debris. The supernatant was used as inoculum for the next passage. The inoculation was performed as mentioned earlier (3.4.2.2). After every

passage, the supernatant was tested for presence of BRV by RT-PCR and RNA-PAGE. The presence of BRV was not detected even after fourth passage, the cell culture supernatant after initial clarification was subjected to ultracentrifugation at 1,00,000x g for two hours in a SW-type rotor to pellet the virus, if present. Then the pellet was reconstituted in 200 µl of PBS (pH 7.2), and subjected to RT PCR. Passaging of virus was stopped when RT-PCR was found to be negative.

3.4.4 Staining of Coverslip Cultures

3.4.4.1 Materials

3.4.4.1a Coverslip cultures

3.4.4.1b May-Grunwald stain

Prepared by dissolving 2.5 g of May-Grunwald stain powder in 100 ml of absolute methanol and allowed to age for one month.

3.4.4.1c Giemsa stain

Prepared by dissolving one gram of stain powder in 66 ml of glycerol and kept at 60°C till the stain powder got dissolved completely. Then added 66 ml of absolute methanol and kept for a day.

3.4.4.1d Methanol

3.4.4.1e Acetone

3.4.4.1f Xylene

3.4.4.1g DPX mountant

3.4.4. 2 Method

The coverslip cultures were fixed overnight in methanol. They were stained for 10 min, in May-Grunwald stain and for 20 min in 1 in 10 diluted Giemsa stain. The coverslips were rinsed rapidly in two changes of acetone and then in two parts of acetone and one part of xylene for five seconds. They were then placed in one part of acetone and two parts of xylene for one minute, cleared in two changes of xylene, two minutes each, dried and mounted with DPX on a clean grease-free glass slide and examined for CPE under microscope. The uninfected coverslips were also fixed as described above and studied in detail.

3.5 PROTEIN PROFILE OF BOVINE ROTAVIRUS

Protein profile of BRV was studied using SDS-PAGE.

3. 5.1 Materials

3.5.1a Acrylamide-bisacrylamide stock (30: 0.8)

Same as **3.2.2.1c**

3.5.1b Tris 1.5 M

Same as **3.2.2.1a**

3.5.1c Tris 0.5 M (pH 6.8)

Same as **3.2.2.1b**

3.5.1d Resolving gel (12.5 per cent)

Acrylamide: bisacrylamide (30: 0.8)	12.5 ml
Tris hydrochloride (1.5 M) pH 8.8	7.5 ml
Sodium dodecyl sulphate (10 per cent)	0.3 ml
Ammonium persulphate (10 per cent)	0.15 ml
N, N, N, N - tetra methyl ethylenediamine (TEMED)	0.01 ml
Distilled water	9.6 ml

3.5.1e Stacking gel (four per cent)

Acrylamide stock solution	0.67 ml
Tris hydrochloride (0.5 M) pH 6.8	1.25 ml
Sodium dodecyl sulphate (10 per cent)	0.05 ml
Ammonium persulphate (10 per cent)	25 µl
N, N, N, N - tetra methyl ethylenediamine (TEMED)	2.5 µl
Distilled water	3 ml

3.5.1f Electrophoresis buffer

Tris base	3.0 g
Glycine	14.4 g
Sodium dodecyl sulphate	1.0 g
Distilled water to make	1000 ml

3.5.1.1g Sample preparation buffer (2X)

0.5 M Tris hydrochloride, pH 6.8	2.5 ml
Glycerol	2.0 ml
Sodium dodecyl sulphate (10 per cent)	4.0 ml
2-mercaptoethanol	0.2 ml
Bromophenol blue	0.5 mg
Distilled water to make	10.0 ml

Distributed in small aliquots and stored at 4°C.

3.5.1.1h Destaining solution I

Glacial acetic acid	70 ml
Methanol	400 ml
Distilled water to	1000 ml

3.5.1.1i Destaining solution II

Glacial acetic acid	70 ml
Methanol	50 ml
Distilled water to	1000 ml

3.5.1.1j Coomassie brilliant blue staining solution

Coomassie brilliant blue (R250)	0.5 g
Methanol	800 ml
Glacial acetic acid	140 ml
Distilled water to	2000 ml

3.5.1.2 Method

The polypeptide pattern of BRV was analyzed by discontinuous system of polyacrylamide gel electrophoresis (Rodger *et al.*, 1977).

Faecal samples were diluted in PBS (1/5) and centrifuged at 5000 rpm for five minutes to sediment coarse materials. The supernatant was ultra centrifuged at 40,000 rpm for 4.5 h in a Servo Combi Plus No. 80 rotor at 4^0C. The clear button like pellet was diluted with a minimum quantity of TNE buffer. The diluted buffer was again centrifuged at 5000 rpm for 10 min. The clear supernatant was transferred to eppendorf tubes. This was later used for SDS- PAGE.

Resolving gel solution, 12.5 per cent was prepared and degassed. Ten per cent ammonium persulphate and TEMED were added and poured between two glass plates so as to form a gel of one millimetre thickness and left for polymerization. Distilled water was layered on the top to ensure uniformity of the gel surface. The set up was left overnight at 4°C for complete polymerization. After polymerization

the distilled water was pipetted out and four per cent stacking gel was prepared and poured between the glass plates. The comb was then inserted and the apparatus left as such for complete polymerization for one and half hours. After polymerization the comb was removed and the wells were washed thoroughly with running buffer. A small quantity of running buffer was added into each of these wells. Twenty micrograms of the sample was mixed with equal volume of sample preparation buffer and kept in boiling water bath for five minutes. The samples were loaded into individual wells under the column of buffer in each well. Standard high range molecular weight marker (GENEI) was loaded in one of the wells. The glass plates were fixed onto the vertical slab gel electrophoresis apparatus and electrophoresis buffer was carefully poured into the top and bottom reservoirs and electrophoresed at 10 mA constant current till the bromophenol blue marker reached near the bottom of the resolving gel. The gel was then removed from the glass plate; the stacking gel was snipped off and transferred to a petri plate containing Coomassie brilliant blue staining solution for two to three hours. The gels were then destained till the background became clear and were viewed in white light and photographed.

The molecular weights of the different protein fractions were obtained by comparing the distance migrated by the different fractions with that of the standard marker proteins of known molecular weights using Bio-Rad gel documentation system with quantity-one software.

RESULTS

4. RESULTS

In the present study, faecal samples from 124 diarrhoeic calves were screened for the presence of rotavirus. Majority of the calves had a history of profuse watery whitish diarrhoea and severe dehydration. The samples were collected mainly from animals brought to Veterinary hospital, Mannuthy and calves maintained in farms attached to Kerala Agricultural University. Samples were also collected from calves reared by some individual farmers and certain private dairy farms in and around Thrissur. Twenty adult diarrhoeic animals and 20 normal healthy calves were also screened for the presence of BRV.

All the faecal samples were subjected to RNA-PAGE, RT-PCR and AGID to detect the presence of BRV. The protein profile of BRV was analyzed using SDS-PAGE. Attempts were made to isolate BRV in MDBK cell line, from samples found to be positive by RNA-PAGE.

4.1 RIBONUCLEIC ACID – POLYACRYLAMIDE GEL ELECTROPHORESIS

The RNA was extracted from faecal samples using TRI reagent. The genome consisting of 11 segments was resolved in eight per cent polyacrylamide gels and visualized after silver staining (fig. 1). The segments were arranged in a 4:2:3:2 patterns. This pattern is typical for Group A rotavirus. The positive control also showed the same arrangement.

Among 124 calves clinically suspected for BRV, 29 (23.39 per cent) were found positive by RNA-PAGE. All of them showed a similar migration pattern. All the faecal samples collected from healthy calves and adult animals with diarrhoea were found to be negative.

Among 34 samples collected from University Livestock Farm, Mannuthy, none were positive by RNA-PAGE. From Veterinary Hospital, Mannuthy, 15 samples were collected and three (20 per cent) of them were found to be positive. Thirty six samples from an organized dairy farm, Pattikkad were screened for the

presence of BRV and 23 (63.89 per cent) were detected as positive by RNA-PAGE. From a private dairy farm, Pallipuram, 11 samples were collected and three (27.27 per cent) of them were positive. Twenty eight samples from diarrhoeic calves reared by individual farmers were screened for the presence of BRV and none of them were found positive (Table 1).

Table 1. Results of RNA-PAGE analysis of faecal samples for BRV

Source	Samples tested by RNA-PAGE	No. of samples positive	Percent positive
University Livestock Farm, Mannuthy	34	0	0
Veterinary Hospital, Mannuthy	15	3	20
Private Farm, Pattikkad	36	23	63.88
Private Farm, Pallippuram	11	3	27.27
Individual Farmers	28	0	0
	124	29	23.39

4.2 REVERSE TRANSCRIPTASE – POLYMERASE CHAIN REACTION

In the present study, VP4 gene specific RT-PCR was done using the primers Con 2 and Con 3. The first step was reverse transcription of the double stranded RNA extracted from faecal samples into cDNA using MMuLV–Reverse transcriptase. The cDNA was subjected to PCR and an amplicon of 877bp was generated. The positive sample also gave the same amplicon. But the negative control did not reveal any amplification (Fig. 2).

Among 124 samples tested, 35 (28.23 per cent) samples were found positive. Thirty four samples from ULF, Mannuthy were screened for the presence of BRV by RT-PCR and none of them were positive. Among 15 samples from Veterinary Hospital, Mannuthy, four samples (26.67 per cent) were positive. From the organized dairy farm, Pattikkad 36 samples were collected and 25 (69.44 per cent) were found to be positive. Eleven samples were collected from a private farm at Pallippuram and four (36.36 per cent) of them were positive. Among 28 samples collected from individual farmers, two (7.14 per cent) were positive (Table 2).

All the samples from healthy calves and adult diarrhoeic animals were found to be negative by RT-PCR.

Table 2. Results of RT-PCR analysis of faecal samples for BRV

Source	Samples tested by RT-PCR	No. of samples positive	Percent positive
University Livestock Farm, Mannuthy	34	0	0
Veterinary Hospital, Mannuthy	15	4	26.67
Private Farm, Pattikkad	36	25	69.44
Private Farm, Pallippuram	11	4	36.36
Individual Farmers	28	2	7.14
Total	124	35	28.23

4.3 AGAR GEL IMMUNODIFFUSION

In the present study, the presence of BRV antigen in faeces of suspected animals were tested using hyper immune serum raised in rabbit against the reference positive antigen. On viewing the gel against diffuse light, a precipitin

line was observed between the reference positive antigen and the central well. The negative control did not give any precipitin line.

Among 124 calves clinically suspected for BRV, 16 (12.90 per cent) were found positive by AGID. The formation of a single precipitin line indicated complete identity with the reference antigen. The faecal samples collected from healthy calves and adult animals with diarrhoea were found to be negative.

Among 34 samples collected from University Livestock Farm, Mannuthy, none of the samples were positive by AGID. From Veterinary Hospital, Mannuthy, 15 samples were collected and one (6.67 per cent) of them was found to be positive. Thirty six samples from an organized dairy farm, Pattikkad were screened for the presence of BRV and 14 (38.89 per cent) were detected as positive by AGID. From a private dairy farm, Pallipuram, 11 samples were collected and only one (9.09 per cent) of them was positive. Twenty eight samples from diarrhoeic calves reared by individual farmers were screened for the presence of BRV and none of them were found positive (Table 3).

Table 3. Results of AGID for BRV antigen in faecal samples

Source	Samples tested by AGID	No. of samples positive	Percent positive
University Livestock Farm, Mannuthy	34	0	0
Veterinary Hospital, Mannuthy	15	1	6.67
Private Farm, Pattikkad	36	14	38.89
Private Farm, Pallippuram	11	1	9.09
Individual Farmers	28	0	0
Total	124	16	12.90

4.4. COMPARISON OF RNA-PAGE, RT-PCR AND AGID

Faecal samples from 124 calves with diarrhoea were screened for the presence of BRV by RNA-PAGE, RT-PCR and AGID. Twenty nine samples (23.39 per cent) were positive by RNA-PAGE. Rotavirus was detected in 35 (28.23 per cent) samples by RT-PCR. Agar Gel Immuno Diffusion could detect only 16 (12.90 per cent) samples as positive. Reverse transcriptase – Polymerase Chain Reaction detected BRV in six samples that were tested negative by RNA-PAGE. From the above results, RT-PCR was found to be more sensitive in detecting BRV.

Table 4. Comparison of RNA-PAGE, RT-PCR and AGID

Test	Number of samples tested	Number of positive samples	Per cent positive
RNA-PAGE	124	29	23.38
RT-PCR	124	35	28.23
AGID	124	16	12.90

4.5 ISOLATION OF BRV IN MDBK CELL LINE

Attempts were made to isolate BRV in MDBK cell line from two samples which were detected as positive by RNA-PAGE. No evidence of CPE could be appreciated in both the samples even after four passages. After the fourth passage, attempts were made to extract RNA from the infected cell line and were subjected to RT-PCR. But no specific amplification was obtained by RT-PCR assay in both the samples.

4.6 AGE-WISE DISTRIBUTION OF BRV INFECTION

The calves suspected for BRV infection were divided into five groups based on age. Thirty three samples were collected from calves of zero to two weeks of age and 13 samples (39.39 per cent) were positive by RT-PCR. From diarrhoeric calves of two to four weeks of age, 27 samples were collected and 10 samples (37.04 per cent) were found to contain BRV. Among 22 samples from calves of four to six weeks, seven samples (31.82 per cent) were detected as positive. Faecal samples from 20 diarrhoeric calves of six to eight weeks of age were tested and five samples (25 per cent) were positive. From calves of eight to fifteen weeks of age, 18 samples were collected and two samples (11.11 percent) were found to be positive. The results of occurrence of BRV infection among different age groups are presented in Table 5.

Table 5. Age –wise distribution of BRV

Age group (weeks)	Samples tested	Samples positive	Percent positive
0 – 2	33	13	39.39
2 – 4	27	10	37.04
4 – 6	22	7	31.82
6 – 8	20	5	25.00
8 – 15	18	2	11.11
Total	124	35	28.23

4.7 PROTEIN PROFILE OF BOVINE ROTAVIRUS

The protein profile of BRV was analysed using SDS-PAGE in 12.5 per cent resolving gel. Nine protein bands of size 131 kDa, 97.5kDa, 92 kDa , 66 kDa , 58 kDa , 43 kDa , 32 kDa , 28.3 kDa and 16.5 kDa were detected. The positive control had an additional band corresponding to 22 kDa (Fig. 4).

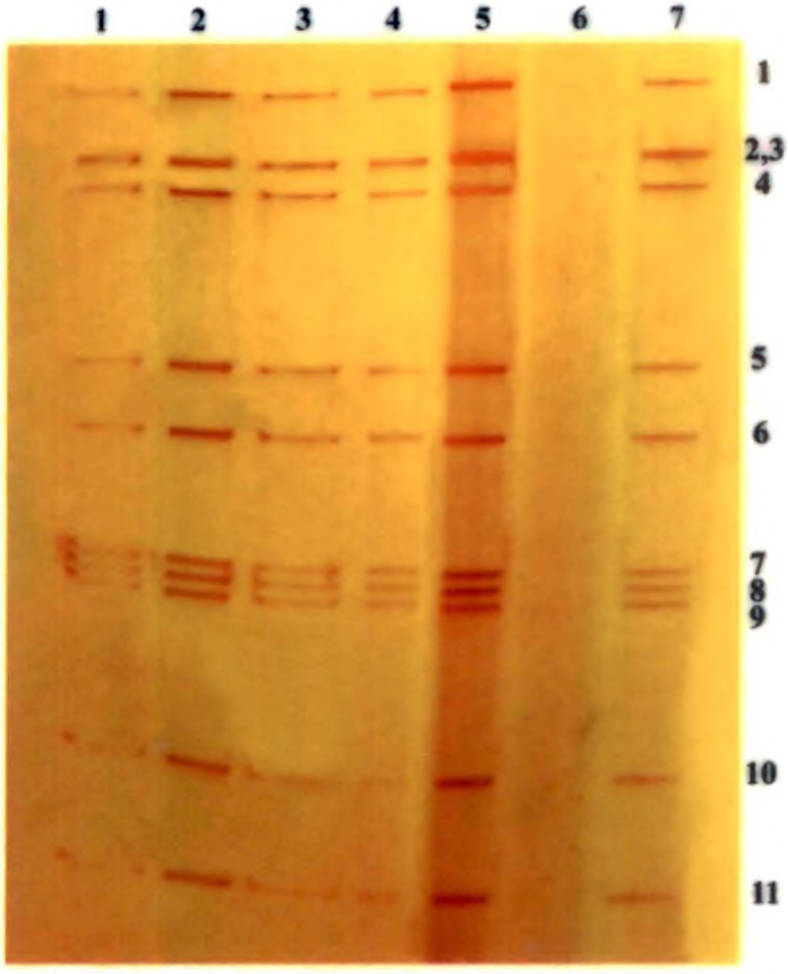

Fig 1. RNA Profile of Bovine Rotavirus (Representation)

Lane 1 to 5 - Samples
Lane 6 - Negative control
Lane 7 - Positive control

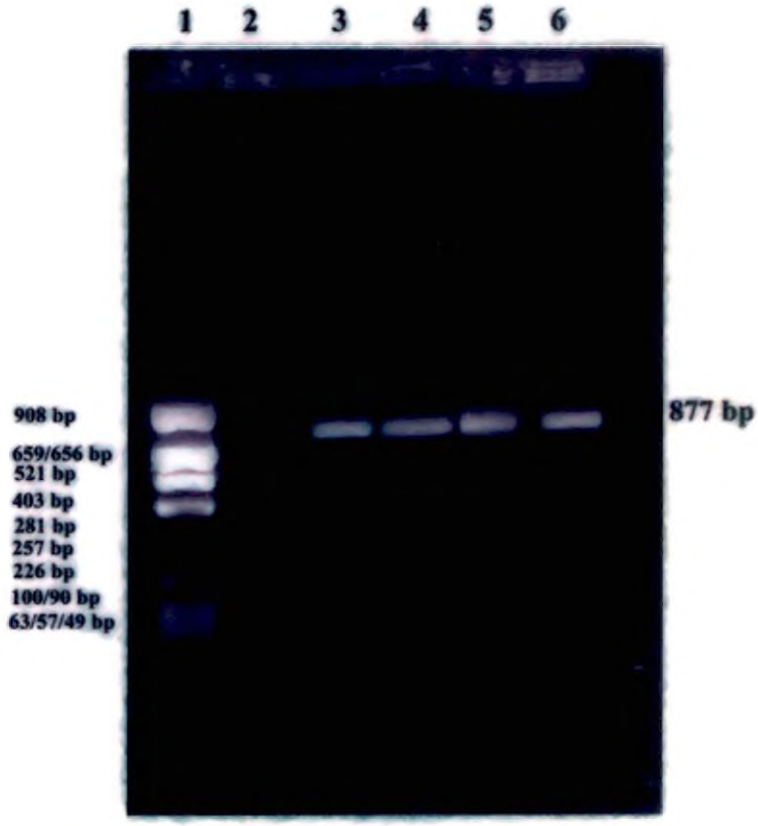

Fig 2. Agrose gel electrophoresis of RT-PCR amplified product of Bovine rotavirus (Representation)

Lane 1 pBR322DNA/Alu I Digest

Lane 2 Negative control

Lane 3 Positive control

Lane 4 to 6 Samples

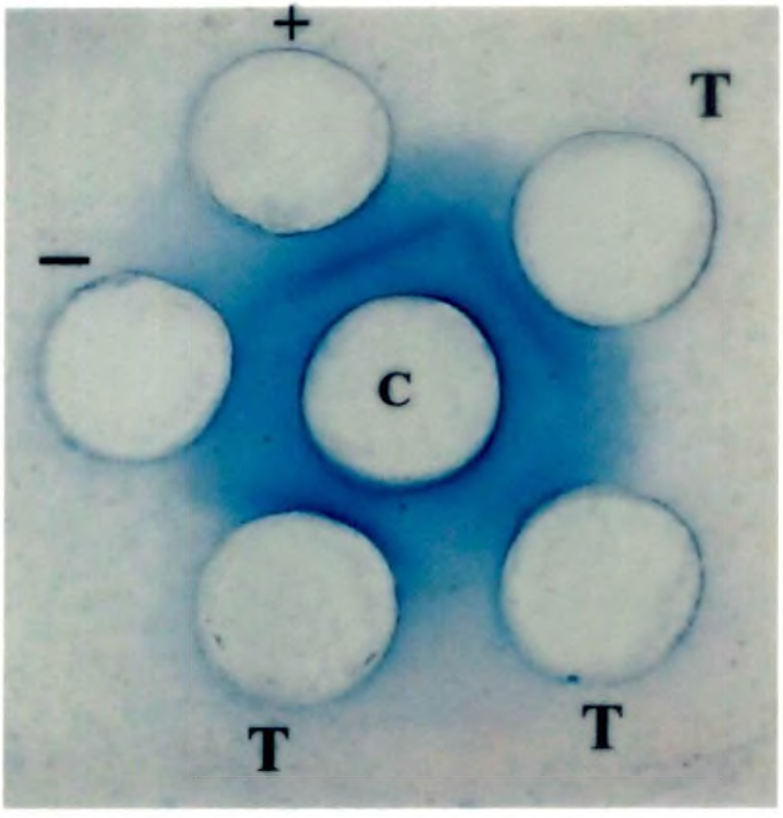

Fig 3. Agar Gel Immunodiffusion Test (Representation)

+ Positive antigen

- Negative control

T Test samples

C Antiserum

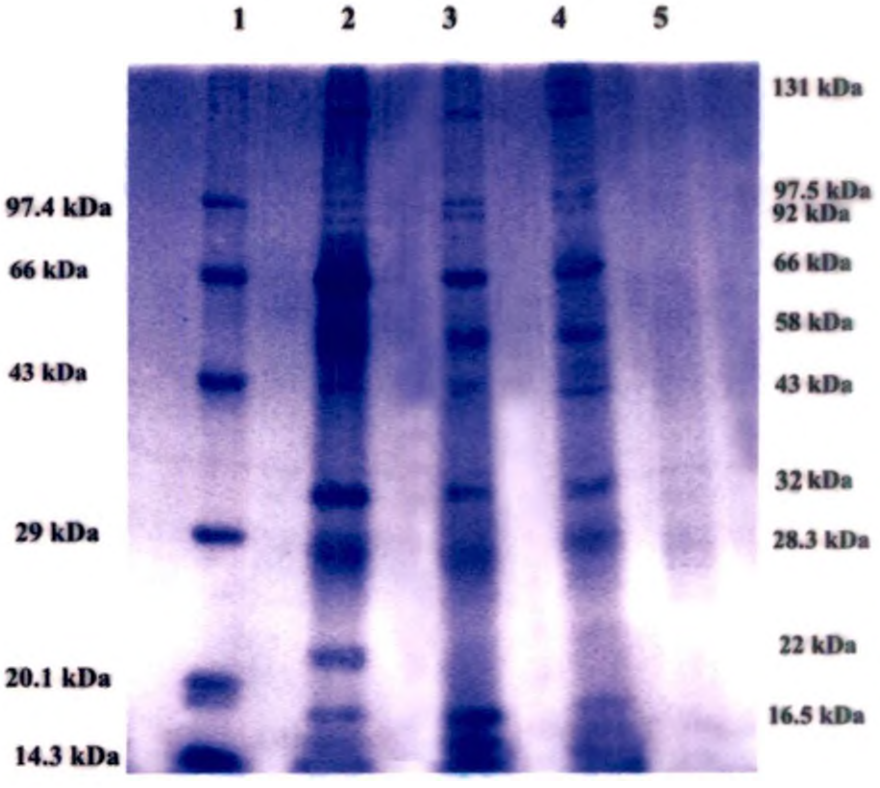

Fig 4. Protein profile of Bovine rotavirus
Lane 1 - Protein Marker
Lane 2 - Positive control
Lane 3,4 - Samples
Lane 5 - Negative control

DISCUSSION

5. DISCUSSION

Bovine rotavirus emerged as a new virus in calves in early 1970's. It is a highly contagious infection which mainly affects calves during first few weeks of their life (Acres and Babuik, 1978; Tzipori *et al.*, 1981 and Wani *et al.*, 2007). The clinical signs include profuse whitish diarrhoea and dehydration (Mebus *et al.*, 1971). It is not possible to diagnose rotaviral infections from clinical signs, since similar signs are shown by various other enteropathogenic bacterial infections (Benfield *et al.*, 1984). In severe cases of rotaviral infection, calves may excrete up to 10^{11} viral particles per millilitre of faeces. Most infections occur as a result of exposure to contaminated faeces. Hence, a rapid, sensitive and specific diagnosis of rotavirus infection is necessary for effective clinical and epidemiological management of the disease.

Previously, confirmatory diagnosis of rotaviral infection was mostly based on the detection of virus by EM and VI in cell cultures. Since conventional methods are time consuming and less sensitive, they are now being replaced by modern molecular biological techniques which allow rapid and presumptive identification of organisms directly from clinical samples. With the introduction of RT-PCR, rapid detection of fewer particles of BRV in faecal samples has become practical. This involves reverse transcription of viral RNA into cDNA followed by specific amplification.

In the present study, the ability of RNA-PAGE, RT-PCR and AGID to detect BRV in faecal samples was compared. The influence of age on the occurrence of rotavirus infection was studied. Sodium Dodecyl Sulphate - Polyacrylamide gel electrophoresis was employed to analyze the protein profile of BRV. The samples which were found positive by RNA-PAGE were inoculated into MDBK cell line. But the attempts to isolate BRV in cell line were found unsuccessful.

RIBONUCLEIC ACID –POLYACRYLAMIDE GEL ELECTROPHORESIS

The segmented RNA genome can be analysed by RNA-PAGE using the discontinuous buffer system as described by Herring *et al.* (1982) with some modifications. Many workers (Clarke and McCrae, 1981 and Herring *et al.*, 1982) employed RNA-PAGE with silver staining to study the electrophoretic migration pattern of rotavirus. The genome consists of 11 segmented RNA which can be separated into discrete bands using RNA-PAGE. The advantage is that it is possible to identify the group of rotavirus from the clustered arrangement of gene segments. It is convenient to analyze the genomic diversity and heterogeneity of the virus. Group A bovine rotavirus showed a migration pattern of 4:2:3:2 in polyacrylamide gels (Rodger *et al.*, 1981).

Adah *et al.* (2001) used 10 per cent acrylamide gels of two millimeter thickness for detection of human rotavirus RNA whereas Herring *et al.* (1982) used a five per cent gel.

In the present study, the extracted RNA was loaded into eight per cent polyacrylamide gels of one millimeter thickness and subjected to electrophoresis at 100 V and 20 mA for 16 h. It was found that when 10 per cent gel was used, the seventh, eighth and ninth segments were seen together as a single thick band. This could be resolved when eight per cent gel was used. The 11 segments showed a clustered arrangement of 4:2:3:2 which is typical of group A rotavirus. The segments were numbered from top. The migration pattern obtained was compared with positive sample. The samples obtained from animals in different farms showed a similar migration pattern which indicated that all of them belonged to group A rotavirus.

5.2 REVERSE TRANSCRIPTASE – POLYMERASE CHAIN REACTION

Reverse Transcriptase-Polymerase Chain Reaction was employed for the rapid detection of rotavirus by many workers (Snodgrass *et al.*, 1990 and Gentsch *et al.*, 1992). By using type specific primers in an RT-PCR, it is also possible to

identify the G serotypes and P genotypes of rotavirus (Gouvea *et al.*, 1994 and Gulati *et al.*, 1999). To identify the G types, VP7 gene specific primers were used. In the present study, the cDNA was synthesized from the extracted rotavirus RNA using genome segment 4 (VP4 gene) specific primers, Con 3 and Con 2 (Gouvea *et al.*, 1994) and Murine Leukemia Virus – Reverse Transcriptase (MMuLV-RT). The amplification of cDNA generated a product of approximately 877 bp size. These results were in accordance with those obtained by Gentsch *et al.* (1992), Fukai *et al.* (1998) and Minakshi *et al.* (2007). Since type specific primers were not used in the study, it was not possible to identify the G and P types of the BRV. If a multiplex RT-PCR assay with a cocktail of primers were used, it could be possible to identify the distribution of G and P types of BRV detected in the present study.

5.3 AGAR GEL IMMUNODIFFUSION

In the present study, AGID test was employed to detect the presence of rotaviral antigen in the suspected faecal samples by using hyper immune serum raised in rabbits. Faecal antigen was subjected to ultra centrifugation for three hours to obtain concentrated viral antigen. This was used to avoid the presence of multiple lines between the wells (Mohammed *et al.*, 1978). The test could detect viral antigen in only 12.90 per cent samples and was found to be the least sensitive among the three.

5.4 COMPARISON OF RT-PCR, RNA-PAGE AND AGID

Conventionally, rotavirus diagnosis was done by antigen detection in faeces using antibody based immunoassays (Flewett *et al.*, 1974 and Snodgrass *et al.*, 1976). Nucleic acid based assays have distinct advantage in the sense that these can detect viral RNA even in the absence of immuno reactive viral antigen. The development of specific and sensitive diagnostic assays is, therefore, becoming increasingly necessary for the detection of rotavirus.

In the present study, the diarrhoeic faecal samples were subjected to RT-PCR, RNA-PAGE and AGID. Among the 124 samples tested, RNA-PAGE could detect 29 (23.39 percent) samples as positive where as RT-PCR could detect BRV in 35 (28.23 per cent) samples. In this study, RT-PCR detected BRV in six samples which were found negative in RNA-PAGE. All the RNA-PAGE positive samples were tested as positive by RT-PCR also. Thus RT-PCR could be considered as more sensitive than RNA-PAGE. These results were in accordance with Arguelles *et al.* (2000). According to them, RT-PCR required 10^4 particles per milliliter of faeces whereas RNA-PAGE required 10^{11} particles per millilitre to be detected as positive. Wani *et al.* (2005) also got similar results. Wani *et al.* (2004) had reported that certain samples failed to produce an amplified viral band in RT-PCR despite being known to contain sufficient viral particles by RNA-PAGE. Some non specific inhibitors might be responsible for this. But in the present study, all RNA-PAGE positive samples were detected as positive by RT-PCR also. Agar Gel Immunodiffusion could detect rotaviral antigen only in 16 (12.90 per cent) samples and hence this test was found to be least sensitive among the three.

The occurrences of the infection in various farms were studied. It was found that the disease was more prevalent in calves in organized dairy farms than in those reared by individual farmers. This was in accordance with Quinn *et al.* (2002) who reported that rotaviruses occurred more frequently in intensively reared animals. Grover *et al.* studied the prevalence of rotaviral diarrhoea in organized dairy farms at Ambala and Meerut and found that BRV could be detected in 21.1 per cent of diarrhoeic cases. Rotavirus infection in organized cattle farms at Hissar was studied by Singh *et al.* (1993) using RNA-PAGE and noticed that the prevalence of BRV was 34.5 percent. In the present work, the prevalences of rotavirus in two private farms were 69.44 and 36.36 per cent respectively. These farms had a history of recurrent diarrhoea and mortality in calves. However, none of the samples collected from ULF, Mannuthy was positive. This might be due to the strict hygienic practices adopted in the farm.

From the diarrhoeic samples of calves kept by individual farmers, two samples out of 28 (7.14 per cent) were found positive by RT-PCR. This could not be detected in RNA-PAGE and AGID. This indicated that the concentration of virus in those samples were very less. Among the 15 samples from Veterinary Hospital, Mannuthy, only one (6.67 per cent) was found positive for BRV antigen by AGID where as RNA-PAGE and RT-PCR could detect rotavirus in two (20 per cent) and four (26.67 per cent) samples respectively.

5.5 ISOLATION OF BRV IN MDBK CELL LINE

In the present study, attempts were made to isolate BRV in MDBK cell line from faecal samples. According to Mebus *et al.* (1971), the established cell lines used for the propagation of BRV were BHK-21, Vero, LLC-MK_2 and He La cells. Bovine rotavirus in faeces of diarrhoeic calves had been isolated in MDBK cell line by Clarke *et al.* (1979). It was found that MDBK cell line produced ten fold more virions than MA-104, LLC-MK_2 and He La cells. The addition of trypsin increased the infectivity of virions (Urquidi *et al.*, 1981). Hence, in the present study, MDBK was chosen as cell line for isolation of BRV. Trypsin was incorporated in the virus inoculum for infectivity. Since faecal samples contain lot of contaminant bacteria, the faecal samples were filtered before inoculation to avoid bacterial contamination of cell cultures.

In the present work, four passages were done. But BRV could not be detected. Many workers had observed discrete foci of CPE in the infected monolayer. Welch and Twiehaus (1972) detected cytoplasmic vacuoles, eosinophilic inclusions and degeneration from the monolayer. Woode *et al.* (1975) reported that CPE was observed only with first passage of field rotavirus and not in subsequent passages in continuous cell lines. However, in the present study, no CPE was observed after four passages. Attempts to propagate human rotavirus in trypsinised bovine intestinal cells were done by Albrey and Murphy (1976) and found that there was no evidence of CPE at any stage. According to Quinn *et al.* (2002), rotaviruses were difficult to be isolated in cell culture from

clinical samples. Bora *et al.* (2007) isolated rotavirus in MA-104 cell line and no CPE was observed up to eight passages.

In the present work, attempts were made to isolate RNA from the cell culture using TRI reagent. Cells grown in monolayer were lysed directly in a culture dish and subjected to the procedures for RNA isolation. The pellet was subjected to RNA-PAGE and RT-PCR. No RNA was detected in polyacrylamide gels. The RT-PCR assay revealed no specific amplification in both the passages indicating the absence of virus replication in infected cell cultures.

5.6 AGE-WISE DISTRIBUTION OF BRV

In the present study, it was found that the occurrence of BRV infection was highest among calves below two weeks of age and lowest among calves above two months of age.

Many workers (Acres and Babuik, 1978; Sharma and Adlakha, 1994) have emphasized that the BRV infection was seen most commonly in calves at three to ten days of age than older age groups. According to Radostits *et al.* (2000), calves of one to three weeks of age were most susceptible to rotaviral diarrhoea. These observations were in accordance with the present study. The occurrence of infection was highest in the age group below two weeks (39.39 per cent) followed by two to four weeks of age (37.04 per cent). Seven out of 22 samples (31.82 per cent) were positive among diarrhoeic calves of four to six weeks of age. Among 20 diarrhoeic calves in the age group of six to eight weeks, only five (25 per cent) were found to be positive. The occurrence of infection was less common (11.11 per cent) in calves of eight to fifteen weeks of age. The age-wise distribution pattern obtained was in accordance with that of Singh *et al.* (1993) who observed that maximum occurrence of rotavirus infection in calves was during the first two weeks. Some other workers (Tzipori *et al.*, 1981) opined that calves were infected with rotavirus only during the first week of their life. In

the present study, 20 adult cows above one year of age with diarrhoea were screened for the presence of BRV, but none of them was found to be positive.

5.7 PROTEIN PROFILE OF BRV

Partially purified BRV when subjected to protein analysis by SDS-PAGE in 12.5 per cent polyacrylamide gels, the various polypeptides of the virus got separated into different bands. These bands were visible when stained with Coomassie brilliant blue dye. The molecular weights of these proteins were calculated by comparing the distance migrated by known molecular weight protein marker using Bio-rad gel documentation system. The positive sample RuBV 128 resolved ten proteins where as the samples under study yielded nine proteins. They lacked the 22 kDa protein. These results were in accordance with Rodger *et al.* (1977) who carried out SDS-PAGE analysis of rotavirus in 10 per cent polyacrylamide gels to yield nine polypeptides having molecular weights in the range of 14.5 to 131 kDa. The protein profile of BRV isolates were analysed using SDS-PAGE by Gulati *et al.*(1997) and five protein bands having molecular weights 116 to 120 kDa, 95kDa, 90kDa, 44kDa and 34kDa were detected. The results obtained in the present study were also in this range.

SUMMARY

6. SUMMARY

Bovine rotavirus, first discovered in 1969, is now recognized as the most important viral agent causing neonatal diarrhoea. The infection has been reported from many countries world-wide and also from different states of India. However, the prevalence of bovine rotavirus infection in Kerala has not been studied so far. Bovine rotavirus affects the younger age groups, most frequently during the first few weeks of their life. The disease has to be differentially diagnosed from other enteropathogenic viral and bacterial infections. Since this virus is highly contagious, a rapid sensitive and specific diagnosis is absolutely necessary to control the spread of disease. The conventional methods employed for the detection of BRV are considered to be relatively less sensitive, more laborious and time consuming. Recently, nucleic acid based techniques like RT-PCR and RNA-PAGE has been introduced for the detection of BRV directly from faecal samples. Hence, the present study was undertaken to detect the presence of BRV in diarrhoeic faeces of calves by RT-PCR, RNA-PAGE and AGID and to compare these three techniques. Attempts for isolation of BRV from faecal samples were also performed in MDBK cell line.

One hundred and twenty four diarrhoeic faecal samples were screened for the presence of BRV. Twenty adult animals having diarrhoea and 20 healthy calves were also tested. University livestock Farm, Veterinary Hospital, Mannuthy and some dairy farms in Thrissur district were the sources. Samples were also collected from calves reared by individual farmers in and around Thrissur. All these samples were subjected to RT-PCR, RNA-PAGE and AGID. The protein profile was analyzed using SDS-PAGE.

Among 124 faecal samples tested for BRV, 29 (23.39 per cent) samples were found to be positive by RNA-PAGE. Silver staining was employed to visualize the 11 segmented genome on eight per cent polyacrylamide gels. The segments were arranged in a 4:2:3:2 pattern, typical of Group A rotavirus. On

RT-PCR assay, 35 (28.23 per cent) samples were detected as positive. The primers used were specific for VP4 gene and a PCR product of 877 bp was generated. In the present study RT-PCR could detect BRV in six samples which were found negative by RNA-PAGE. Agar Gel Immunodiffusion could detect rotaviral antigen in 16 (12.90 per cent) samples. From the present study, RT-PCR can be considered not only as a sensitive and specific, but also as a rapid and simple technique for routine diagnosis of BRV infection in calves.

The occurrence of rotavirus infection was most common in calves in the age group of zero to two weeks (39.39 per cent) followed by two to four weeks (37.04). All the faecal samples collected from healthy calves and adult diarrhoeic calves were tested negative for BRV by RNA-PAGE, RT-PCR and AGID. The protein profile of BRV was studied using SDS-PAGE in 12.5 per cent polyacrylamide gels and the gels were stained using Coomassie Brilliant Blue. Nine polypeptides having molecular weights 131 kDa, 97.5 kDa, 92 kDa, 66 kDa, 58 kDa, 43 kDa, 32 kDa, 28.3 kDa and 16.5 kDa were detected. Attempts to isolate BRV from faecal samples in MDBK cell line were found unsuccessful.

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* Originals not seen

DETECTION OF ROTAVIRUS IN THE FAECES OF DIARRHOEIC CALVES BY REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION AND SILVER STAINING

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ABSTRACT

A study was undertaken to detect the presence of rotavirus in the faeces of diarrhoeic calves by RT-PCR and RNA-PAGE. Agar Gel Immune Diffusion was performed to detect the presence of rotaviral antigens in faecal samples using hyperimmune serum raised in rabbits. The protein profile of BRV was analyzed using SDS-PAGE. Attempts were made to isolate BRV from faecal samples in MDBK cell line.

One hundred and twenty four faecal samples of diarrhoeic calves were collected from University Livestock Farm, Mannuthy, Veterinary Hospitals of Kerala Agricultural University, some dairy farms in Thrissur district and also from individual farmers in and around Thrissur. Twenty samples each were collected from adult cattle above one year of age with diarrhoea and normal healthy calves. All these samples were screened for the presence of BRV by RNA-PAGE, RT-PCR and AGID. Among 124 faecal samples collected 29 (23.39 per cent) samples were detected as positive by RNA-PAGE. The clustered arrangement of the 11 segments of the genome showed a 4:2:3:2 migration pattern, typical of group A bovine rotavirus. Reverse Transcriptase – Polymerase Chain Reaction could detect BRV in 35 (28.23 per cent) samples. By using AGID, only 16 (12.90 per cent) samples were found positive. Among the various tests employed, RT-PCR was found to be more sensitive in the diagnosis of BRV infections. All the 20 faecal samples from adult cattle with diarrhoea were tested negative by the three methods. Rotavirus could not be detected in the faeces of healthy calves by any of the tests employed. The protein profile of BRV revealed nine polypeptides having molecular weight in the range of 16.5 to 131 kDa. The age-wise distribution of BRV infection in calves was studied. It was found that the occurrence of infection was most common in zero to two weeks of age (39.39 per cent) followed by two to four weeks of age (37.04 per cent). The faecal samples which were found positive by RNA-PAGE was inoculated into MDBK cell line. But the attempts to isolate BRV were found unsuccessful.