# MOLECULAR DETECTION OF CAUSATIVE AGENTS ASSOCIATED WITH DIARRHOEA IN CALVES

**T H E S I S**

**Submitted**

**in partial fulfillment of the requirements for the Degree of**

**MASTER OF VETERINARY SCIENCE**

**IN**

**VETERINARY MICROBIOLOGY**

**BY**

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**(INDIA)**

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## DECLARATION OF STUDENT

I hereby declare that the experimental research work and interpretation of the thesis entitled "**MOLECULAR DETCTION OF CAUSATIVE AGENTS ASSOCIATED WITH DIARRHOEA IN CALVES"** or part thereof has not been submitted for any other degree or diploma of any university, nor the data have been derived from any thesis/publication of any university or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

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**SRUTHY S** has satisfactorily prosecuted her course of research for a period of not less than one semester and that the thesis entitled, "**MOLECULAR DETCTION OF CAUSATIVE AGENTS ASSOCIATED WITH DIARRHOEA IN CALVES"** submitted by her is the result of research work and is sufficient to warrant its presentation to the examination in the subject of **Veterinary Microbiology** for the award of **Master of Veterinary Science** degree by the Maharashtra Animal and Fishery Sciences University, Nagpur.

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This is to certify that the thesis entitled, "**MOLECULAR DETECTION OF CAUSATIVE AGENTS ASSOCIATED WITH DIARRHOEA IN CALVES"** submitted by **SRUTHY S** to the Maharashtra Animal and Fishery Sciences University in partial fulfillment of the requirement for the degree of **Master of Veterinary Science** has been approved by the Student's Advisory Committee after examination in collaboration with the External Examiner.

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***Nagpur*** ***Sruthy S***

***Date***

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| | |
|---|---|
| % | Percentage |
| µg | Microgram |
| µg/ml | Microgram Per Millilitre |
| µl | Microliter |
| µM | Micromolar |
| $^0$C | Degree Celsius |
| AGE | Agarose Gel Electrophoresis |
| BHI | Brain Heart Infusion |
| BLAST | Basic Local Alignment Search Tool |
| BoCV | Bovine coronavirus |
| BoRV | Bovine rotavirus |
| bp | Base pairs |
| cDNA | Complementary deoxy ribonucleic acid |
| CL | Control Line |
| CTAB | Cetyltrimethyl ammonium bromide |
| DNA | Deoxy ribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| E.coli | Escherichia coli |
| e.g. | For example |
| EAggEC | Enteroaggresive *E. coli* |
| EDTA | Ethylene Diamine Tetra Acetic acid |
| EHEC | Enterohaemorrhagic *E. coli* |
| EIA | Enzyme Immuno Assay |
| EIEC | Enteroinvasive *E. coli* |
| ELISA | Enzyme Linked Immunosorbent Assay |
| EMB | Eosine Methylene Blue |
| EPEC | Enteropathogenic *E.coli* |
| *et al.* | *Et alia* |
| Et Br | Ethidium Bromide |
| ETEC | Enterotoxigenic *Escherichia coli* |

| | |
|---|---|
| ETEC | Enterotoxigenic *E. coli* |
| For | Forward |
| g | Grams |
| HCl | Hydrochloric Acid |
| HE | Haemagglutinin -Esterase |
| Hrs | Hours |
| IMViC | Indole Methyl Red Voges Proskauer Citrate |
| LAT | Latex Agglutination Test |
| LF | Lateral Flow |
| LT | Heat Labile Toxin |
| MEGA | Molecular Evolutionary Genetics Analysis |
| mg | Milligrams |
| $MgCl_2$ | Magnesium Chloride |
| MHA | Muller Hinton Agar |
| Min. | Minute |
| Ml | Millilitre |
| mM | Millimolar |
| MR | Methyl Red |
| N gene | Nucleocapsid gene |
| NA | Nutrient Agar |
| NaCl | Sodium chloride |
| NFW | Nuclease Free Water |
| NSP | Non-structural Protein |
| OIE | Office International Des Epizooties |
| PAGE | Polyacrylamide Gel Electrophoresis |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| Rev | Reverse |
| RNA | Ribonucleic acid |
| RNA-PAGE | Ribonucleic acid- Polyacrylamide Gel Electrophoresis |

| | |
|---|---|
| RPM | Revolutions Per Minute |
| rRNA | Ribosomal Ribonucleic Acid |
| RT-PCR | Reverse Transcription Polymerase Reaction. |
| SBM | Sample Buffer Mixture |
| SDS | Sodum Dodecyl Sulphate |
| SN-PCR | Semi nested Polymerase Chain Reaction |
| Spp. | Species |
| ST | Heat Stable Toxin |
| STEC | Shiga toxin-producing *E. coli* |
| Stx 1 | Shigatoxin 1 |
| Stx 2 | Shigatoxin |
| TBE | Tris Borate Ethylene diamine tetra acetic acid |
| TE | Tris- Ethylene diamine tetraacetic acid |
| TL | Test Line |
| TSI | Triple Sugar Iron |
| VP | Voges-Proskauer |
| VP | Viral Protein |
| VTEC-RPLA | Verotoxigenic *Escherichia coli*- Rapid Plate Latex Agglutination |

# INTRODUCTION

In any livestock system, the success of the finished animal is dependent upon the quality and management of the newborn. The calf rearing period is a most crucial period for dairy and beef enterprises as development of healthy calves play major role in making the enterprise profitable. In the first year of life of calves, 50% mortality occurs within the first six weeks. The high morbidity and mortality rates of the diseases in turn reduce farm profitability. The mortality of neonatal calves in the first month of age is more than 80% of the total mortality (Jenny *et al*., 1981). Neonatal diarrhea is one of the most important diseases in calves worldwide causing large economic losses to cattle herds (Stanton *et al*., 2013).

Various infectious agents like viruses, bacteria and protozoa are responsible for calf diarrhea (Smith, 2009). Among these agents, bovine rotavirus group A and bovine coronavirus (BCoV) act as viral agents; *Salmonella* species and *E.coli* K99+ species act as bacterial agents and *Cryptosporidium* species act as protozoan agent (Bhat *et al*., 2012; Bhat *et al*., 2013; Singla *et al*., 2013).

Bovine rotavirus is a very important infectious agent that causes neonatal calf diarrhea (Alferi *et al.*, 2004) and it belongs to the genus *Rotavirus* of the family *Reoviridae*. It is a non-enveloped virus possessing eleven double-stranded RNA segments (16-21kbp) (Fenner, 2011). Based on antigenic and genetic similarity of intermediate capsid protein (VP6), there are 7 serogroups (A through G) among rotavirus (Steele *et al*., 2004). Major cause of rotaviral infection in domestic animals is Group A rotavirus. It is the Group A where most of the bovine rotavirus (95%) belong to .Groups B and C rotaviruses have conjointly been known to be present in the field cases (Ghosh *et al*., 2007; Lucchelli *et al*., 1992; Tsunemitsu *et al*., 1992). Virus classified as group A rotavirus (RV-A), together with BoRV-A, possess two independent neutralization antigens. They are VP4 and VP7, on the outer capsid, that specify the P type (protease-sensitive protein) and G type (glycoprotein), respectively (Estes, 2001; Kapikan *et al.*, 2001). So it has been proposed that serotypic classification of rotaviruses accounts

for both VP4 (P) (Snodgrass *et al.,* 1992) and VP7 (G) (Hoshino *et al.,* 1984) specificities. 27 P types and 16 G types have been reported in domestic animals. Bovine rotavirus belongs to G1, G6, G8, or G10 types (Gulati *et al.,* 2007; Khamrin *et al*., 2007; Martella., 2007; Rao *et al*., 2000).

The rotavirus genome encodes six structural and six non-structural polypeptides. The nomenclature of viral proteins designates structural proteins as VP and non-structural proteins as NSP followed by number indicating the proteins molecular weight (Arias *et al.,* 1982; Mason *et al.,* 1983). The genome segment 6 encodes VP6 protein and is most abundant structural protein forming the inner capsid of the virion. The protein contains epitopes, common for all group 'A' rotaviruses and epitopes for subgroup I or II (Greenberg *et al.,* 1983). VP6 protein plays an important role in transcriptase activity by maintaining the proper conformation of viral core or transcriptional complex (Estes and Cohen, 1989). The protein is highly antigenic and immunogenic and a target protein in most diagnostic assays for detection of rotaviruses. Replication defective recombinant adenovirus vectors which express the group specific antigen VP6 have been used for studying the immune response induced by this protein *in vivo* (He *et al.,* 2002). The genome segment 4 encodes a non-glycosylated protein VP4. The protein shares neutralization specific epitopes with VP7, the major protein involved in neutralizing immune response. Though minor, this protein is an important structural component of outer capsid. The gene segment 7 (can be genome segment 7, 8 or 9 depending upon the rotavirus strain) encodes VP7 protein, which is the major outer capsid protein. This protein is highly immunogenic and induces neutralizing antibodies and is major protein responsible for serotype specificity.

While VP4, VP6 and VP7 play the major role in maintaining the viral structure, virus attachment and antigenicity, nonstructural glycoprotein 4 (NSP4) is having a special role as viral enterotoxin and it interferes with cellular homeostasis. It is done by elevating the calcium ion influx into cytoplasm (Ball *et al*., 2005) which accounts for the drastic change in movement of water and nutrients across the intestinal epithelium. Bovine rotavirus causes diarrhea in

calves of 1 to 2 weeks of age (Chinsangaram *et al*., 1995). The milk uptaken by the calf provides a good environment for the survival of rotavirus at the gastrointestinal pH and the virus thus infects the intestinal epithelial cell (Dhama *et al*., 2009).This may explain the susceptibility of unweaned calves to calf diarrhea than weaned calves. The incubation period is very short (12-24 hours) (Steele *et al*., 2004) and induces per acute diarrhea infected calves. The virus replication occurs in the cytoplasm of epithelial cells of villi (Holland, 1990) and causes destruction of mature enterocytes in the villi. This results in the secretion of a viral enterotoxin (e.g., NSP4) caused by activation of the enteric nervous system by vasoactive components from the damaged cells. This results in maldigestive/ malabsorptive diarrhea. Evidence for interspecies transmission and genetic reassortment between human and animal rotaviruses has raised the zoonotic concern of rotavirus (Martella *et al*., 2010).

Bovine coronavirus (BCoV) is only second to rotavirus in causing neonatal diarrhea (Clark, 1993; Saif, 1990). Bovine coronavirus has been identified in both diarrheic and healthy calves, complicating assessment of its role as a primary pathogen (Duckmanton *et al.*, 1998; Snodgrass *et al*., 1986). Bovine coronavirus is a member of the genus *Betacorona virus* which was formerly classified as group 2a coronaviruses and belongs to the family *Coronaviridae,* order *Nidovirales* and possess a single-stranded, enveloped, non-segmented RNA of 27-32 kb genome with positive polarity (Decaro *et al*., 2008; De Vries *et al*., 1997; Hasoksuz *et al*., 2007; Van Regenmortel *et al*., 2000). The viral genome includes thirteen open reading frames. Its non-segmented RNA acts as a messenger RNA (mRNA) in infected cells and directly exploits the cell protein machinery to produce viral proteins (Saif, 2004; Clark, 1993; Almeida *et al*., 1968). The coronavirus particles are helical in symmetry, about 120 nm in diameter. Their pleomorphic to spherical shape with prominent surface projections is just like the corona of the sun (Lai, 1990). Virion contains five structural proteins: the nucleocapsid (N), the transmembrane (M), the haemagglutinin/esterase (HE), the spike (S) and the small membrane (E) proteins (Saif, 1993).

The bovine coronavirus infection can cause diarrhea in calves at 1-2 weeks of age, winter dysentery in adults and bovine respiratory disease complex in both adults and young (Cho *et al*., 2001; Cho *et al*., 2013 and Liu *et al*., 2006). Cases of bovine coronavirus enteritis occur in both beef and dairy herds where clinical signs develop between 5 and 30 days of life and the peak of incidence will be between days 7 and 10 (Boileau *et al*., 2010).

The spike (S) protein of bovine coronavirus has a major role in virus entry and subsequent pathogenesis (Lin *et al*., 2000). The spike protein consists of 2 subunits (S1 and S2) and has a crucial role in virus-host interaction. S1 subunit functions in attachment of virus to the host cell receptor and S2 subunit helps in the infusion of viral envelope to the membranes of host cells (Reuter *et al*., 2009; Yoo *et al*., 1991). The infection starts in the small intestine and colon will be affected later. Initially, the spike protein and hemagglutinin-esterase (HE) protein of the virus attach and fuse onto epithelial cells of intestine. (Payne *et al*., 1990; Schultze *et al*., 1991). The virus replication occurs in enterocytes and the release of progeny viruses occurs through normal secretory mechanism and cell lysis. The clinical signs have a longer duration and this is due to the damage done by the virus to crypt enterocytes.

The organisms isolated from the faeces of neonates and first described by Escherich as bacterium coli commune (Escherich, 1885) have become the most fully documented organisms currently known. *Escherichia coli* belongs to the bacterial family Enterobacteriaceae. It is a Gram-negative, rod-shaped, motile, non-spore forming bacteria that are often motile by means of flagella. Almost all strains of *E.coli* grow well on usually used laboratory media in both the presence and absence of oxygen. Its metabolism can be either by respiration or fermentation. It serves as the major facultative anaerobe as a harmless saprophyte throughout its life but Larulle (1889) was the first to suggest the role of *E. coli* as a pathogenic organism. About 50% cases of neonatal diarrhoea are ascribed to *E. coli* (Tripathi and Soni, 1984). *E. coli* can mainly be classified into 6 pathogroups based on their virulence scheme such as enterotoxigenic *E. coli* (ETEC); shiga toxin-producing *E. coli* (STEC); enteropathogenic *E.coli* (EPEC); enteroinvasive

*E. coli* (EIEC); enteroaggresive *E. coli* (EAggEC); and enterohaemorrhagic *E. coli* (EHEC) (Kaper *et al*., 2004; Nataro and Kaper, 1998). Among these pathogroups, ETEC strain is the most common cause of neonatal diarrhea and this is due to the K99 (F5) adhesion antigen which is commonly referred to as *E. coli* K99+ and also the heat-stable enterotoxin (Nataro and Kaper, 1998). Neonatal calves are most susceptible to ETEC infection during first 4 days after birth and results in "watery" diarrhea (Foster and Smith, 2009). Following ingestion, the gut epithelium gets infected by ETEC and multiplication occurs in enterocytes of intestinal villi. In enterotoxic colibacillosis, the pathogenic *E. coli* gets adhered to the mucosa and proliferate in the lumen of intestine and results in the production of a potent enterotoxin, which stimulate excessive secretion of fluid from intestinal mucosa (Moon, 1974). Due to this fluid loss, animal undergoes dehydration which in turn leads to death. The most favorable environment of ETEC colonization is distal portion of small intestine and is due to a pH less than 6.5. The bacterium expresses the K99 antigen which helps in the attachment (Francis *et al*., 1989). After the colonization, heat stable toxin is evoked by ETEC and results in secretary diarrhea. Enterotoxigenic *E. coli* produce severe diarrhea in calves during the first two weeks of life with highest frequency of pathogenicity in calves less than 3 days old (Snodgrass, 1986; Jones and Hunt, 1985).

Diarrhoeagenic *E. coli* should be differentiated from non-diarrhoeagenic E. coli and it is mainly done on the basis of 'O', 'H' and 'K' antigens, which together constitute the serotype. Serotyping of O antigen is not enough to identify a strain as diarrheic, because it does not correlate the presence of virulence factors (Sunabe and Honma, 1998). Thus, identification of diarrheic *E. coli* strains should be done by the detection of factors that determine the virulence of these organisms.

Molecular diagnostic methods were developed initially for the detection of diarrheic *E. coli* strains. Indeed, molecular methods remain the most reliable and popular techniques for the differentiation of diarrhoeagenic strains from nonpathogenic members of the intestinal flora and for distinguishing one category

from another. Substantial progress has been created each within in the development of PCR methods and nucleic acid-based probe technologies.

The polymerase chain reaction is widely used for the detection of enterotoxin genes and verocytotoxin genes. The PCR is a fast and versatile technique. The technology is sensitive and provides a powerful set of tools for diagnosis and detection of *E. coli* (Patel, 2009). An additional advantage of the PCR assay is that it allows the amplification of a product that can be sequenced to get further epidemiologic information. Jadhav (2009) and Tumlam (2018) have worked on Rotavirus in other parts of Maharashtra state, however no work has been done in the Vidharbha region for molecular detection of bacterial and viral causative agents associated with diarrhea in calves and it is necessary to utilize and evaluate highly sensitive PCR based tools for detection and differentiation of locally circulating types of bovine rotavirus & coronavirus, and bacterial strains which would eventually help in disease diagnosis and to formulate control strategies. The data generated will be useful for development of a vaccine of local prevalent strains. Hence, looking to the paucity of the information and importance of the neonatal calf diarrhea and considering the above facts, the present study is planned with the objectives,

1. To detect and characterize bovine rotavirus & coronavirus by RT-PCR and nested PCR, recovered from diarrheic calves.

2. To detect and characterize predominant bacterial isolates recovered from diarrheic calves.

3. To study sequencing and phylogenetic analysis of bovine rotavirus, coronavirus and predominant bacterial isolates

# REVIEW OF LITERATURE

Neonatal calf diarrhea poses a threat to the dairy industry. It is of multifactorial etiology, which creates a complex situation. The common causes are bovine rotavirus, bovine coronavirus, enterotoxigenic *E. coli* (ETEC), *Salmonella*, coccidia and cryptosporidia. Amongst these, bovine rotavirus, bovine coronavirus and enterotoxigenic *E. coli* (ETEC) are commonly encountered in neonatal calf diarrhea.

The present review of literature has been dealt under the following broad heads:

A. Detection of bovine rotavirus and coronavirus infection in calves by Latex agglutination Test & Antigen Detection Test kit respectively.

B. Molecular detection of bovine rotavirus and coronavirus infection in calves by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

C. Characterization of predominant bacterial isolate E.coli using cultural, biochemical and molecular techniques.

D. Sequencing and phylogenetic analysis of bovine rotavirus, coronavirus, predominant bacterial isolates.

## 2.1. Detection of bovine rotavirus and coronavirus infection in calves by Latex Agglutination Test & antigen detection test kit

### 2.1.1. Detection of bovine rotavirus by Latex Agglutination Test

**Work done in India**

Singh (2009) processed a total of 53 faecal samples collected from bovine (cattle/buffalo) calves showing signs of diarrhea. Latex Agglutination Test detected rotavirus in 17 (32.08%) samples. Sex wise, female calves (38.46%) were more susceptible than males (25.93%). Age wise, highest incidence was observed in calves of age group 0-2 weeks (35.00%), followed by 4-6 weeks (33.33%), 2-4 weeks (31.25%) and 6-8 weeks (20.00%).

Hashem *et al*. (2012) determined the success of LA and EIA by screening 450 fecal specimens for rotavirus. In a test, about 94 samples (20.8%) were reactive, 45.7% as non- reactive and 33.3% remain indeterminate. Further all LA positive also been positive in EIA tests and 25 LA negative samples were positive by EIA.

Ade *et al.* (2019) reported that a total of 35 out of 288 (12.15%) fecal samples were found positive for bovine rotavirus in Maharashtra state, India by latex agglutination test. A higher incidence (14.50%) in cattle was observed as compared to buffalo (8.04%) calves of 0 to 6 months of age. Further higher incidence was noted in male and female both in cattle as compared to buffalo calves.

**Work done abroad**

Hammami *et al*. (1990) reported that ELISA and agglutination had higher sensitivity (84%) for rotavirus than PAGE (79%) when a total of 77 bovine faecal samples, collected from young calves with diarrhea were screened from California Veterinary Laboratory Diagnostic System at Davis or the Veterinary Medicine Teaching and Research Centre at Tulare, California USA.

Yousif *et.al*. (2001) compared latex agglutination with ELISA showed sensitivity 100% and specificity 96.3% from 63 faecal samples of calves with clinical diarrhea due to gastroenteritis were tested for Bovine rotavirus by the Rota test latex slide agglutination test comprising 40 specimens (63%)showed positivity by LAT.

Erdogan *et al*. (2003) studied to determine the frequency of rotavirus and coronavirus in neonatal calves using Latex agglutination and ELISA respectively. The prevalence of bovine rotavirus and coronavirus in neonatal calves was determined to be 31.0% and2.2% respectively.

### 2.1.2 Detection of bovine coronavirus by rapid antigen detection test kit

**Work done abroad**

Izzo *et al.* (2012) reported that 30 out of 132 (22.73%) diarrheic fecal samples were positive for bovine coronavirus on lateral flow test using dipstick.

Abou El-Ella *et al*. (2013) reported that 21 out of 124 fecal samples collected from diarrheic calves were positive on immune- chromatographic rapid tests (FASTest® Strips)

## 2.2 Molecular detection of bovine rotavirus and corona virus infection in calves by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

### 2.2.1 Molecular characterization of bovine rotavirus by RT-PCR

**Work done in India**

Dadawala (2008) screened 76 fecal samples for the detection of rotavirus antigen using Latex agglutination test and RT-PCR was employed for the detection of rotavirus genome. Of these, 14 (18.42%) and 6 (7.89%) were found positive in LAT and RT-PCR, respectively.

Malik *et al.* (2013) studied rotavirus genotype distribution in human, buffalo, cattle, swine and poultry by nested PCR and reported genotypes of G10 and G3 specificity and P [1] and P [3] types in Madhya Pradesh. Whereas, only the G1 genotype dominated and for the first time group B rotavirus was also detected in an adult. The overall prevalence rate of rotavirus was 17.19% (126/733), maximum in humans (30.23%), followed by buffaloes (22.01%), cattle (13.33%), swine (13.04%) and poultry (6.47%).

Udaykar *et al.* (2013) reported that out of 116 fecal samples collected from diarrheic cow and buffalo calves, rotavirus was detected in 5 (4.3%) samples by RT–PCR VP7 gene based amplification by RT–PCR confirmed the presence of rotavirus in Malwa region of Madhya Pradesh. The higher rotavirus incidences in

buffalo calves (4.76%) than cow calves (3.77%) when targeted for VP7 by using RT-PCR analysis.

Malik *et al.* (2014) selected cattle calves below 6 months of age, having diarrhea for study and 87 samples were collected from organized dairy farm at Mukteswar, Nainital (n=20) and Tunja, BajwarDharanaula, Bhaishori villages of Almora districts (n=67) of Uttarakhand, India during winters of 2010-2012. Out of 87 samples, only 7 samples (8.04%) were found positive for the amplification of partial length VP4 gene (864 bp).

Sravani *et al.* (2014) reported that a total of 120 samples were collected from cattle and buffalo calves upto 3 months of age from July 2012 to March 2013 and conducted RT-PCR for the identification of VP4 gene amplicon size of 864 base pairs. Out of 120 samples, 4 samples (3.33%) were positive for VP4 gene.

Deswal *et al.* (2015) reported that out of 85 specimens, 11 (12.94%) were detected positive with characteristic migration patterns (4:2:3:2) of long electropherotype of group A rotavirus. One out of eleven samples (B29 isolate) was amplified in VP4 and VP7 gene specific RT-PCR. The agarose gel electrophoresis of VP4 and VP7 gene PCR products of B29 isolate showed 864 and 1062 bp amplification, respectively.

Prasad *et al.* (2015) examined a total of 103 diarrheic fecal samples from calves of 0-1 month of age which were collected from organized dairy farms of Hisar district of Haryana during 2007-2008 and found out that 11 (10.67%) out of 103 samples were showing amplicons of 864 bp VP4 gene by RT-PCR. The amplification of cDNA was carried out using VP4 gene specific published oligonucleotide primers (Isegawa *et al*., 1993)

Ahmed *et al.* (2017) studied 196 samples of diarrheic and non-diarrheic calves ageing about 4 months by using RNA-PAGE assay. They recorded about 13.26% of sample positive for bovine rotavirus having characteristics migration pattern of group A RV. But when same samples screened by RT-PCR, about 71

(36.22%) of positive cases for RV found which narrates the success of this technique.

Tumlam *et al*. (2018) reported that out of 44 samples screened, 43 (97.72%) samples were found positive for rotavirus. Samples from bovine calves, piglets, lambs, kids and pups were found cent percent positive for group A Rotavirus except for the human infants, where 17 out of 18 samples were found positive by VP6 gene based RT–PCR assay.

Nayak *et al*. (2018) reported that 3 out of 100 (3%) diarrheic fecal samples from buffalo calves were found positive by RT-PCR.

Das *et al.* (2018) reported that 39 out of 175 (22.28%) diarrheic bovine fecal samples were for found positive for bovine rotavirus. All the samples were screened by RT-PCR. For RT-PCR two published primer sets were used which were amplified to produce 309 and 304 bp sized amplicon for VP6 and VP7 gene on 1.7% agarose gel electrophoresis and 39 (22.28%) numbers were found to positive for both VP6and VP7 genes of bovine rotavirus. Both Electrophero typing and RT-PCR were suitable for detection of group a rotaviral infection though RT-PCR was found to be more sensitive than electropherotyping.

Gill *et al.* (2017) reported that 15 out of 198 (7.57%) diarrheic fecal samples collected from bovine calves were positive for the presence of bovine rotavirus. The RNA was subjected to two step reverse transcriptase PCR with VP7 gene specific primers as per cycling conditions described by Husain *et al.* (1995). The resultant PCR products (304 bp) was analyzed by agarose gel electrophoresis and digitally recorded by gel documentation system.

**Work done abroad**

Thais *et al.* (2011) reported that the rotavirus was detected in 80% (16 out 20) of diarrheic fecal samples analyzed by RT-PCR assay amplified 1,062 bp and 876 bp products of VP7 and VP4 genes, respectively.

Mukhtar *et al.* (2016) reported that 12 out of 200 diarrheic fecal samples were positive for the presence of bovine rotavirus by ELISA. After RNA extraction and cDNA synthesis, the PCR was done for amplification of VP4 gene and VP6 gene of all ELISA positive bovine rotavirus samples. The PCR resulted in amplification of 880 bp VP4 gene in 5 samples, when visualized on 1.2% agarose gel. Out of these 5 samples, 3 were from buffalo calf and 2 were from cattle calf. The PCR resulted in amplification of 250 bp VP6 gene in 4 samples, when visualized on 1.2% agarose gel. Out of these 4 samples, 3 were from buffalo calf and 1 was from cattle calf.

Yilmaz (2016) reported that of the 112 feces samples examined by ELISA 10 samples were identified as positive (8.92%). The 10 samples that were rotavirus antigen positive by ELISA were also positive on RT-PCR technique.

### 2.2.2 Molecular characterization of bovine coronavirus by RT-PCR

**Work done in India**

Bardhan (2007) reported that none of the diarrheic (56) bovine fecal samples has been found positive for Bovine Corona virus by RT-PCR with N-gene specific primers using all the conditions described by Khalilli *et al.* (2006) for amplification of N-gene.

Hansa et al. (2012) reported that out of total 101 samples screened by RT-PCR, 20 (19.80%) samples were found positive for BCoV. The virus incidence rate in clinical cases at military dairy farm and LPM farm, IVRI, both in Bareilly district was found to be 21.05% (8/38) and 16.67% (5/30), respectively, with an overall prevalence of 19.11% (13/68). In dead cases from Post Mortem Facility, IVRI, Bareilly, the incidence rate of 27.27% (3/11) was observed. At dairy farm of Raebareli district, an incidence rate of 18.18% (4/22) was recorded by RT-PCR

Santhosh Kumar *et al*. (2013) reported that out of total 160 samples screened by RT-PCR, 15 (9.38%) samples were found positive for, bovine coronavirus i.e., 11 diarrheic fecal samples of clinical cases and 4 intestinal contents of necropsy cases were shown positive for bovine coronavirus.

**Work done abroad**

Brandao *et al*. (2003) developed a nested PCR targeted to the Si ectodomain gene of the spike (S) glycoprotein of corona viruses to detect bovine coronavirus in fecal samples from cattle. The nested PCR assay was tested in 22 fecal samples from calves with or without diarrhea and 1 sample from a diarrheic cow, resulting in 10 positive samples both from diarrheic and non-diarrheic individuals. The RT-PCR was found to be more specific and sensitive tool for bovine coronavirus.

Takiuchi *et al.* (2005) developed and evaluated a semi-nested PCR (SN-PCR) to amplify a 251 bp fragment of bovine coronavirus N gene from 25 fresh and frozen diarrheic fecal samples of naturally infected calves. To improve detection of bovine coronavirus in faecal samples by the SN-PCR an internal control was developed, and the results were compared with a conventional RT-PCR assay. The rates of positive samples by SN-PCR and RT-PCR were 24% (12/50) and 8% (4/50), respectively. Only fresh samples were positive in RT-PCR while the SN-PCR detected bovine coronavirus in both fresh and frozen faecal samples.

Khalili *et al.* (2006) used RT-PCR assay for detection of bovine coronavirus, targeting a 730 bp fragment of the N-gene that could amplify all bovine coronavirus strains and evaluated presence of bovine coronavirus in diarrheic and non-diarrheic samples. The 108 fecal samples from diarrheic calves and 80 fecal samples from non-diarrheic calves collected. In 13 of 108 diarrheic samples both ELISA and RT-PCR detected bovine coronavirus. In 4 of 80 samples second group (non-diarrheic) bovine coronavirus was detected by RT-PCR only not capture ELISA. They reported that RT-PCR is more sensitive than ELISA to detect bovine coronavirus, especially in subclinical cases.

Park *et al.* (2006) detected 83 out of 184 (45.10%) fecal samples for bovine coronavirus positive by a nested PCR assay, which targeted a 407 bp fragment of N gene.

Klein *et al*. (2009) reported that RT-PCR was used for the detection of bovine coronavirus using the nucleotides 92-480 of the bovine coronavirus nucleocapsid (N) gene. RT-PCR for bovine coronavirus was positive in 70/180 (38.9%) samples, of which 42/70 (60%) were also positive using the rapid assay.

Gomez *et al.* (2017) reported that bovine coronavirus was detected in 157 samples out of 286 samples (55%) collected from diarrheic calves, using RT-PCR for which amplification of an 81 bp fragment of the bovine coronavirus nucleocapsid protein was performed.

## 2.3 Characterization of predominant bacterial isolate *E.coli* using cultural, biochemical and molecular techniques

**Work done in India**

Hussain and Saikia (2000) examined a total of 93 fecal samples from diarrheic calves and isolated 68 isolates of *E. coli*. Single infection only with *E. coli* in majority of the cases (73.12%) and mixed infection with two or three species was recorded in few cases of calf diarrhea.

Anand *et al.* (2006) designed molecular characterization of *Escherichia coli* using PCR single strand confirmation polymorphism analysis of 16S rRNA gene. Out of 22 SSCP patterns, "A" was found in maximum number in 77 (51.33%) of the samples tested. Second most frequent pattern was "C" found in 14 (9.33%) samples.

Dadawala *et al.* (2008) confirmed 45 *E. coli* isolates by PCR using SRV3-I and SRV3-II primer sequences and showed the 100% specificity of PCR technique.

Patel (2009) processed 64 fecal samples and found 51 samples positive by both cultural and PCR technique. Result showed 98.04% specificity and 100% sensitivity of PCR using Eubacterial universal primers as per the comparison with gold standard.

Srivani *et al*. (2017) reported that the prevalence of *E. coli* associated diarrhea in buffalo calves was 85.04%, of which 35.01% was STEC origin. In STEC, the combination of *eaeA* and, *hlyA* virulence genes was highest (42.45%) followed by *stx1* (16.04%), *stx 1, stxs2* and *hlyA* (13.21%), *stx 2* (12.64%), *stx1, eae* and *hlyA* (9.43%) and *stx1* and *hlyA* (6.6%) genes were detected.

**Work done abroad**

Merchant and Packer (1967) reported that E. coli in fecal samples are most often recovered on Mac Conkey or Eosin Methylene Blue (EMB) agar and considered EMB agar as a suitable medium for isolation of *E. coli* from feces and foods because of the unique ability to produce distinctive colonies having greenish metallic sheen. For epidemiological or clinical purposes *E. coli* strains are often selected from Mac Conkey agar plates after presumptive visual identification of lactose fermenting pink colonies.

Edwards and Ewing (1972) reported that there are no specific media recommended for isolation of enterotoxigenic *E. coli* but like other *E. coli* strains they can grow on nutrient agar, Mac Conkey agar, and Eosin methylene blue (EMB) agar. Blood agar plates can also be used for isolation of diarrhoeagenic *E. coli*. However, this method should be used only with caution because only 90 per cent of *E. coli* is lactose positive, some diarrhoeagenic *E. coli* strains are typically lactose negative.

Nataro and Kaper (1998) reported that the indole test which was positive in 99 per cent of *E. coli* strains, is the single best test for differentiation from other members of *Enetrobacteriaceae*.

Chapman *et al.* (2001) studied the comparison of culture, PCR and immunoassay for detecting *E. coli* O157 following enrichment. Immunomagnetic separation was performed on naturally contaminated raw meat products. Out of 120 naturally contaminated 80 (67%) were positive by PCR.

Salvadori *et al.* (2003) isolated 205 *E. coli* strains from calves with diarrhea from mid-western Brazil. They screened them for the presence of

virulence factors associated with bovine colibacillosis using specific oligonucleotide primers for shiga toxins (1 and 2), LT and ST variants. They detected 9.7 and 6.3 per cent of *E. coli* strains positive for shigatoxin 1 and 2 respectively; 8.3 and 3.9 per cent *E. coli* strains positive for LT-II and STa enterotoxins respectively as detected by PCR.

Irino *et al*. (2005) reported a total of 202 individual shiga toxin producing *E. coli* (STEC) isolates among 1471 *E. coli* colonies screened for *stx*. 140 (69.3%) of them were typable. The great majority of the isolates carried *stx* 2 (40.6%) or *stx 1 stx* 2 (56.4%) sequences. Only few isolates harboured *stx 1* sequence alone (3%).

Zweifel *et al*. (2005) reported a total of 42 STEC strains from slaughtered healthy cattle in Switzerland. The PCR analysis showed that 18 (43%) strains carried the *stx 1* gene, 20 strains (48%) had the *stx* 2 gene and four (9.5%) strains had both *Stx 1* and *Stx* 2 genes.

Al-Ajmi *et al.* (2006) studied the evaluation of a PCR detection method for *E. coli* O157:H7 in bovine fecal sample and found that PCR based detection was highly specific and sensitive for the detection of *E. coli.*

Sepehriseresht *et al.* (2009) studied comparison of polymerase chain reaction (PCR) and serologic (through verotoxigenic *Escherichia coli*- rapid plate latex agglutination (VTEC-RPLA kit) methods to detect shiga-toxigenic *E.coli* and isolated 328 (82%) and 72 (18%) from diarrheic and non-diarrheic calves, respectively 400 samples. Thirty four isolates (8.5%) which were positive in their PCR results showed that 24 (70.5%) carried *stx 1* gene, eight (23.5%) carried *stx 2* gene and two (6%) carried both *stx 1* and *stx 2* genes. Again all 400 isolates were evaluated through verotoxigenic *Escherichia coli*- rapid plate latex agglutination for shiga-toxin production, which showed 100 per cent concordance between PCR and VTEC-RPLA results.

Vu-Khac and Cornick (2008) investigated the prevalence of Shiga toxin producing E. coli (STEC) in 568 healthy domestic animals (buffaloes, cattle, and

goats) in 18 the central region of Vietnam. They reported *stx* 1c genes were widely distributed amongst buffalo, cattle, and goat isolates, accounted for 80% of the *stx* 1 variants.

## 2.4 Sequencing and phylogenetic analysis of bovine rotavirus & coronavirus, predominant bacterial isolates

**Work done in India**

Jagannath *et al*. (2000) carried out comparison study of nucleotide and amino acids sequences belonging to VP4, VP7, NSP1 and NSP3 of MP 409 with public domain database sequences of different serotypes of bovine rotavirus and it is observed that VP4 and VP7, and NSP1 share much more homology with P6 [1], G8 type bovine rotavirus A5 rotavirus from a calf with diarrhea.

Malik *et al*. (2012) found that VP7 sequence analysis of both the group A bovine rotavirus isolates revealed maximum homology (95.6 % at nucleotide level and 98.3% at amino acid level) with the bovine group A rotavirus isolate of Punjab. Similarly, VP7 sequence homology analysis of P-43 isolate of Uttarakhand revealed maximum homology (95.5 % at nucleotide level and 98.2% at amino acid level) with the bovine group A rotavirus isolate of Punjab. The close homology of VP7 gene of both the bovine rotavirus isolates with the bovine rotavirus isolate of Punjab suggests the frequent migration of infected animals across the geographical boundaries.

Sharma *et al*. (2012) reported that the majority of strains were demonstrated to belong 1 (54.5%) or P [8] (77.8%) on the basis of nucleotide sequencing of fragments from their VP7 and VP4 genes. The other genotypes detected included G2, G8, G9, G12, and P [4]. A G8P [6] strain, strain DS108, was detected for the first time in northern India. The VP7 gene of DS108 was most homologous with the VP7 gene of a bovineG8 strain, strain A5 (98.9%), indicating its bovine parentage. In contrast, the VP4 gene had a high degree of nucleotide sequence homology (92.9% to 99.1%) with the VP4 genes of human P [6] strains. The VP6 gene and nonstructural genes (NSP1 to NSP3 and NSP5) were most homologous with the VP6 gene and non-structural genes of human

rotaviruses belonging to the DS1 geno group. Interestingly, the NSP4 gene of DS108 clustered within genotype E6 that until now had only two representative strains, both with G12P (6) specificity (strainsRV176-00 and N26-02).

Deswal *et al.* (2015) reported that by VP4 and VP7 gene based RT-PCR and in sequence analysis, the VP4 gene showed up to 99.3% sequence identity at nucleotide as well as amino acid levels with P[3] rotavirus genotypes. Similarly, VP7 gene showed a maximum nucleotide and amino acid identity of 99.1 and 98.7%, respectively with G3 genotypes of group A rotavirus (RVA) from several host species and places. The phylogenetic analysis of VP4 gene also revealed close relatedness with other P [3] genotype of rotaviruses from bovine, goat, canine and feline origin. Similarly, VP7 gene revealed close relation with several G3 rotaviruses from bovine and equine origin.

**Work done abroad**

Drancourt *et al.* (2000) reported 16s ribosomal DNA (rDNA) based identification of bacteria as a useful alternative to phenotypic characterization methods, based on the similarity score in Gene Bank. The similarity score values ($\geq$ 97-99%) identified bacteria to the genus and species level.

Xiang *et al.* (2002) studied amplification of ribosomal gene sequences with universal primers. The amplified ribosomal gene was cloned and subjected to temporal temperature gradient gel electrophoresis (TTGE). Partial *16S rRNA* gene sequences were determined from clones and from major bands in TTGE gels. A total of 1,656 partial *16S rRNA* gene sequences were obtained and compared to sequences in the Gene Bank. The comparison indicated that 243 different sequences were present in the samples.

Fukushima *et al.* (2002) reported that the alignment of *16S rRNA* nucleotide sequence with nucleotide homology of *16S rRNA* for *E.coli, Shigella, Salmonella, Enterobactor* and *Klebsiella* with *Yersinia. S.sonnei* and *S.flexneri* with 99.9% similarity to each other and 99.7% to *S.boydii.*

Edgar (2004) and Kumar *et al*. (2016) reported that multiple-sequence alignment, including 20 bovine coronavirus and 10 clade A beta coronaviruses closely related to bovine coronavirus from North America, two DcCoVs from the United Arab Emirates, and two Human coronavirus OC43 (HCoV-OC43) strains from France, was performed using the Muscle algorithm implemented in MEGA7.

Park *et al*. (2006) reported that phylogenetic and bootstrap (1,000 replicates) analyses based on nucleotide and amino acid alignments of bovine coronavirus were constructed by the neighbor-joining method and the unweighted-pair group method using average linkages of Molecular Evolutionary Genetics Analysis (MEGA, version 3.1) with pair wise distance and reported that all the S, HE, M, and E genes of the nine KWD strains of bovine coronavirus contained open reading frames of 4,092, 1,272, 690, and 252 nucleotides, respectively. These nucleotide sequences encoded predicted proteins of 1,363, 424, 230 and 84 amino acid (aa) residues, respectively.

Park (2010) had done sequencing of the rotavirus positive RT-PCR products. Each gene fragment was purified using QIAEX II Gel Extraction Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. DNA sequencing was carried out using an automated DNA sequencer (ABI System 3700; Applied Biosystems, Inc., Foster City, CA). Each gene sequence of the 31 strains was compared with those of the other known group A rotaviruses using the DNA basic module (DNAsis; MiraiBio, Alameda, CA)

Kin *et al*. (2016) carried out the complete genome sequencing of bovine coronavirus with overlapping PCR products in both directions, using original primers and Sanger's dideoxy sequencing. Sequences were assembled and annotated using the Geneious software (version 5.1.6) and obtained a sequence counting 30,847 nucleotides.

Mukhtar *et al*. (2016) reported that phylogenetic analysis of the VP4 gene of bovine rotavirus was done with the 12 VP4 genes of bovine rotavirus reported on NCBI using the MEGA 5.1 software. The neighbor-joining tree having the bootstrap values (1000 replicates) was constructed and analyzed. Bootstrap values

above 50 were shown. From the phylogenetic tree, it was evident that the Pakistani bovine rotavirus VP4 gene (BRV/QOL/13; Accession no. KX537745) has a maximum identity of 98% with Indian bovine rotaviruses VP4 gene (accession No. AB625614, AB625613, AB625615, AB625616).

# MATERIALS AND METHODS

## 3.1 MATERIALS

### 3.1.1 Place &Facilities

The present research work was conducted in the Department of Veterinary Microbiology and Animal Biotechnology Teaching and Research Cell, Nagpur Veterinary College, Nagpur.

### 3.1.2 Biological, chemical and scientific instruments.

In the current study of molecular biology, analytical grade chemical and reagents used were acquired from Sigma (USA), Himedia, Promega (USA), Invitrogen and Qiagen. Glassware equipments used in study were obtained from Borosil$^{TM}$, India.

### 3.1.3 Hiculture Collection Device (VS)

Hiculture Collecting Device was used for collection of diarrheic fecal samples. The device consist of sterile cotton swab in screw capped polypropylene tube with size 75 mm x 12 mm diameter. (M/S Himedia Labortories Pvt. Limited, Mumbai, India).

### 3.1.4 Equipments

Centrifuge machine (Thermo scientific, India)

Incubator (M.C.Dalal, India)

Autoclave (Tanco, India)

Hot air oven (M.C.Dalal, India)

Refrigerator $4^0$C (LG, India)

Deep freezer ($-20^0$C) (Blue star, India)

ULT freezer (-70$^0$C) (Thermo scientific, India)

Hot water bath (Memmert, Germany)

Micropipettes (Eppendrof, Germany)

Spinvin (Genie, India)

Thermo cycler pros (Eppendrof, Germany)

Laminar air flow (Microfit, India)

### 3.1.5 Plasticware and other consumables

Pipette tips (Germany, India)

PCR tubes (Axygen, USA)

Centrifuge tubes 15 ml and 50 ml (Himedia, India)

Para flim (bio basic inc., USA)

Syringes and needles (Dispoven, India)

## 3.2 METHODOLOGY

### 3.2.1 Collection of Faecal Samples

A total of 54 fecal samples were collected from diarrheic calves of 0-3 months of age from organized and unorganized farms in and around Nagpur (Table3.1). Samples were collected using sterile rectal swabs and kept on ice and transported to laboratory. Two swabs were used for each animal for collecting feces for bacterial and viral work separately. Samples were then stored at -20$^0$ C.

**Table 3.1: Details of the samples collected from diarrhoeic calves.**

| Sr. No | Lab No | Date of collection | Breed of the animal | Age of the animal | Sex of the animal |
|---|---|---|---|---|---|
| 1 | 1 | 10/01/2019 | Gir | 3 months | Male |
| 2 | M102 | 10/01/2019 | Gir | 3 months | Male |
| 3 | CB629 | 10/01/2019 | Gir | 2 months | Female |
| 4 | CB636 | 10/01/2019 | Gaolao | 2.5 months | Male |
| 5 | G1 | 13/01/2019 | CB | 20 days | Male |
| 6 | G2 | 13/01/2019 | CB | 3 months | Female |
| 7 | G3 | 13/01/2019 | CB | 20 days | Female |
| 8 | G4 | 13/01/2019 | CB | 3 months | Female |
| 9 | G5 | 13/01/2019 | CB | 1 month | Female |
| 10 | G6 | 13/01/2019 | CB | 1 month | Male |
| 11 | G7 | 13/01/2019 | CB | 2 months | Female |
| 12 | G8 | 20/01/2019 | CB | 2.5 months | Male |
| 13 | G9 | 20/01/2019 | CB | 2 months | Male |
| 14 | G10 | 20/01/2019 | CB | 20 days | Male |
| 15 | G11 | 20/01/2019 | CB | 3 months | Female |
| 16 | G12 | 20/01/2019 | CB | 1 month | Male |
| 17 | G13 | 20/01/2019 | CB | 1 month | Male |
| 18 | G14 | 20/01/2019 | CB | 2.5 months | Female |
| 19 | B1 | 04/02/2019 | CB | 3 days | Male |
| 20 | B2 | 04/02/2019 | CB | 2 months | Male |

| | | | | | |
|---|---|---|---|---|---|
| 21 | B3 | 04/02/2019 | CB | 2 months | Female |
| 22 | B4 | 04/02/2019 | CB | 2.5 months | Male |
| 23 | B5 | 04/02/2019 | CB | 1.5 months | Female |
| 24 | B6 | 05/02/2019 | CB | 3 months | Male |
| 25 | B7 | 05/02/2019 | CB | 1 month | Male |
| 26 | B8 | 05/02/2019 | CB | 1 month | Female |
| 27 | B9 | 05/02/2019 | CB | 7 days | Male |
| 28 | B10 | 05/02/2019 | CB | 3 months | Female |
| 29 | B11 | 05/02/2019 | CB | 3 months | Female |
| 30 | B12 | 05/02/2019 | CB | 1 month | Female |
| 31 | S1 | 10/02/2019 | CB | 15 days | Male |
| 32 | S2 | 10/02/2019 | CB | 3 months | Female |
| 33 | S3 | 10/02/2019 | CB | 4 days | Female |
| 34 | S4 | 10/02/2019 | CB | 1 month | Male |
| 35 | S5 | 10/02/2019 | CB | 10 days | Female |
| 36 | S6 | 10/02/2019 | CB | 1 month | Male |
| 37 | S7 | 10/02/2019 | CB | 1.5 months | Male |
| 38 | S8 | 10/02/2019 | CB | 1.5 months | Female |
| 39 | S9 | 10/02/2019 | CB | 3 months | Female |
| 40 | S10 | 10/02/2019 | CB | 3 months | Male |
| 41 | S11 | 10/02/2019 | CB | 1 month | Male |
| 42 | S12 | 10/02/2019 | CB | 12 days | Male |
| 43 | S13 | 10/02/2019 | CB | 3 months | Female |

| | | | | | |
|---|---|---|---|---|---|
| 44 | S14 | 10/02/2019 | CB | 1.5 months | Male |
| 45 | S15 | 10/02/2019 | CB | 2.5 months | Female |
| 46 | S16 | 10/02/2019 | CB | 1 month | Male |
| 47 | S17 | 10/02/2019 | CB | 2 months | Female |
| 48 | S18 | 10/02/2019 | CB | 15 days | Male |
| 49 | CBF1 | 11/02/2019 | Gaolao | 2 months | Male |
| 50 | CBF2 | 11/02/2019 | Gir | 2 months | Female |
| 51 | CBF3 | 11/02/2019 | Gir | 3 months | Female |
| 52 | CBF4 | 11/02/2019 | Gir | 2.5 months | Male |
| 53 | CBF5 | 11/02/2019 | Gir | 1 month | Female |
| 54 | CBF6 | 11/02/2019 | Gir | 1 month | Male |

CB: Crossbred

## 3.3 Screening of fecal samples

### 3.3.1 Processing of fecal samples

For virus isolation, a 10% fecal suspension was prepared in phosphate buffer saline (PBS) (pH 7.2), mixed and centrifuged at 10000 x g for 15 min to remove coarse particles. The clear suspension was transferred to fresh tubes and stored at -20ºC.

For bacterial isolation and identification, rectal swabs were inoculated in nutrient broth. Depending on the staining reaction, they were inoculated in differential media and selective media.

### 3.3.2 Detection of rotavirus by using Latex Agglutination Test kit

Collected fecal samples were screened for presence of bovine rotavirus using Latex Agglutination Test kit and bovine coronavirus using rapid antigen detection

test kit (Lateral flow test) available commercially. Latex agglutination test was performed using LK08-HiRotavirus Latex Test kit according to manufacturer's instructions. Test reagents are coated with rabbit antibodies raised against a pool of different Rotavirus isolates, including human. When a fecal extract is mixed with the Test Reagent any Rotavirus antigens present will react with the sensitizing antibodies, resulting in visible agglutination of the latex particles. A Control Reagent, latex particles coated with normal rabbit globulin, is included to identify non-specific reactions which may occur with some faecal specimens. The test is read with naked eye within 2 minutes. The reagents were allowed to reach room temperature. Specimen extract was centrifuged at approximately 1000g for 10 minutes. 50 µl of clear supernatant was pipetted onto each of the two wells on the reaction card. 20 µl of well mixed Test Reagent (LK08a) to one well and 20 µl of well mixed Control Reagent (LK08b) to the other. The contents of each well was mixed using a separate mixing stick for each sample, covering the entire area of the well. The card was gently rocked and the agglutination was observed for up to two minutes

### 3.3.3 Detection of coronavirus by using Rapid Antigen Detection test/Lateral Flow Test

Lateral Flow Test was performed using FASTest® BCV Strips which is the Test-kit for the qualitative detection of Bovine Coronavirus (BCV) antigens in faeces of cattle. The FASTest® BCV Strip is based on the latest rapid immunochromatographic technique. The Bovine Coronavirus (BCV) antigens in the faecal sample will react at the conjugate pad with mobile monoclonal antibodies bound to gold particles. Migrating ("lateral flow", LF) along the nitrocellulose membrane, these specific antigen-antibody complexes are bound by fixed monoclonal anti-BCV antibodies (mAbs) producing a pink purple Test line (TL). These monoclonal antibodies guarantee a high level of specificity for the etiologic detection of Bovine Coronavirus. A correct test procedure will be indicated by a second, pink purple control line (CL).

The faecal sample was mixed homogenously using a vortexer. Then the required amount of faecal sample (pulpy-compact feces: 1 level spoon, fluid watery faeces: 2-3 level spoons) was added steadily to the sample tube containing buffer diluent. The sample tube was closed tightly and rotated it to get the mixture as homogenous as possible. The sample tube was placed on a flat and horizontal surface for 1-5 minutes for the sedimentation of gross fecal particles.

The dipstick was removed from its coil pouch shortly before use. The dipstick was introduced vertically and with the arrows pointing downwards into the sample tube for at least one minute. Care was taken not to increase the liquid level more than 50%of the absorption pad. The dipstick was removed from the sample tube when the sample buffer mixture (SBM) reached the control line (CL) which was indicated by the development of pink-purple colour of CL. Then the dipstick was placed on a flat and horizontal surface. The test result was read after 5 (max. 10) minutes.

### 3.4 Extraction of ds-RNA by Trizol method

The viral RNA was extracted using TRIzole method

#### 3.4.1 TRIzole method

250 µl of virus suspension was taken in a 1.5 ml micro centrifuge tube. 750 µl of TRIzole reagent was added and mixed with pipette. It was vortexed several times and then incubated at room temperature for 5 minutes. 200 µl of chloroform per 750 µl TRIzole reagent was added. It was shaken vigorously for 15 seconds and incubated at room temperature for 10 minutes. It was centrifuged at 13000 rpm for 15 minutes at $4^0$ C in cooling centrifuge. The aqueous phase was transferred in a fresh labeled tube. 500 µl isopropanol was added per 750 µl of TRIzole reagent. It was incubated at room temperature for 15 minutes. It was centrifuged at 13000 rpm for 10 minutes at $4^0$ C in cooling centrifuge. The supernatant was discarded carefully to avoid dislodging of pellet. 1 ml of 70% chilled ethanol was added to the pellet and mixed by taping. It was centrifuged at 1000 rpm for 8 minutes $4^0$ C in cooling centrifuge.

The ethanol was removed carefully to avoid dislodging of pellet. The tubes were placed upside down on paper towel for 1-2 minutes to drain the residual ethanol. Over drying was avoided. 20 µl Nuclease Free Water was added and mixed well. Incubated at room temperature for 10 minutes. The RNA was quantified and stored at-$20^0$ C.

## 3.5 Reverse-Transcriptase Polymerase Chain Reaction (RT–PCR) for rotavirus and coronavirus

### 3.5.1 cDNA synthesis

The cDNA was synthesized using cDNA synthesis kit according to the instructions.

| | |
|---|---|
| Viral RNA | 10 µl |
| Random hexamers | 2 µl |

This mixture was heated at $65^0$C for 5 minutes and then snap chilled immediately on ice.

The following reagents were added after the snap chilling:

| | |
|---|---|
| RNase inhibitor/ RNase out | 1 µl |
| 10x RT buffer | 2 µl |
| 25x dNTP | 0.8 µl |
| Multiscript RT enzyme | 1 µl |
| Nuclease free water | 3.2 µl |

Thus the total volume was made to 20 µl.

This mixture was allowed to undergo one cycle of annealing (at $25^0$ C for 10 minutes), reverse transcription (at $37^0$ C for 120 minutes) and the final heat

inactivation (at $85^0$ C for 5 minutes) in the thermal cycler. The cDNA was stored for further use at $-20^0$ C.

### 3.5.2 Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) for VP6 gene of bovine rotavirus

The cDNA synthesized is used for amplification of VP6 gene (379 bp) of bovine rotavirus. The published primer sequences were used for RT-PCR. The details of primers are listed in (Table 3.3)

| | |
|---|---|
| cDNA | 2 µl |
| Forward primer | 1 µl |
| Reverse primer | 1µl |
| Nuclease free water | 8.5 µl |

This mixture was heated at $97^0$ C for 5 minutes in thermal cycler and then snap chilled on ice. After snap chilling, 12.5 µl of PCR master mix was added and the mixture was allowed to undergo the following cycling condition as shown in (Table.3.2). The reaction was of 35 cycles and was carried out in a thermal cycler.

**Table 3.2: Cyclic conditions for PCR for genotyping of VP6 gene of bovine rotavirus**

| Step | Temperature | Time |
|---|---|---|
| Initial denaturation | $94^0$ C | 5 minutes |
| Denaturation | $94^0$ C | 30 seconds |
| Annealing | $50^0$ C | 1 minute |
| Extension | $72^0$ C | 1 minute |
| Final extension | $72^0$ C | 7 minutes |

**Table 3.3: Details of the primers used for the detection of VP6 gene of bovine rotavirus**

| Primers | Sequence (5'-3') | Location | Expected product size (bp) | Reference |
|---|---|---|---|---|
| VP6-F | 5'-GACGGVGCRACTACATGGT- 3, | 747-766 | 379 bp | Falcone (1999) |
| VP6-R | 5'-GTCCAATTCATNCCTGGTGG-3' | 1126-1106 | 379 bp | |

### 3.5.3 RT-PCR for N- gene of bovine coronavirus

The cDNA synthesized is used for amplification of N-gene (730 bp) of bovine coronavirus. The published primer sequences were used for RT-PCR. The details of primers are listed in (Table 3.5).

| | |
|---|---|
| cDNA | 5 µl |
| Forward primer | 1 µl |
| Reverse primer | 1µl |
| Nuclease free water | 5.5 µl |

This mixture was heat denatured at 94-96$^0$ C for 5 minutes in thermal cycler and then snap chilled on ice. After snap chilling, 12.5 µl of PCR master mix was added and the mixture was allowed to undergo the following cycling condition as shown in (Table.3.4). The reaction was of 35 cycles and was carried out in a thermal cycler.

**Table 3.4: Cyclic conditions for PCR for genotyping of N- gene of bovine coronavirus**

| Step | Temperature | Time |
|---|---|---|
| Initial denaturation | $94^0$ C | 5 minutes |
| Denaturation | $94^0$ C | 1 minute |
| Annealing | $58^0$ C | 1 minute |
| Extension | $72^0$ C | 2 minute |
| Final extension | $72^0$ C | 7 minutes |

**Table 3.5: Details of the primers used for the detection of N- gene of bovine coronavirus**

| Primers | Sequence (5'-3') | Location | Expected product size (bp) | Reference |
|---|---|---|---|---|
| N gene-F | 5'-GCAATCCAGTAGTAGAGCGT- 3, | 21-40 | 730 bp | Khalili *et al.(* 2006) |
| N gene-R | 5'-CTTAGTGGCATCCTTGCCAA-3' | 731-750 | | |

### 3.6 Agar Gel Electrophoresis

The PCR products were resolved by agarose gel electrophoresis (AGE) using 1.5% agarose gels containing 0.5 µg/ml ethidium bromide in 0.5x Tris-Borate-EDTA (TBE) buffer. 10 µl of PCR product was mixed with 5 µl of 6x gel loading dye and loaded into the wells of the gel. 100 bp DNA ladder was loaded along with PCR products in one well of each set. Electrophoresis was carried out at 85 Volts in 0.5 x TBE buffer in gel electrophoresis apparatus. Power supply was given until the indicator dye reached last one third of the gel. The gel was the read using Gel Documentation system.

## 3.7 Isolation and identification of bacteria from diarrheic fecal samples

### 3.7.1 Preparation of glass wares:

All the glass wares used in this study were soaked overnight in detergent solution and then washed with tap water followed by distilled water. All the glass wares were sterilized in hot air oven at $160^0$ C for 1 hr.

### 3.7.2 Preparation of media:

All the media employed for the identification of secondary bacteria from fecal swab were prepared as per the guidelines of Cowan and Steel (1970) and Cruickshank *et al*. (1975). Collected swabs were subjected for isolation of bacterial pathogens as per the standard methods described in Bergey's Manual of Systematic Bacteriology, 1986. From the duplicate diarrheic sample collected from each animal using sterile rectal swabs, one swab from each was inoculated in a test tube containing 3 ml of sterile nutrient broth. It was incubated at $37^0$C for 24 hours. After 24 hours, the test tubes were checked for turbidity/sedimentation/ pellet formation. It was then streaked on sterile nutrient agar plate and was incubated at $37^0$ C for 24 hours. After 24 hours, the plates were checked for growth of bacterial colonies. It was then stained by Gram's staining for preliminary morphological identification. Specific identification and biochemical characterization of the isolates was done as per the standard techniques described by Cruickshank *et al*. (1975) and Cowan and Steel (1993).

### 3.7.3 Morphological identification

The smear was prepared from colonies grown on nutrient agar plate and was prepared on clean grease free microscopic glass slide. The smear was then stained with Gram's Method and observed under microscope to study morphological characteristics and staining reaction.

### 3.7.4 Cultural characteristics

The colonies from each nutrient plate were streaked on Mac Conkey Agar plate and were incubated at $37^0$ C for 24 hours. The colonies were examined for colony characteristics like lactose fermenting, non-lactose fermenting, mucoid, etc. The lactose fermenting colonies indicated by pink color colonies were then streaked on Eosin-Methylene Blue Agar plates (EMB Agar) and were incubated at $37^0$ C for 24 hours. Colonies showing characteristic metallic sheen on EMB agar plates after the incubation were considered as presumptive of *E.coli* isolates.

### 3.7.5 Biochemical Characteristics

Presumptive identification of *E.coli* based on IMViC test and Triple Sugar Iron test were carried out. The biochemical characterization was done as per the methods described by Agarwal *et al*. (2003) and Cruickshank *et al*. (1975).

### 3.7.6 Indole test

This test was performed as per the method described by Agarwal *et al.* (2003). A loop full of bacterial culture was inoculated into a test tube containing peptone water and incubated at $37^0$ C for 48-96 hours. After incubation, 0.5 ml of Kovac's reagent was added to the test tube. Development of a red/ purple coloured ring at the junction of two liquids was considered to be positive and yellow coloured ring at the junction of two liquids were considered to be negative. Kovac's reagent contains p-dimethylaminobenzaldehyde which reacts with indole to produce a red colored compound.

### 3.7.7 Methyl Red (MR) test

This test was performed as per the method described by Cruickshank *et al.* (1975). A loop full of bacterial culture was inoculated into a test tube containing glucose phosphate broth and incubated at $37^0$ C for 2-5 days. After incubation, 5 drops of 0.04% methyl red solution were added and mixed well. Development of red

colour was considered to be positive and that of yellow colour was considered to be negative.

### 3.7.8 Voges- Proskauer (VP) test

This test was performed as per the method described by Cruickshank *et al.* (1975). A loop full of bacterial culture was inoculated into a test tube containing glucose phosphate broth and incubated at $37^0$ C for 48 hours. 1 ml of 40% KOH and 3 ml of 5% solution of α-naphthol were added. Development of pink colour within 2-5 minutes was considered to be positive and the reaction was considered negative if it remains colourless upto 30 minutes.

### 3.7.9 Citrate Utilization test

Citrate utilization test was performed using Koser's medium. A loop full of bacterial culture was inoculated into a test tube containing Koser's medium and incubated at $37^0$ C for 18-24 hours. Development of turbidity was taken as positive reaction.

### 3.7.10 Triple Sugar Iron test

This test was performed to determine the ability of an organism to attack specific carbohydrates incorporated in a growth medium with or without the production of gas along with the determination of possible hydrogen sulphide production. The incorporated carbohydrates are glucose, lactose and sucrose. The medium was inoculated with the bacterial culture and given incubation of 18-24 hours. Development of yellow colour was considered to be due to fermentation of carbohydrate and that of red colour indicates the carbohydrates were not fermented.

## 3.8 *Invitro* Antibiotic Sensitivity Test

All the 54 fecal samples were subjected to *in vitro* antibiotic sensitivity testing for the detection of antimicrobial drug resistance. This was done by disc diffusion

technique as per the method of Bauer *et al.* (1966) wherein which the diameter of the zone of inhibition was compared with the standard known value against each specific antimicrobial agent as suggested in the product information (interpretation guideline) from manufacturer. The antibiotic discs were produced from HiMedia Laboratories Ltd., Mumbai**.** The details of antibiotics used are listed. (Table 3.6)

**Table 3.6 : Details of antibiotics used *in vitro* antibiotic sensitivity testing**

| Sr. No. | Name of the antibiotic | Symbol on the antibiotic disc | Concentration of antibiotic in the disc |
|---|---|---|---|
| 1 | Amoxycillin | AMX | 30 mcg |
| 2 | Amoxycillin-Clavulanic acid | AMC | 30 mcg |
| 3 | Amoxycillin- Sulbactam | AMS | 30/15 mcg |
| 4 | Ampicillin-Sulbactam | A/S | 10/10 mcg |
| 5 | Cefotaxime | CTX | 30 mcg |
| 6 | Ceftazidime | CAZ | 30 mcg |
| 7 | Ceftriaxone | CTR | 30 mcg |
| 8 | Ceftriaxone- Tazobactam | CIT | 30/10 mcg |
| 9 | Enrofloxacin | EX | 5 mcg |
| 10 | Furazolidone | F | 50 mcg |
| 11 | Gentamicin | GEN | 50 mcg |
| 12 | Levofloxacin | LE | 5 mcg |
| 13 | Metronidazole | MT | 5 mcg |
| 14 | Norfloxacin | NX | 5 mcg |
| 15 | Oxytetracycline | O | 30 mcg |
| 16 | Penicillin | P | 2 units |

### 3.8.1 Antibiotic sensitivity test protocol

- Inoculum from pure culture slant (BHI agar slant) was picked up and added into 3 ml of Nutrient broth and mixed properly. This was incubated at $37^0$C for 24 hours.

- 1 ml of the broth culture was uniformly spread on the Muller Hinton Agar (MHA) plate with a sterile L-shaped loop and it was allowed to get dried for 1-2 minutes. 2 MHA plates were used for each sample.

- Antibiotic discs were placed on the agar surface containing the inoculum at about 2 cm apart.

- The plates were then incubated at $37^0$C for 24 hours and were examined for zones of inhibition.

- Diameter of the zones of inhibition was measured for each antibiotic and this was then compared with zone size interpretation chart published by the manufacturer and the zones were graded as sensitive (S), Resistant (R) or Intermediate (I)

### 3.9 Isolation of genomic DNA

Isolation of genomic DNA was carried out as per the method described by Sambrook and Russel (2001)

The bacterial isolates were grown in 2 ml of BHI broth overnight at 37ºC. The cultures were harvested by centrifugation at 10,000 rpm for 5 min. and suspended in 400 µl of 1X TE solution (10 mM tris HCL; 1 mM EDTA, pH 8.0). After that 40 µl of the lysozyme solution (10 mg/ml) was added, gently mixed and incubated in water bath at 37ºC for 2 hrs. Bacteria was lysed by addition of 5 µl proteinase K (10 mg/ml) and 45 µl of 10% SDS and followed by incubation at 50º C for 1 hr in water bath. The cell wall debris, denatured proteins, polysaccharides and polymeric matrix were

eliminated by precipitation with addition of 60 µl of 5M NaCl and 64 µl of CTAB solution (10% CTAB in 0.7M NaCl) and incubated at 56ºC for 30 min. DNA was purified by two extractions with phenol : chloroform (1:1) and chloroform : isoamyl alcohol (24:1). DNA was precipitated by adding 0.1 volume of 3M sodium acetate (pH 5.2) and 0.6 volume of isopropanol and kept at -20 ºC for 1 hr and later centrifuged at 10,000 rpm for 15 min. The pellet was washed in 70% alcohol and air dried to remove the alcohol and re-suspended the in 50 µl TE buffer. 2.5 µl of suspended pellet was used as the DNA template.

**3.10 Polymerase chain reaction**

Polymerase chain reaction was carried out to detect the presence of *stx 1* and *stx 2* genes in *E.coli.* The details of primers are listed in (Table 3.7).

The reaction was carried out after adding the following reagents:

DNA template: 3 µl

Forward primer: 1 µl

Reverse primer: 1 µl

PCR master mix: 12.5 µl

Nuclease free water: 7.5 µl

Thus the total volume of reaction mix was 25 µl the mixture was allowed to undergo the following cycling condition as shown in (Table.3.8). The reaction was of 35 cycles and was carried out in a thermal cycler.

**Table 3.7 : Details of the primers used for the detection of *stx 1* and *stx 2* genes of *E.coli.***

| Primers | Sequence (5'→3') | Virulence factor (target gene) | PCR product (bp) | Reference |
|---|---|---|---|---|
| *stx 1* A (LP30)- F | 5'- CAGTTAATGTCGTGGCGAAGG-3' | Shigatoxin 1 | 348 bp | Cebula *et al.* (1995) |
| *stx 1* A (LP31)- R | 5'CACCAGACAATGTAACCGCTG3' | Shigatoxin 1 | 348 bp | |
| *stx 2* A (LP43)- F | 5'ATCCTATTCCCGGGAGTTTACG3' | Shigatoxin 2 | 584 bp | |
| *stx 2* A (LP44)- R | 5'GCGTCATCGTATACACAGGAGC3' | Shigatoxin 2 | 584 bp | |

**Table 3.8 Cycling conditions for PCR for of *stx 1* and *stx 2* genes of *E.coli***

| Step | Temperature | Time |
|---|---|---|
| Initial denaturation | $94^0$ C | 5 minutes |
| Denaturation | $94^0$ C | 1 minute |
| Annealing | $54^0$ C | 1 minute |
| Extension | $72^0$ C | 2 minute |
| Final extension | $72^0$ C | 10 minutes |

### 3.11 Agar Gel Electrophoresis

The PCR products were resolved by agarose gel electrophoresis (AGE) using 1.5% agarose gels containing 0.5 µg/ml ethidium bromide in 0.5x Tris-Borate-EDTA

(TBE) buffer. 10 µl of PCR product was mixed with 5 µl of 6x gel loading dye and loaded into the wells of the gel. 100 bp DNA ladder was loaded along with PCR products in one well of each set. Electrophoresis was carried out at 85 Volts in 0.5 x TBE buffer in gel electrophoresis apparatus. Power supply was given until the indicator dye reached last one third of the gel. The gel was the read using Gel Documentation system.

### 3.12 Phylogenetic analysis and sequencing

A total of two randomly selected PCR products including the 2 gene VP6 & *stx* 2 were got sequenced from Eurofins Genomics India Pvt. Ltd, Bangalore, India. The sequences of VP6 & *stx* 2 genes were analyzed using BLAST (Basic Local Alignment Search Tool) and the Clustal-W (CLUSTAL2.1 multiple sequence alignment) to generate sequence alignment reports. Molecular Evolutionary Genetic Analysis (MEGA) version 7.0 was used for construction of phylogenetic tree. The bootstrapped phylogenetic tree was constructed using Neighbor-joining method.

# RESULTS AND DISCUSSION

In the present study, bovine rotavirus, coronavirus and *E. coli* were recovered either singly or in association with each other in the form of mixed infection. In the present study 54 diarrheic fecal samples from bovine calves (0-3 months of age) were collected (Plate 4.1 and 4.2) and screened for the presence of bovine rotavirus and corona viruses by latex agglutination test and lateral flow test respectively. Among these, 11 (20.37%) samples were found positive for bovine rotavirus by latex agglutination test and 01 (1.85%) sample was found positive for bovine coronavirus by lateral flow test. All the samples were subjected for the further detection of group A Rotavirus by VP6 gene based RT–PCR assay and N-gene based RT-PCR for corona virus. Of the 54 samples, 11 (20.37%) samples were positive for bovine rotavirus by VP6 gene based RT-PCR. However none of the 54 diarrheic fecal samples were found positive for corona virus by N-gene based RT-PCR.

All the 54 samples were subjected for the isolation and identification of predominant bacteria. Among them, *E.coli* could be recovered from 41 samples. All the *E.coli* positive samples were then subjected for PCR for the detection of *stx* 1 and *stx* 2 genes. 10 (24.39%) and 13 (31.70%) isolates out of 41 isolates were found to be positive for *stx* 1 and *stx* 2 genes, respectively.

Two positive PCR purified products were subjected for sequence analysis, one for bovine rotavirus and one for *E.coli.* All the sequences from the PCR positive samples were exactly (100%) similar.

## 4.1 Screening of bovine rotavirus by Latex Agglutination Test

A total of 54 diarrheic fecal samples suspected for rotavirus were screened by Latex Agglutination test. Out of 54 diarrheic fecal samples tested for rotavirus, 11 (20.37%) were found positive and was indicated by the agglutination reaction formed as a result of the reaction between the sample and 8a reagent (Table 4.1) (Plate 4.3).

**Plate 4.1: Diarrheic cow calf**

**Plate 4.2: Diarrheic feces of calf**

The present findings are in agreement with findings of Ade *et al*. (2019) who found that out of 288 samples, 35 (12.15%) samples were detected positive for rotavirus by Latex Agglutination test from 5 districts of Amravati region. Jindal *et al*. (2000) reported the incidence of rotavirus ranging from 10% to 52% in cattle calves and 11% to 24% in buffalo calves respectively. Hassan *et al.* (2014) who reported overall incidence of the bovine rotavirus 12.8% (37/290) in calf diarrheic fecal samples by latex agglutination test. Khafagi *et al.* (2010) found that 12.3% of incidence of rotavirus by Latex agglutination test (LA) and Enzyme Linked Immunosorbant Assay (ELISA) for the rotavirus with diarrhea positive lambs and kids in Egypt. Hashem *et al*. (2012) screened 450 fecal specimens by Latex agglutination (LA) and Enzyme Immuno Assay (EIA). In a test, about 94 (20.8%) samples were reactive, 45.7% as non- reactive and 33.3% remained indeterminate. Further all LA positive also been positive in EIA tests, and 25 LA negative samples were positive by EIA. Paesi *et al*. (2012) reported that the use of Rotavirus Latex Kit (Richmond immunosystems Diagnostics) was more sensitive in 38 samples (11.6%) and found to be positive when compared to PAGE where only 26 samples (7.9%) found positive for detecting rotavirus antigen in diarrheic feces of piglets and human.

## 4.2 Screening of Bovine corona virus by lateral flow test kit

Bovine corona virus (BCoV) is only second to rotavirus in causing neonatal diarrhea (Saif, 1990). During the present study, 54 fecal samples were processed for detection of Bovine corona virus using lateral flow test kit.

In the present study, all the 54 samples collected from diarrheic calves of 0-3 months of age were subjected to screening by lateral flow test kit to detect the presence of bovine corona virus (Plate 4.4). Only one (1.85%) out of 54 diarrheic fecal samples was positive for bovine corona virus. (Table 4.2). But the result was observed after the time insisted by the manufacturer. The present study findings confirms the finding of Izzo *et al*. (2012) who reported that 30 out of 132 (22.73%) diarrheic fecal samples were positive for bovine corona virus on lateral flow test

using dipstick and Abou El-Ella *et al*. (2013) found that 21 out of 124 faecal samples collected from diarrhoeic calves were positive on immune- chromatographic rapid tests (FASTest® Strips). Klein *et al*. (2009) found that out of 70 fecal samples 42 (60%) were also positive using the rapid assay for the bovine corona virus

## 4.3 Molecular characterization of bovine rotavirus

### 4.3.1 Targeting of VP6 gene by RT-PCR

RT-PCR was employed for the confirmation of the presence of bovine rotavirus from the samples collected. RT-PCR is a highly sensitive and specific method for the detection of causative organisms from the suspected samples. This method is based on the amplification of nucleic acids present in the sample. In RT-PCR, cDNA is synthesized from RNA of the organism and is then subjected to PCR for the amplification.

A total of 11 rotavirus positive samples were subjected to amplification of VP-6 gene based RT-PCR. Out of 54 samples, 11(20.37%) samples were positive for bovine rotavirus. Amplification of VP6 gene yielded 379 bp product on agarose gel. Out of 11 samples which were positive for bovine rotavirus by Latex Agglutination Test, 10 samples were found positive by RT-PCR and one sample which was negative by LAT was found positive by RT-PCR. The results of present study confirm that RT-PCR method was more sensitive tool for rapid molecular detection and identification of *Rotavirus.* (Table 4.1, Plate 4.5).Thus a total of 12 (22.22%) samples out of 54 diarrheic samples were found positive for bovine rotavirus.

The present study findings are in agreement with the findings of Das *et al*. (2018) who analyzed that RT-PCR to be more effective method for rotavirus diagnosis. They used two published primer sets which were amplified to produce 309 and 304 bp sized amplicons for VP6 and VP7 gene on 1.7% agarose gel electrophoresis and 39 (22.28%) out of 175 samples were found to be positive for both VP6 and VP7 genes of bovine rotavirus . Similarly Tumlam *et al*. (2018),


**Plate 4.3: Screening of Group A rotavirus by Latex Agglutination Test**
**1: Positive control**
**2: Negative control**
**3, 4, 5, 6: Positive test samples**


**Plate 4.4: Screening of bovine coronavirus by Rapid Lateral Flow Test**
**A: Positive test**
**B, C: Negative test**

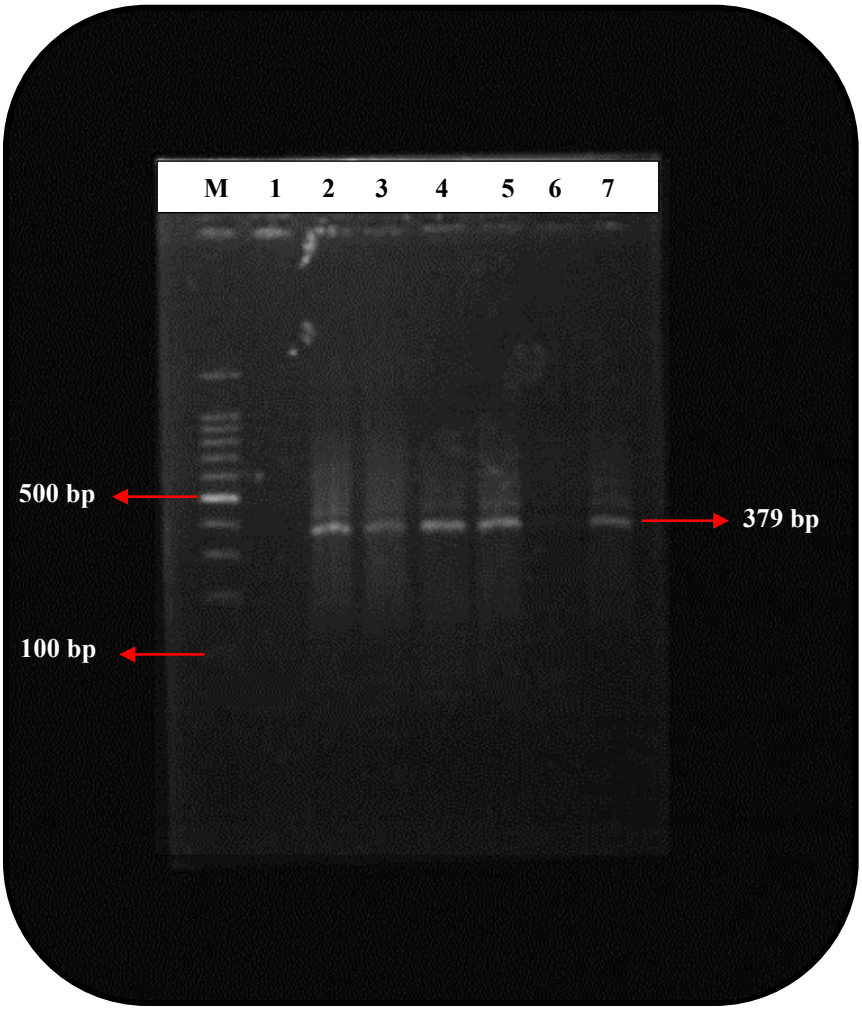


**Plate 4.5: Detection of VP6 gene of bovine rotavirus by RT-PCR**

**Lane M: Molecular weight marker (100 bp ladder)**
**Lane 1: Negative control**
**Lane 2: Positive control**
**Lane 3, 4, 5, 7: Positive samples**
**Lane 6: Negative sample**

reported that out of 44 samples screened, 43 (97.72%) samples were found positive for rotavirus. 17 out of 18 samples were found positive by VP6 gene based RT–PCR assay from bovine calves, piglets, lambs, kids and pups were found cent percent positive for group A rotavirus . Mondol *et al*. (2013) revealed that out of 211 samples collected from diarrheic bovine, porcine and human infants (below 6 months of age), 26 (12.32%) samples were found positive for group A rotavirus by VP6 gene based RT–PCR assay. A total of 11.23% (10/89) bovine, 10.97% (10/82) porcine fecal samples and 17.5% (6/40) human stool samples were positive by VP6 based RT–PCR assay.

## 4.4 Molecular characterization of bovine corona virus

### 4.4.1 Targeting of N- gene by RT-PCR

RT-PCR was employed for the confirmation of the presence of bovine coronavirus from the samples collected. In the present study, N gene with a product size of 730 bp was the target gene for RT- PCR product 730 bp. None of the 54 diarrheic bovine fecal samples were found positive for the presence of N gene of bovine coronavirus by RT-PCR and nested PCR using all the conditions described by Khalilli *et al.* (2006) for amplification of N-gene (Table 4.2, Plate 4.6). Similarly, in the study conducted by Bardhan (2007), none of the diarrheic 56 bovine fecal samples had been found positive for BCoV by RT-PCR for amplification of N-gene. In contrast to the present study, Klein *et al*. (2009) found that RT-PCR was used for the detection of N gene of bovine corona virus. Out of 180 fecal sample 70 (38.9%) samples were positive for bovine corona virus RT-PCR.

## 4.5 Isolation and Identification of *E.coli*

*E. coli* normally inhabits the intestinal tract of man and animals and has a potential to produce mild to severe pathological conditions and it is considered to be one of the major causes of diarrhea in cattle. Enterotoxigenic *E. coli* (ETEC) expressing K99 (F5) fimbriae and heat stable type Ia (STa) toxin is the leading

bacteria causing calf diarrhea (Osman *et al*., 2013). The predominant bacterial isolates were identified by morphological, biochemical, cultural and molecular characterization.

In the present study, all the 54 samples collected from diarrheic calves were subjected to the isolation and identification of the bacteria and out of 54 samples, 41 samples (75.92%) were identified as *E.coli* based on morphological, biochemical, cultural characteristics. On morphological examination using Gram's staining, Gram negative short rods, non-spore forming bacilli could be observed in all the 41 samples. On biochemical characterization, all the 41 isolates showed the IMViC pattern of Indole and Methyl red tests positive and Voges Proskauer and citrate tests negative. On Triple Sugar Iron Test, yellow color of butt and slant could be observed. (Table 4.3; plate 4.7, 4.8, 4.9, 4.10 and 4.11).

On cultural examination, all the 41 samples developed lactose fermenting colonies indicated by pink colonies on Mac Conkey Agar plate (Plate 4.12) and colonies with metallic sheen on EMB agar plate which was indicative of *E.coli*. (Plate 4.13).

The present study was in agreement with the study of Merchant and Packer (1967**)** where in which they found out that *E. coli* in fecal samples are most often recovered on Mac Conkey or Eosin Methylene Blue (EMB) agar and considered EMB agar as a suitable medium for isolation of *E. coli* from faces and foods because of the unique ability to produce distinctive colonies having greenish metallic sheen. For epidemiological or clinical purposes, Mac conkey agar plates are used for selecting *E. coli* strains after presumptive visual identification of lactose fermenting pink colonies. These findings are in concurrence with the report of Barnes *et al.* (2003) and Cowan and Steel (1993) who found that 49 out of 100 fecal samples from the diarrheic calves were positive for bacterial isolation on to media like Mac Conkey agar and EMB. All the 49 isolates produced pink coloured colonies indicating lactose fermentation on Mac Conkey agar plate and metallic green sheen colonies on EMB

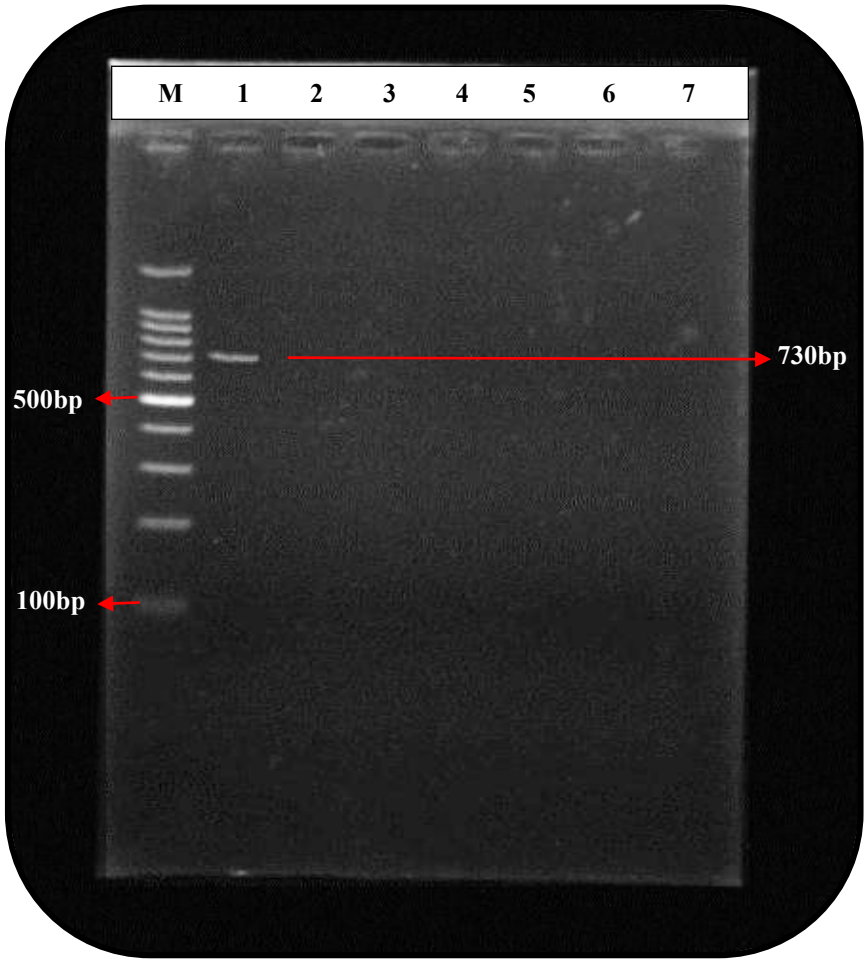


**Plate 4.6**: **Detection of N gene of bovine coronavirus by RT-PCR**

**Lane M: Molecular weight marker (100 bp ladder)**
**Lane 1: Positive control**
**Lane 2: Negative control**
**Lane 3, 4, 5, 6, 7: Negative samples**

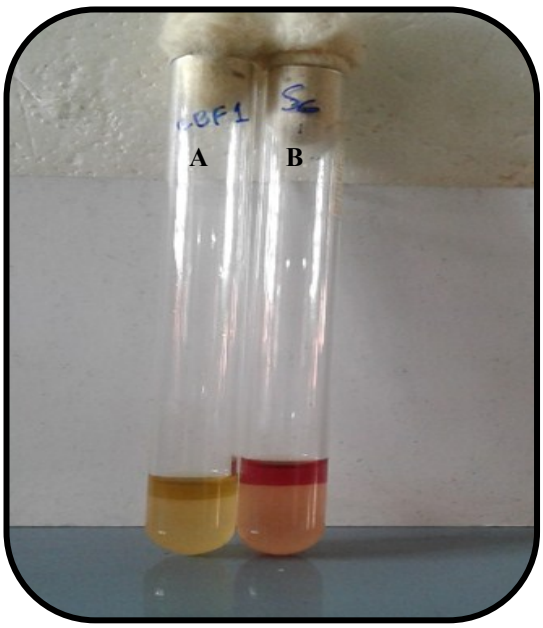


**Plate 4.7: Indole Test**
**A: Negative Test**
**B: Positive Test**

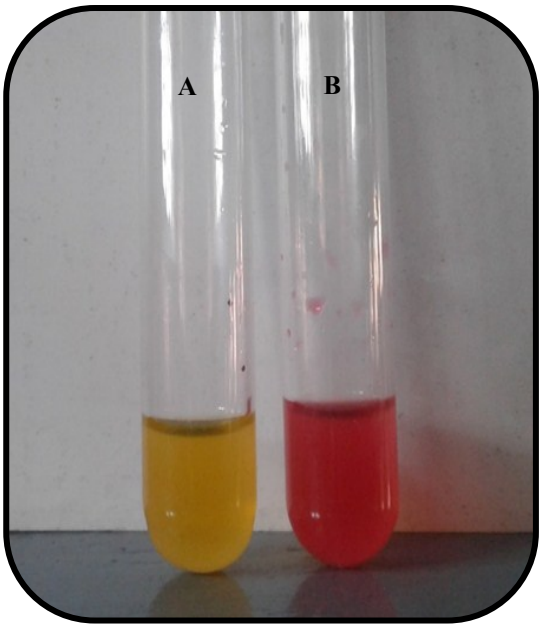


**Plate 4.8: Methyl Red Test**
**A: Negative Test**
**B: Positive Test**

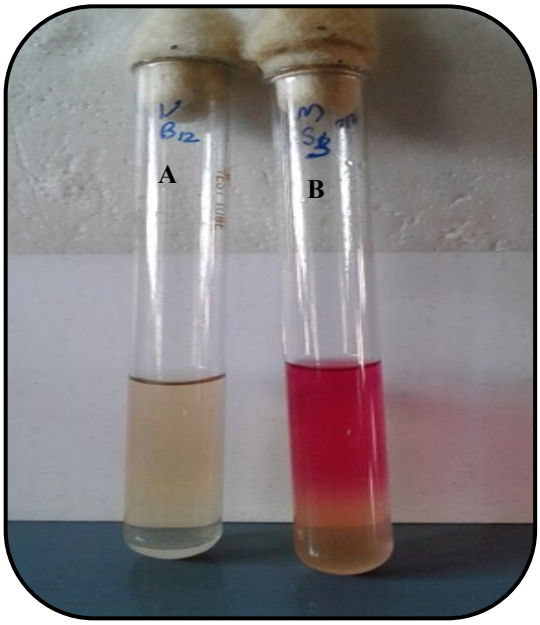


**Plate 4.9: Voges Proskauer Test**
**A: Negative Test**
**B: Positive Test**


**Plate 4.10: Citrate Utilization Test**
**A: Negative Test**
**B: Positive Test**


**Plate 4.11: Triple Sugar Iron Test**
**A, B, C, D: Colonies of *E.coli* on TSI slant**

**Plate 4.12: Pink coloured lactose fermenter colonies of *E.coli* on Mac Conkey Agar plate**

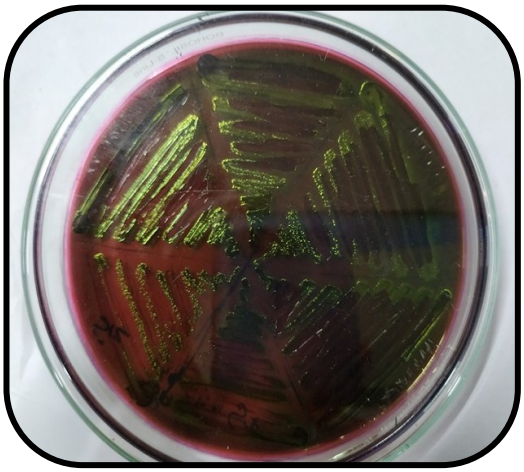

**Plate 4.13: Metallic sheen of *E.coli* colonies of on EMB Agar plate**

plates. Similarly Paul *et al.* (2010) examined that upon cultural, morphological and biochemical examinations 23 (45%) samples were found to be positive for *E. coli* from buffalo calves.

### 4.6 Sample wise occurrence of different isolates in the fecal samples of diarrheic calves.

The fecal samples of diarrheic calves were examined for bovine rotavirus, coronavirus and predominant bacteria, *E.coli* by different methods as mentioned in 3.3, 3.4, 3.5 and 3.7. Sample wise occurrence of different isolates in the fecal samples of diarrheic calves is presented in Table 4.4. The fecal samples were collected from 54 diarrheic calves out of which 5 samples showed mixed infection of rotavirus and *E.coli* and only one sample showed mixed infection of rotavirus, coronavirus and *E.coli*.

These results are in agreement with the study of Hansa *et al.* (2012) who reported that microorganisms like *E.coli, Salmonella*, rotavirus, *Cryptosporidium* species are responsible for diarrhea in neonatal calves. Similarly, Aydin *et al.* (2001) reported the high occurrence of *E.coli* infection in diarrheic calves.

This study is also in agreement with the study conducted by Malaviya (2012) in which out of 62 faecal samples, 45 (72.58%) and 7 (11.29%) samples were found positive for *E. coli* and rotavirus infections, respectively. Only 5 (8.06%) samples were detected positive for both rotavirus and *E. coli.*

Similarly, Sharma (2013) reported that out of 100 diarrheic bovine fecal samples, 76 and 24 samples were found positive for *E.coli* and mixed infection of *E..coli*, rotavirus, *Salmonella, Cryptosporidium* respectively.

### 4.7 Determination of i*n vitro* antibiotic resistance pattern of *E. coli* isolates

*In vitro* antibiotic resistance pattern of the 41 isolates was determined by disc diffusion method described by Bauer *et al.* (1966). The *E.coli* positive samples were

subjected to antibiotic sensitivity test to study about the antimicrobial resistance. For this study, 16 antibiotics which are commonly used in the field cases for the treatment of calf diarrhea were used. (Plate 4.14)

In our study the result of antibiogram pattern of 41 *E.coli* isolates when tested against commonly used antimicrobial agents reflected varying sensitivity. The highest sensitivity was observed for Gentamicin (82.92%) followed by Ampicillin-Sulbactam (78.08%), Enrofloxacin and Norfloxacin (73.17%), Levofloxacin and Cefotaxime (68.29%), Ceftriaxone-Tazobactam (63.41%) and Oxytetracycline (60.97%) (Figure 4.1) Results also showed that all the 41isolates were resistant to Metronidazole (100% resistance) followed by Penicillin (82.93%), Ceftazidime and Amoxycillin-Clavulanic acid (80.49%), Furazolidone (75.61%), Ceftriaxone (70.74%), Amoxycillin (68.30%) and Amoxycillin-Sulbactam (56.10%) (Table 4.4). Our findings were found similar with the findings of Islam *et al.* (2015) who reported that Antibiotic resistance patterns of antibiotics were 100% resistant to Penicillin, Oxacillin and Rifampicin followed by Erythromycin which was more than 80% resistant. Amoxicillin and Tetracycline were found to be having 59.65% and 61.40% resistance respectively. Ayis *et al*. (2015) found that 150 isolates out of 260 *E. coli* isolates were subjected to sensitivity test of *E. coli* to ten antimicrobial agents used for treatment of calf diarrhea in Sudan. They exhibited high susceptibility to Gentamicin, Ampicillin, Ciprofloxacin, Sulphamethoxazole-trimethoprim, Cephalothin, Kanamycin, Chloramphenicol and Tetracycline but they demonstrated less sensitivity to Erythromycin and high resistance to Penicillin. Mulik (2006) tested 64 isolates of *E. coli* recovered from diarrhoeic calves. All the *E. coli* isolates showed multiple drug resistance 100% to ampicillin and erythromycin, 94.87 % to oxytetracycline, 89.74 % to cephalexin, 61.53% to chloramphenicol, 20.51 % to nitrofurantoin and nalidixic acid, 2.56 % to gentamicin while cephalothin and amikacin were 100 % sensitive. But Verdier *et al.* (2012) reported that antimicrobial resistance was common in *E. coli* particularly from in calves from herds experiencing problems with neonatal diarrhea. Resistance could not be associated to the use of

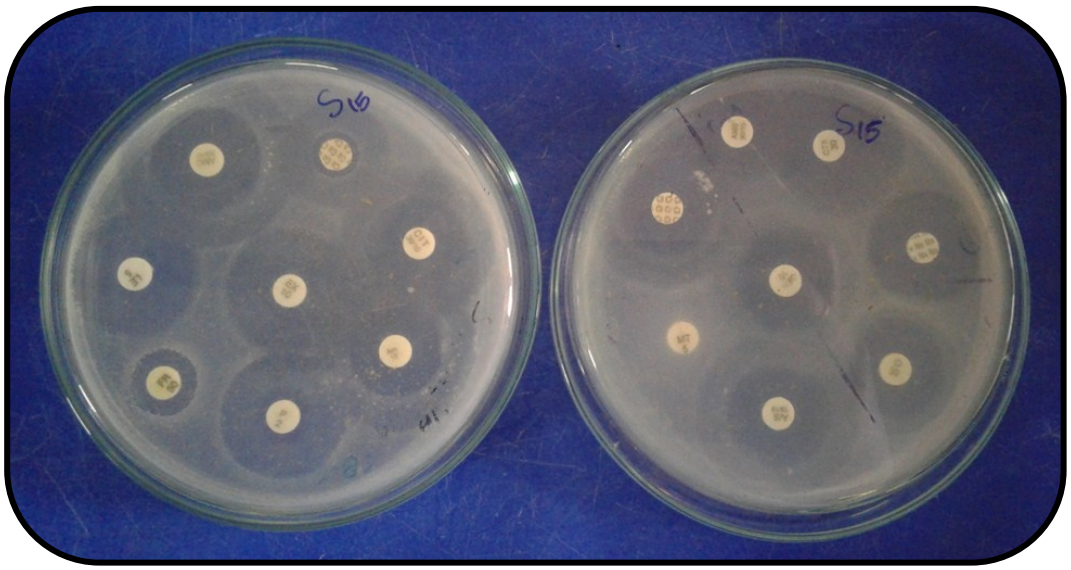


**Plate 4.14: *In vitro* Antibiotic Sensitivity Test of *E.coli* isolates**

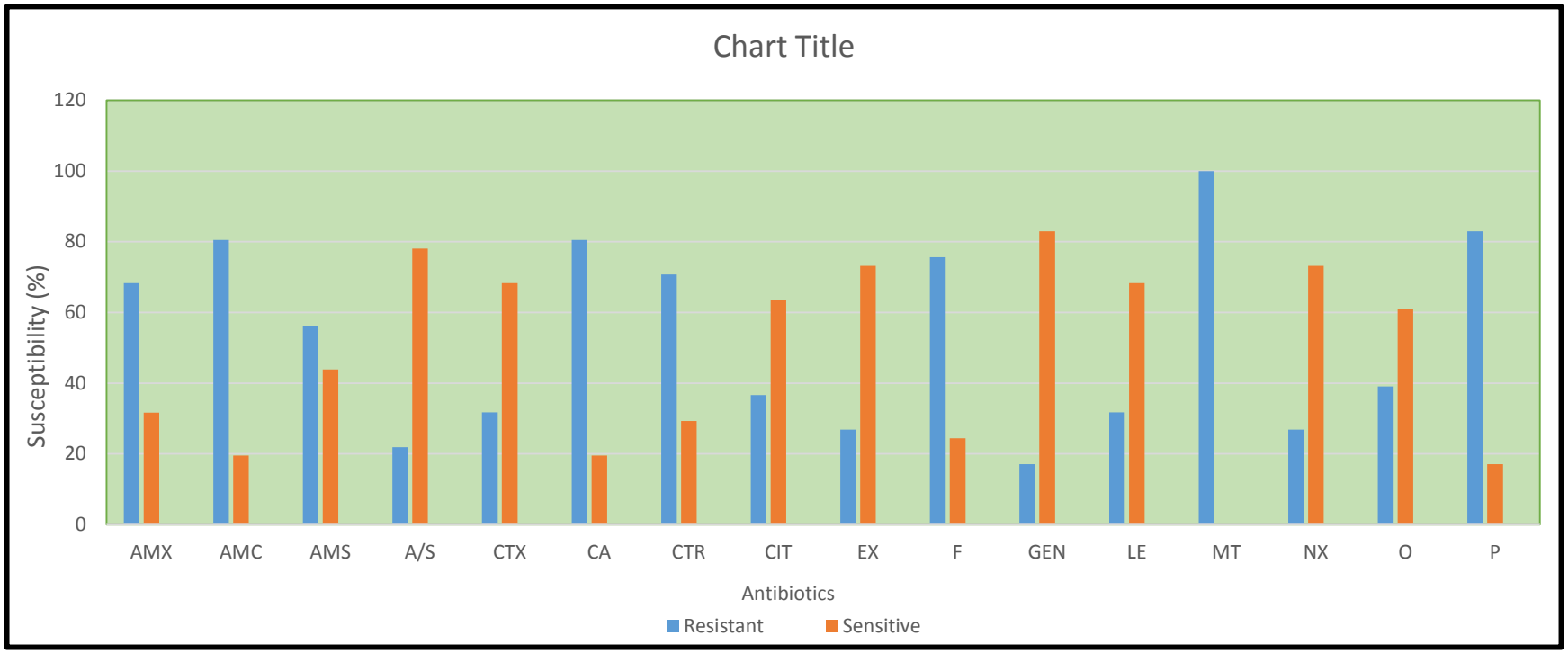


**Figure 4.1. *In-vitro* susceptibility pattern of *E.coli* isolates**

| | |
|---|---|
| AMX- Amoxycillin | EX- Enrofloxacin |
| AMC- Amoxycillin- Clavulanic Acid | F- Furazolidone |
| AMS- Amoxycillin- Sulbactam | GEN- Gentamicin |
| A/S- Ampicillin-Sulbactam | LE- Levofloxacin |
| CTX- Cefotaxime | MT- Metronidazole |
| CA- Ceftazidime | NX- Norfloxacin |
| CTR- Ceftriaxone | O- Oxytertacycline |
| CIT- Ceftriaxone- Tazobactam | P- Penicillin |

antimicrobials, implying that other factors can also influence the epidemiology of resistant *E. coli*. Isolates with virulence genes were common in calves with or without clinical signs. Srivani *et al.* (2016) revealed that the antimicrobial susceptibility testing 69.81% of the STEC isolates were resistant to three or more of the antimicrobial agents tested. Among the STEC isolates, highest percentage of antimicrobial resistance was observed for tetracycline (63.21), followed by ampicillin (48.11%), aztreonam (36.79%), cefotaxime, ceftazidime, and streptomycin, (31.13%), nalidixic acid (29.25%), sulfisoxazole (28.30%), cotrimoxazole (26.42%), amoxicillin clavulanic acid (20.75%), piperacillintazobactem (18.87%) meropenem (17.92%) kanamycin and nitrofurantoin (12.26%) ciprofloxacin (4.72), chloramphenicol and gentamycin (3.77%) while lowest % of 0.94 was observed for imipenem antibiotics. Malik *et al*. (2013) also observed highest antimicrobial resistance to the tetracycline and ampicillin and highest susceptibility to gentamicin for the *E.coli* isolated from diarrhoeic calves in UP, India. The observation of highest sensitivity of *E.coli* isolates from calves in Bangladesh (Hossain *et al*., 2013) to chloramphenicol and gentamicin also corroborate the findings in the present study.

In this study, the high prevalence of multidrug-resistant bacteria observed may lead to treatment failures and will result in economic losses for the dairy producer. With the continuing emergence of antibiotic resistance, it is imperative that actions should be taken to prolong the effectiveness of existing antibiotics while maintaining levels of food animal production. Multidrug resistance showed by these isolates in this study is alarming. This may be due to indiscriminate use of antibiotics in clinical practice. This study detected that most of the isolates are sensitive to Gentamicin, Ampicillin-Sulbactam, Enrofloxacin and Norfloxacin, Levofloxacin and Cefotaxime, Ceftriaxone-Tazobactam and Oxytetracycline antibiotics, because these are seldom used in calf diarrhea cases in this area.

Hence present investigation emphasizes on judicious selection of antibiotics or antimicrobial agents, preferably after *in vitro* antimicrobial susceptibility testing

and using such antimicrobials at an adequate dose for sufficient duration for effective treatment and control of various diseases caused by *E. coli*.

### 4.8 Multiple antibiotic resistance (MAR) indexing of *E.coli* isolates

Multiple Antibiotic Resistance indices of individual isolates were calculated as per the method described by Krumperman (1983). In the current study, MAR index for the isolates was calculated for antibiotics as shown in (Table 4.6) and a high incidence of *E. coli* strains with MAR, was observed. None of the strains had MAR value < 0.2. All the strains were having the MAR value > 0.2 and up to 1, showing very high degree of resistance originating from diarrheic fecal samples of bovine calves. This study has also brought out that there may be possibility of indiscriminate use of large number of antibiotics for the treatment of diarrhea. This findings correlates with the study of Bhardwaj *et al*. (2015) who revealed that 100% of the isolates exhibited multi drug resistance (MDR) character and all the isolates having very high multiple antibiotic-resistance (MAR) index, due to high antibiotic usage. The high incidence of *E. coli* strains with MAR value > 0.2 and up to 1, showing very high degree of resistance, was observed and none of the strains having MAR value < 0.2. Odonkor and Addo (2018) found that sixty-three percent (63%) of the multidrug resistant *E. coli* strains having multiple antibiotic resistance (MAR) index value >0.2 which indicates the high level of risk of contamination. Tambekar *et al.* (2008) reported that high incidence of multiple resistance of *E. coli* isolated from feces with high multi drug resistance (MAR) index.

### 4.9 Molecular characterization of *E.coli* isolates to detect the presence of virulence genes *stx*1 and *stx* 2

The phenotypic association between antibiotic resistance and virulence genes of *E.coli* isolates from fecal samples of diarrheic calves is presented in (Table 4.5). The *E.coli* isolates carrying *stx1* gene showed highest resistance of 100% against Metronidazole followed by 90.00% against Amoxycillin, 80% against Amoxycillin-Clavulanic acid, 70% against Amoxycillin- Sulbactam, Ceftazidime , Ceftriaxone.

*stx-2* gene showed highest resistance of 100% was detected in isolates carrying Metronidazole followed by 92.30% against Ceftazidime and Ceftriaxone and 84.62% against Amoxycillin-Clavulanic acid, 76.92% against Amoxycillin antibiotics. (Table 4.4).

Multiple virulence genes are associated with the pathogenicity of Shiga toxin associated *E.Coli* (STEC). In the present study, the virulence genes associated with the production of shigatoxins (*stx* 1 and *stx* 2 genes) were examined using PCR technique. All the 41 *E.coli* isolates were subjected to PCR for the detection of *stx 1* and *stx 2* genes as per the standard protocol describes by Cebula *et al.* (1995). In this study *stx 2* gene was detected in highest number, ie. in 13 samples (31.70%) found to be positive with 584 bp as compared to *stx1* gene in 10 samples (24.39%) with expected product size of 348 bp. (Plate 4.15 and 4.16). The results of the present study were somewhat in agreement with the study conducted by Wani *et al.* (2003) reported more prevalence of *stx 2* than *stx 1* gene in diarrhoeic calves and lambs in India. As there is a strong association between the presence of the *stx 2* gene and the capacity of STEC strains to cause severe human disease, production of the *stx 2* toxin is an index for serious clinical consequences in infected patients, (Bielaszewska *et al*., 2006). Similar occurrence of *stx 1* and higher occurrence of *stx 2* was observed in goats in previous studies (Oliveira et al., 2007; Vu-Khac and Cornick, 2008).

These findings are consistent with the observations of Sepehriseresht *et al.* (2009) who found out that 24 (70.5%) and 8 samples (23.5%) out of 34 samples were positive for *stx1* gene and *stx2* gene respectively. A similar result was also observed in the study conducted by Srivani *et al*. (2016) where in which they reported that *stx* 1 and *stx* 2 genes could be detected in 16.04% and 12.26% of *E.coli* isolates respectively. Wani *et al.* (2003) determined the prevalence and molecular characteristics of shiga toxin producing *E. coli* (STEC) and enteropathogenic *E. coli* (EPEC) in calves and lambs with diarrhoea in India. Zweifel *et al*. (2005) reported that a total of 42 STEC strains from slaughtered healthy cattle in Switzerland. The

PCR analysis showed that 18 (43%) strains carried the *stx1* gene, 20 strains (48%) had the *stx*2 gene and four (9.5%) strains had both *stx 1* and *stx* 2 genes.

Therefore, screening of larger number of animals is required in order to establish precisely the identity and prevalence of virulence factors associated with neonatal calf diarrhea. Such studies will enable to reveal the actual magnitude of the problem caused by VTEC and ETEC. This will provide an important epidemiological data about this disease and also give an early warning regarding any outbreaks in future.

### 4.10 Sequence homology and Phylogenetic analysis

After confirming the positive amplification, two samples were sequenced by Sanger's method to obtain sequences of VP6 and *stx 2* genes. Phylogeny tree was constructed to identify evolutionary relationship in VP6 genes segment of rotavirus samples and *stx 2* gene of *E. coli* and sent for the sequencing. Sequencing data of these rotavirus & *E. coli* genes were validated by BLAST analysis and further used for comparison along with already reported gene sequence from rotavirus and *E. coli* from various regions for phylogenetic analysis.

Phylogeny tree constructed for VP6 gene after retrieving the already reported sequences from the NCBI viral genomes resource (Brister *et al*., 2015). All sequences were first aligned by using ClustalW 1.6 software. Further aligned sequences were converted into MEGA file for Phylogeny construction. Phylogenetic analysis was done by using MEGA 7.0 software.

*stx 2* gene of *E. coli* was sequenced. The forward and reverse sequence was scrutinized for the validation using MEGA 7.0 software. Further, sequence was BLAST for pairwise alignment. Then the aligned sequence was used for phylogeny tree construction by using NCBI open source facility with Neighbor Joining tree construct, 0.75 maximum sequence difference and Jukes-Cantor distance method.

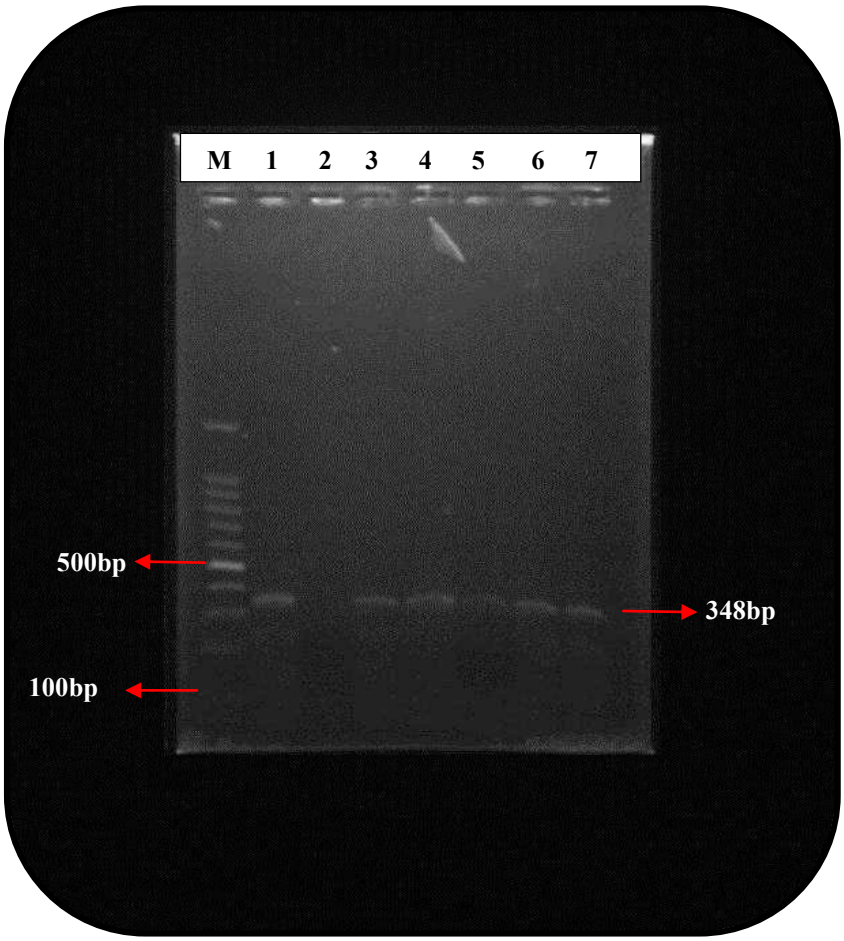


**Plate 4.15: Detection of *stx* 1 gene of *E.coli* by PCR**
**Lane M: Molecular weight marker (100 bp ladder)**
**Lane 1: Positive control**
**Lane 2: Negative control**
**Lane 3, 4, 5, 6, 7, : Positive samples**

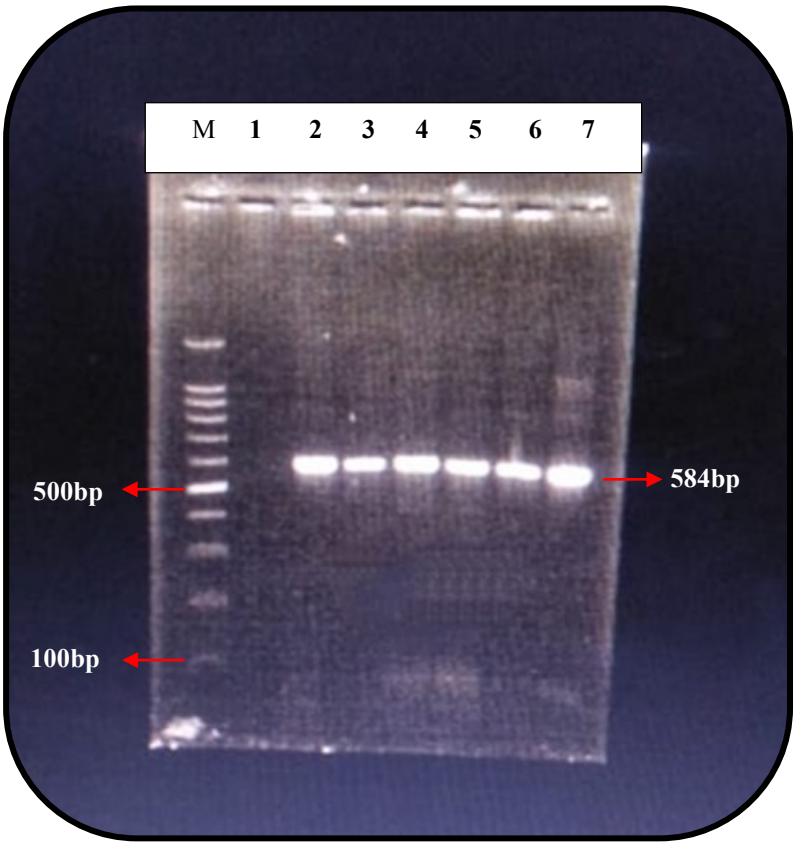


**Plate 4.16: Detection of *stx* 2 gene of *E.coli* by PCR**
**Lane M: Molecular weight marker (100 bp ladder)**
**Lane 1: Negative control**
**Lane 2: Positive control**
**Lane 3, 4, 5, 6 ,7: Positive samples**

### 4.10.1 Phylogenetic analysis of VP6 gene of cattle calves

In the present study, the phylogenetic analysis of VP6 gene of rotavirus of cattle calves showed 100% homology with already reported sequence from Maharashtra. And this was observed with first cluster whereas the remaining clusters have 80% identity with human rotavirus VP6 protein as per the phylogenetic tree (Figure 4.2). The result of the present study was similar to study conducted by Sharma *et al.* (2012) they found out that the VP6 gene and nonstructural genes (NSP1 to NSP3 and NSP5) were most homologous with the VP6 gene and non-structural genes of human rotaviruses belonging to the DS1 group. Banerjeee *et al*. (2007) sequence analysis of a 379-bp fragment of the gene encoding VP6 were closely related to VP6 sequences of other common human rotavirus strains and BLAST homology show maximum identity to the HRV sequences rj8200/04 (accession number DQ498163) and RMC/G66 (accession number AY601553) reported from Rio Di Janeiro and Manipal (nucleotide level identity of 99–98%). Molinari *et al*. (2014) detected 100% nucleotide and amino acid identities of rotavirus VP6 gene sequences, suggesting Rota virus group B.The sequence analysis of the VP6 gene showed that they are different from other Rotavirus Group B. Tumlam (2018) showed phylogenetic sequence analysis of VP6 gene of bovine rotavirus were independent taxonomic unit under same cluster with human spp. and showed interspecies cross relationship 79% & 81% homology with human rotavirus. Phylogenetic analysis showed not much sequence variation between rotavirus from human infants and cattle calf.

### 4.10.2 Phylogenetic analysis of *stx 1* & *stx 2* gene of *E.coli*

In our study total 582 nucleotides were recovered from the analysis. There were no any mutational differences like insertion, deletion or frame shift mutation. BLAST analysis confirmed the *stx* gene of *E. coli.* Furthermore, distance tree suggest that there were two clustered of different *E. coli Stx* gene sequences. Aligned *stx* gene sequenced of *E. coli* isolate was clustered with already reported sequence like strain

TS07/07 and strain c466-01B which was shown in tree (Figure 4.3). This study correlates with Tahamtan *et al*. (2010) who found that c466-01B strains of *stx 2* show 98% homology and potentially more virulent than strains *stx 1* or even strains carrying both *stx1* and *stx 2*. Ludwig *et al*. (2002) revealed that strains possessing *stx 2* are potentially more virulent than strains harboring *stx 1*.

**Table 4.1 : Details of the samples positive for bovine rotavirus.**

| Sr. No | Lab No | Breed of the animal | Age of the animal | Sex of the animal | Latex Agglutination Test | RT-PCR |
|---|---|---|---|---|---|---|
| 1 | G3 | CB | 20 days | Female | + | + |
| 2 | G5 | CB | 1 month | Female | + | - |
| 3 | B1 | CB | 3 days | Male | + | + |
| 4 | B2 | CB | 2 months | Male | + | + |
| 5 | B4 | CB | 2.5 months | Male | + | + |
| 6 | B6 | CB | 3 months | Male | + | + |
| 7 | B10 | CB | 3 months | Female | - | + |
| 8 | B11 | CB | 3 months | Female | + | + |
| 9 | S1 | CB | 15 days | Male | + | + |
| 10 | S6 | CB | 1 month | Male | + | + |
| 11 | S12 | CB | 12 days | Male | + | + |
| 212 | S13 | CB | 3 months | Female | + | + |

**Table 4.2. Details of the samples positive for bovine coronavirus.**

| Sr. No | Lab No | Breed of the animal | Age of the animal | Sex of the animal | Lateral Flow Test | RT-PCR |
|---|---|---|---|---|---|---|
| 1. | S12 | CB | 12 days | Male | + | - |

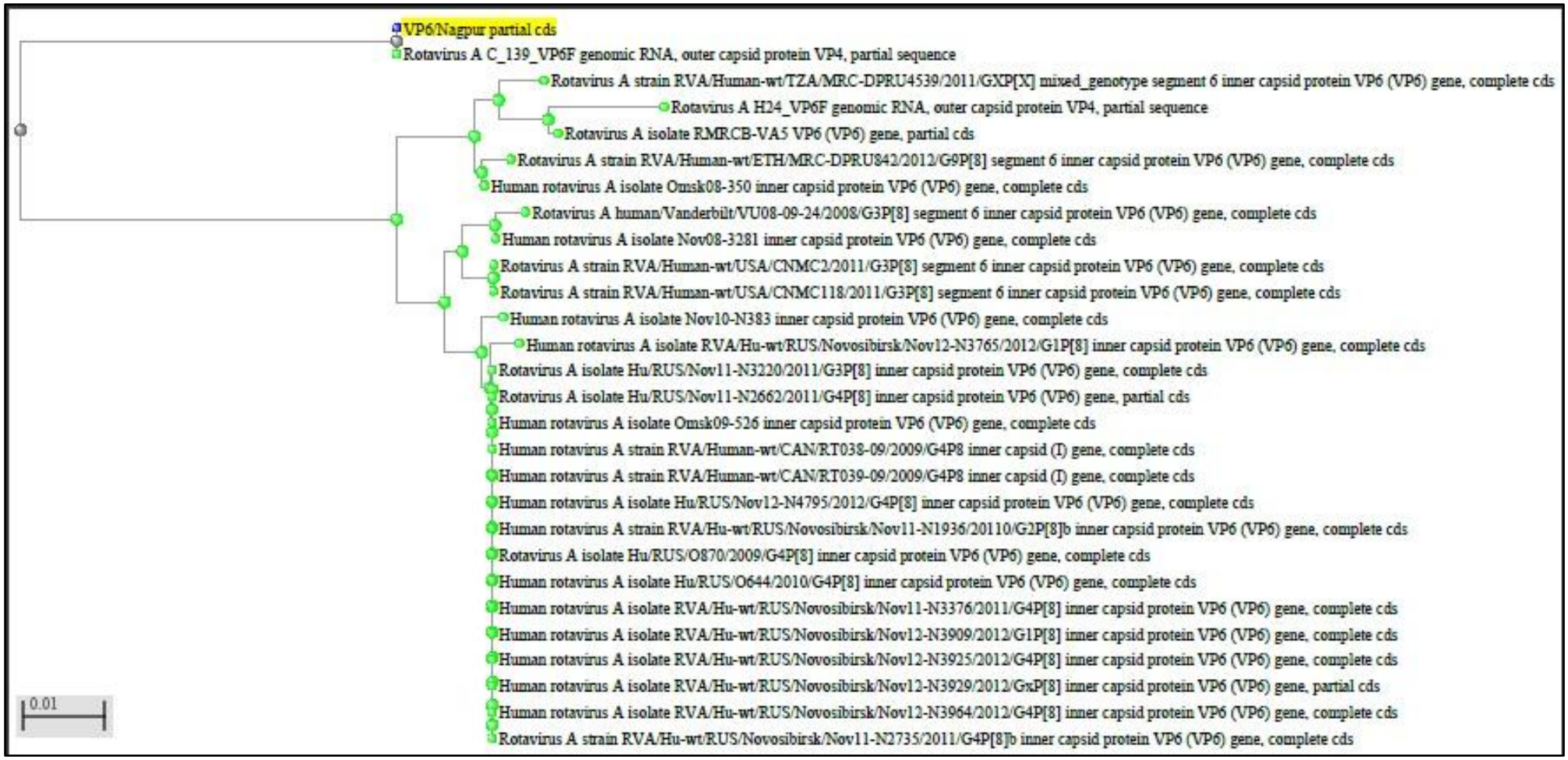


**Figure 4.2: Phylogenetic tree and sequence based analysis of VP6 gene of bovine rotavirus**


**Figure 4.3: Phylogenetic tree and sequence based analysis of *stx* 2 gene of *E.coli***

**Table 4.3. Biochemical characterization of calf *E.coli* isolates.**

| Isolate No. | Indole | MMR | VVP | Citrate | Malonate Utilization | Isolate No. | Indole | MR | VP | Citrate | Malonate Utilization |
|---|---|---|---|---|---|---|---|---|---|---|---|
| M102 | + | + | - | - | - | S1 | + | + | - | - | - |
| CB629 | + | + | - | - | - | S2 | + | + | - | - | - |
| CB636 | + | + | - | - | - | S4 | + | + | - | - | - |
| G2 | + | + | - | - | - | S5 | + | + | - | - | - |
| G3 | + | + | - | - | - | S6 | + | + | - | - | - |
| G5 | + | + | - | - | - | S7 | + | + | - | - | - |
| G6 | + | + | - | - | - | S8 | + | + | - | - | - |
| G8 | + | + | - | - | - | S9 | + | + | - | - | - |
| G9 | + | + | - | - | - | S10 | + | + | - | - | - |
| G10 | + | + | - | - | - | S11 | + | + | - | - | - |
| G11 | + | + | - | - | - | S12 | + | + | - | - | - |
| G12 | + | + | - | - | - | S14 | + | + | - | - | - |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| G13 | + | + | - | - | - | S15 | + | + | - | - | - |
| G14 | + | + | - | - | - | S16 | + | + | - | - | - |
| B2 | + | + | - | - | - | S17 | + | + | - | - | - |
| B6 | + | + | - | - | - | S18 | + | + | - | - | - |
| B7 | + | + | - | - | - | CBF2 | + | + | - | - | - |
| B8 | + | + | - | - | - | CBF3 | + | + | - | - | - |
| B9 | + | + | - | - | - | CBF4 | + | + | - | - | - |
| B10 | + | + | - | - | - | CBF6 | + | + | - | - | - |
| B11 | + | + | - | - | - | | | | | | |

**Table 4.4. Sample-wise Occurrence of Different Isolates in the Fecal Samples in Diarrhoeic Calves**

| Sample No. | Bovine rotavirus | | Bovine coronavirus | | *E.coli* | | |
|---|---|---|---|---|---|---|---|
| | Latex Agglutination Test | RT-PCR | Lateral Flow Test | RT-PCR | Conventional methods | *stx* 1 on PCR | *stx* 2 on PCR |
| 1 | - | - | - | - | - | - | - |
| M102 | - | - | - | - | + | - | - |
| CB629 | - | - | - | - | + | + | - |
| CB636 | - | - | - | - | + | - | + |
| G1 | - | - | - | - | - | - | - |
| G2 | - | - | - | - | + | + | - |
| G3 | + | + | - | - | + | + | - |
| G4 | - | - | - | - | - | - | + |
| G5 | + | - | - | - | + | - | - |
| G6 | - | - | - | - | + | - | + |
| G7 | - | - | - | - | - | - | - |
| G8 | - | - | - | - | + | + | - |
| G9 | - | - | - | - | + | - | + |
| G10 | - | - | - | - | + | + | - |
| G11 | - | - | - | - | + | - | + |
| G12 | - | - | - | - | + | + | - |
| G13 | - | - | - | - | + | - | + |
| G14 | - | - | - | - | + | - | + |
| B1 | + | + | - | - | - | - | - |
| B2 | + | + | - | - | + | - | - |
| B3 | - | - | - | - | - | - | - |
| B4 | + | + | - | - | - | - | - |
| B5 | - | - | - | - | - | - | - |
| B6 | + | + | - | - | + | - | - |
| B7 | - | - | - | - | + | - | + |
| B8 | - | - | - | - | + | - | + |
| B9 | - | - | - | - | + | - | + |
| B10 | - | + | - | - | + | - | - |
| B11 | + | + | - | - | + | - | - |
| B12 | - | - | - | - | - | - | - |
| S1 | + | + | - | - | + | - | - |
| S2 | - | - | - | - | + | - | - |
| S3 | - | - | - | - | - | - | - |
| S4 | - | - | - | - | + | - | - |
| S5 | - | - | - | - | + | - | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| S6 | + | + | - | - | + | - | - |
| S7 | - | - | - | - | + | - | - |
| S8 | - | - | - | - | + | - | - |
| S9 | - | - | - | - | + | - | + |
| S10 | - | - | - | - | + | - | - |
| S11 | - | - | - | - | + | + | - |
| S12 | + | + | + | - | + | - | - |
| S13 | + | + | - | - | - | - | - |
| S14 | - | - | - | - | + | - | + |
| S15 | - | - | - | - | + | - | - |
| S16 | - | - | - | - | + | + | - |
| S17 | - | - | - | - | + | + | - |
| S18 | - | - | - | - | + | - | - |
| CBF1 | - | - | - | - | - | - | - |
| CBF2 | - | - | - | - | + | - | - |
| CBF3 | - | - | - | - | + | - | - |
| CBF4 | - | - | - | - | + | + | - |
| CBF5 | - | - | - | - | - | - | - |
| CBF6 | - | - | - | - | + | - | + |

**Table 4.5 : Details of the Antibiotic susceptibility pattern of *E.coli* isolates from diarrheic calves.**

| Sr. No | Name of Antibiotic | Symbol of Antibiotic | Diarrhoeic (n=41) | |
|---|---|---|---|---|
| | | | Resistant | Sensitive |
| 1 | Amoxycillin | AMX | 28 (68.30) | 13 (31.70) |
| 2 | Amoxycillin-Clavulanic acid | AMC | 33 (80.49) | 8 (19.51) |
| 3 | Amoxycillin- Sulbactam | AMS | 23 (56.10) | 18 (43.90) |
| 4 | Ampicillin-Sulbactam | A/S | 9 (21.92) | 32 (78.08) |
| 5 | Cefotaxime | CTX | 13 (31.71) | 28 (68.29) |
| 6 | Ceftazidime | CAZ | 33 (80.49) | 8 (19.51) |
| 7 | Ceftriaxone | CTR | 29 (70.74) | 12 (29.26) |
| 8 | Ceftriaxone- Tazobactam | CIT | 15 (36.59) | 26 (63.41) |
| 9 | Enrofloxacin | EX | 11 (26.83) | 30 (73.17) |
| 10 | Furazolidone | F | 31 (75.61) | 10 (24.39) |
| 11 | Gentamicin | GEN | 7 (17.08) | 34 (82.92) |
| 12 | Levofloxacin | LE | 13 (31.71) | 28 (68.29) |
| 13 | Metronidazole | MT | 41 (100) | 0 (0) |
| 14 | Norfloxacin | NX | 11 (26.83) | 30 (73.17) |
| 15 | Oxytetracycline | O | 16 (39.03) | 25 (60.97) |
| 16 | Penicillin | P | 34 (82.93) | 7 (17.07) |

(Figures in the parenthesis indicates percentage)

**Table 4.6 : Correlation between resistance antibiotic and virulence genes (STEC) of *E.coli* isolates from diarrheic calves.**

| Sr. No | Name of Antibiotic | Symbol of Antibiotic | *stx* 1 (n= 10) | *stx* 2 (n= 13) |
|---|---|---|---|---|
| 1 | Amoxycillin | AMX | 9 (90) | 10 (76.92) |
| 2 | Amoxycillin-Clavulanic acid | AMC | 8 (80) | 11 (84.61) |
| 3 | Amoxycillin- Sulbactam | AMS | 7 (70) | 7 (53.84) |
| 4 | Ampicillin-Sulbactam | A/S | 2 (20) | 4 (30.76) |
| 5 | Cefotaxime | CTX | 4 (40) | 5 (38.46) |
| 6 | Ceftazidime | CAZ | 7 (70) | 12 (92.30) |
| 7 | Ceftriaxone | CTR | 7 (70) | 12 (92.30) |
| 8 | Ceftriaxone- Tazobactam | CIT | 2 (20) | 8 (61.53) |
| 9 | Enrofloxacin | EX | 3 (30) | 3 (23.07) |
| 10 | Furazolidone | F | 6 (60) | 8 (61.53) |
| 11 | Gentamicin | GEN | 1 (10) | 3 (23.07) |
| 12 | Levofloxacin | LE | 4 (40) | 3 (23.07) |
| 13 | Metronidazole | MT | 10 (100) | 13 (100) |
| 14 | Norfloxacin | NX | 3 (30) | 2 (15.38) |
| 15 | Oxytetracycline | O | 3 (30) | 3 (23.07) |
| 16 | Penicillin | P | 9 (90) | 11 (84.61) |

(Figures in the parenthesis indicates percentage)

**Table 4.7 Multiple Drug Resistance (MDR) index of the *E. coli* isolates**

| Sample No | Antibiotics (n=16) | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **O** | **MT** | **F** | **AMC** | **EX** | **CTX** | **CA** | **LE** | **CTR** | **GEN** | **NX** | **AMS** | **CIT** | **A/S** | **AMX** | **P** | **MDR index value** |
| M102 | R | R | R | R | R | S | R | R | S | S | R | R | S | S | R | R | 0.6875 |
| CB629 | S | R | S | R | S | R | R | S | R | S | S | R | S | S | R | R | 0.5 |
| CB636 | R | R | S | R | R | R | R | R | R | S | R | R | S | S | R | R | 0.75 |
| G2 | R | R | R | R | S | S | R | S | R | S | S | S | S | S | R | R | 0.5 |
| G3 | S | R | R | R | R | S | S | S | R | S | S | S | S | S | R | R | 0.4375 |
| G5 | R | R | S | R | R | S | R | R | S | S | S | R | S | S | R | R | 0.5625 |
| G6 | R | R | R | R | S | S | R | R | R | S | S | R | R | R | R | R | 0.75 |
| G8 | S | R | R | R | R | R | R | R | R | S | R | R | R | S | R | R | 0.8125 |
| G9 | S | R | S | R | S | R | R | S | R | S | S | R | R | R | R | R | 0.625 |
| G10 | S | R | R | R | S | S | S | S | S | S | R | R | S | S | R | R | 0.4375 |
| G11 | S | R | S | R | S | R | R | R | R | S | S | R | R | R | R | R | 0.6875 |
| G12 | S | R | S | R | S | R | R | S | R | S | S | R | R | R | R | R | 0.625 |
| G13 | S | R | R | R | S | S | S | S | R | S | S | S | R | S | S | S | 0.3125 |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| G14 | S | R | R | R | S | S | R | S | R | R | S | R | S | S | R | R | 0.5625 |
| B2 | S | R | R | R | S | S | R | S | R | S | S | R | S | S | S | S | 0.375 |
| B6 | S | R | R | R | S | S | S | S | R | S | R | S | R | S | S | S | 0.375 |
| B7 | S | R | R | S | S | S | R | S | S | S | S | S | S | S | R | R | 0.3125 |
| B8 | S | R | R | S | S | S | R | S | R | S | S | S | R | S | S | S | 0.3125 |
| B9 | S | R | R | R | R | S | R | S | R | R | R | S | R | S | S | R | 0.625 |
| B10 | R | R | R | R | R | S | R | S | R | S | R | S | R | S | S | S | 0.5625 |
| B11 | R | R | R | R | S | R | R | S | R | S | S | R | S | S | R | R | 0.625 |
| S1 | R | R | R | R | R | R | R | R | R | S | R | R | S | S | R | R | 0.8125 |
| S2 | R | R | R | R | R | R | R | R | R | S | S | R | R | S | R | R | 0.8125 |
| S4 | S | R | R | R | S | R | R | S | R | S | S | R | R | S | R | R | 0.625 |
| S5 | S | R | R | R | S | S | R | S | R | S | S | R | R | S | R | R | 0.5625 |
| S6 | R | R | R | S | S | S | S | S | S | S | S | S | S | S | S | R | 0.25 |
| S7 | R | R | S | R | S | S | S | S | S | R | S | S | S | S | S | R | 0.3125 |
| S8 | S | R | S | R | S | S | R | S | R | S | S | R | S | S | R | R | 0.4375 |
| S9 | R | R | R | R | R | S | R | S | R | R | S | S | S | S | R | R | 0.625 |
| S10 | S | R | R | R | S | S | R | S | R | S | R | S | S | S | R | R | 0.5 |
| S11 | R | R | R | S | S | S | S | R | S | S | S | R | S | S | R | R | 0.4375 |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S12 | S | R | R | R | S | S | R | S | S | S | S | S | S | S | S | R | 0.3125 |
| S14 | S | R | S | R | S | R | R | S | R | S | S | R | R | R | R | R | 0.625 |
| S15 | S | R | R | S | S | S | S | S | S | S | S | S | S | S | S | S | 0.125 |
| S16 | S | R | S | S | S | S | R | S | S | S | S | S | S | S | S | S | 0.125 |
| S17 | S | R | R | R | S | S | R | R | R | R | S | R | S | R | R | R | 0.6875 |
| S18 | R | R | R | R | S | R | R | R | R | R | R | R | S | R | R | R | 0.875 |
| CBF2 | R | R | R | R | R | S | R | R | R | S | R | S | S | S | R | R | 0.6875 |
| CBF3 | S | R | R | R | S | S | R | S | R | S | S | R | R | R | R | R | 0.625 |
| CBF4 | S | R | R | S | S | R | R | S | S | S | S | S | S | R | S | R | 0.375 |
| CBF6 | R | R | R | S | S | S | R | R | S | R | S | S | S | S | S | R | 0.4375 |
| Resistant | 16 (39.02) | 41 (100) | 31 (75.6) | 33 (80.48) | 11 (26.82) | 13 (31.7) | 33 (80.48) | 13 (31.7) | 29 (70.73) | 7 (17.07) | 11 (26.82) | 23 (56.09) | 15 (36.58) | 9 (21.95) | 28 (68.29) | 34 (82.92) | |
| Sensitive | 25 (60.97) | 0 (0) | 10 (24.4) | 8 (19.52) | 30 (73.18) | 28 (68.3) | 8 (19.52) | 28 (68.3) | 12 (29.27) | 34 (82.93) | 30 (73.18) | 18 (43.91) | 26 (63.42) | 32 (78.05) | 13 (31.71) | 7 (17.08) | |

(Figures in the parenthesis indicates percentage)

O: Oxytetracycline
F: Furazolidone
EX: Enrofloxacin
CA:Ceftazidime
CTR: Ceftriaxone
NX: Norfloxacin
CIT: Ceftriaxone- Tazobactam
AMX: Amoxycillin
MT: Metronidazole
AMC: Amoxycillin- Clavulanic acid
CTX: Cefotaxime
LE: Levofloxacin
GEN: Gentamicin
AMS: Amoxycillin-
A/S: Ampicillin- Sulbactam
P: Penicillin

## SUMMARY

The present study was carried out for the detection of causative agents associated with diarrhea in calves. For this study, 54 faecal samples were collected from diarrheic calves of 0-3 months of age. Samples were collected in duplicate in order to facilitate the detection of both viral and bacterial agents. Screening of the faecal samples for the presence of bovine rotavirus and coronavirus was carried out by employing Latex Agglutination Test using commercially available Rotavirus antigen detection test kit and Lateral Flow Test using FASTest® BCV Strips, respectively. The samples were then processed for molecular characterization of bovine rotavirus and coronavirus by RT-PCR. Amplification of VP6 gene of 379 bp and N-gene of 730 bp was carried out for the molecular detection of bovine rotavirus and coronavirus respectively. One of the duplicate samples collected from each animal was also processed for the isolation and identification of predominant bacteria by morphological, biochemical and cultural characterization. The predominant bacterial isolates identified by the conventional methods were then subjected to PCR for the detection of virulence genes.

In the present study, 11 (20.37%) out of 54 samples were found positive for bovine rotavirus by Latex agglutination Test. Out of theses 11 samples which were positive for bovine rotavirus by LAT, 10 were found positive by RT-PCR. One sample which was found negative by LAT was positive for bovine rotavirus by RT-PCR. Thus a total of 12 (22.22%) samples out of 54 diarrhoeic samples were found positive for bovine rotavirus.

Only 1 (1.85%) out of 54 samples was found positive for bovine coronavirus by lateral flow test. But none of the bovine fecal samples were found positive for bovine coronavirus for the presence of N gene by RT-PCR.

In the present study, 41 (75.92%) out of 54 diarrhoeic faecal samples were positive for the presence of *E.coli*. The presence of virulence genes associated with

the production of shiga toxins (*stx* 1 and *stx* 2 genes) was undertaken using PCR with the product size of 348 bp for *stx* 1 gene and 584 bp for *stx* 2 gene. 10 (24.39%) and 13 (31.70%) isolates out of 41 isolates were found to be positive for *stx* 1 and *stx* 2 genes respectively.

All the 54 samples were subjected to Antibiotic Sensitivity Test to study about the antimicrobial resistance. The result of antibiogram pattern of 41 *E.coli* isolates when tested against commonly used antimicrobial agents reflected varying sensitivity.The highest sensitivity was observed for Gentamicin with 34 (82.92%) out of 41 samples sensitive, followed by Ampicillin-Sulbactam (78.08%), Enrofloxacin and Norfloxacin (73.17%), Levofloxacin and Cefotaxime (68.29%), Ceftriaxone-Tazobactam (63.41%) and Oxytetracycline (60.97%). All the 41isolates were resistant to Metronidazole (100%) followed by Penicillin (82.93%), Ceftazidime and Amoxycillin-Clavulanic acid (80.49%), Furazolidone (75.61%), Ceftriaxone (70.74%), Amoxycillin (68.30%) and Amoxycillin- Sulbactam (56.10%).

Sequencing and phylogenetic analysis of VP6 RT-PCR positive samples showed 100% homology with already reported sequence from Maharashtra and 80% identity with human rotavirus VP6 protein. Similarly, sequencing of *stx* 2 gene showed analogy with already reported sequence like strain TS07/07 and c466-01B strain of world and Indian isolates.

## CONCLUSIONS

From the present study, the following conclusions are drawn.

- The Latex Agglutination Test is a useful and sensitive technique for the preliminary screening of bovine rotavirus.
- RT-PCR is a sensitive and specific molecular method for the detection of bovine rotavirus.
- *E.coli* is the major causative agent for diarrhoea in calves below 3 months of age.
- The *E.coli* isolates were highly sensitive to Gentamicin and Ampicillin-Sulbactam whereas complete resistance was observed with Metronidazole.
- Sequencing and phylogenetic analysis of rotavirus revealed 100% homology with already reported sequence from Maharashtra and 80% identity with human rotavirus VP6 protein and sequencing of *stx* 2 gene showed analogy with sequences like strain TS07/07 and c466-01B strain of world and Indian isolates.

## BIBILIOGRAPHY

Abou El-Ella, G. A., A. M. Mohamed and A. A. Aamer (2013) Prevalence of enteropathogens associated with neonatal calf scour in cattle and buffalo calves using (FASTest® Strips) rapid field test, Assiut Vet. Med. J. 59 (138): 19-26.

Acres, S. D., J. R. Saunders and O. M. Radostits (1997). Acute undifferentiated neonatal diarrhea of beef calves: the prevalence of enterotoxigenic *E. coli*, reo- like (rota) virus and other enteropathogens in cow-calf herds. Canadian Vet. J. 18:113-121.

Ade, M. J., V.C. Ingle, P. A. Tembhurne, U. M. Tumlam, A. S. Kadam, G. R. Bhojne and P. M. Sonkusale (2019) Incidence and Molecular Characterization of Rotaviruses of Cattle and Buffalo Calves in Amravati Region, Maharashtra, Ind. J. of Vet. Sci. & Biotech. 14 (3):20-23.

Agarwal, R. K., K. N. Bhilegaonkar, D. K. Singh, A. Kumar and R. S. Rathore (2003) Laboratory manual for the isolation and identification of food borne pathogens, 1st Edn. ICAR, New Delhi.

Ahmed, S. P., R. A. Hazarika, D. P. Bora and S. Tamuly (2017) Detection and Genotypic Characterization of Rotavirus from Bovine Calves of Assam, A North Eastern State of India, The J. of Ani. & Pl. Sci. 27(2): 439-445 Issn: 1018-7081.

Al-Ajmi, D., J. Padmanabha, S. Denman, R. A. Gilbert, R. A. M. Al Jassim and C. S. McSweeney (2006). Evaluation of a PCR detection method for *Escherichia coli* O157: H7/H bovine faecal samples. App. Microbiol, 42(4)**:** 386-391.

Almeida, J.D., D.M. Berry, C.H. Cunningham, D. Hamre, M.S. Hofstad, L. Mallucci, K. McIntosh, and D.A.J. Tyrrell (1968). Coronaviruses. Nature 220:650.

Anand, S. K., S. Fatima, C. G. Joshi and J. H. Purohit (2006). Molecular characterization of *E. coli* using PCR-SSCP analysis of *16S rRNA* gene. Ind. J. Poul. Sci. 41(1)**:** 1-5.

Arias, C.F., S. Lopez and R.T. Espejo (1982). Gene protein products of SA-11 simian rotavirus genome. J. of Virol. 41: 42-50.

Aydin, F., S. Umer, G. Gocke, O. Geno and M. A. Guler (2001) The isolation and identification f bacteria and parasites from diarrheic calves in Kars district. Kafkas Universitesi Veteriner Fakultesi Dergisi, 7(1): 7-14.

Ayis A. A., A. A. Elgaddal and Y. A. Almofti (2015) Isolation, Identification and Enterotoxin Detection of *Escherichia Coli* Isolated from Calf Diarrhea and their Virulence Characteristics. J. of App. and Industr. Sci. 3 (4): 141-149, ISSN: 2328-4595, ISSN: 2328-4609 (ONLINE)

Ball, J. M., D. M. Mitchell, T. F. Gibbons, and R. D. Parr. (2005) Rotavirus NSP4: a multifunctional viral enterotoxin. Viral. Immunol. 18:27-40.

Banerjee, I., S. Ramani, B. Primrose, M. I. Gomara, J. J. Gray, D. W. Brown and G. Kang (2007) Modification of Rotavirus Multiplex RT-PCR for the Detection of G12 Strains Based on Characterization of Emerging G12 Rotavirus Strains From South India. J. of Med. Virol. 79:1413–1421

Bardhan, K. H (2007) Nucleic Acid Based Approach for the Detection and Characterization of Neonatal Calf Diarrheic Viruses. Thesis submitted to Jawaharlal Nehru Krishi Vishwa Vidyalaya Jabalpur.

Barnes, H. J., J. P. Vaillancourt and W. B. Gross (2003). Colibacilllosis, diseases of poultry, lowa State Press, lowa, USA, 11: 631-652

Bauer, A. W., M. M. Kirby, J. C. Sherris and M. Turck (1966) Antibiotic susceptibility testing by a standardized single disc method. Ameri. J. of Clin. Pathol. 45: 493-496.

Bergey's Manual of Systematic Bacteriology (1986) Volume (2), Williams and Wilkins Publication. pp: 418-423

Bharadwaj, R., A. Gupta and J. K. Garg (2015) Prevalence of multidrug resistance in *Escherichia coli* strains isolated from river Yamuna, Delhi stretch Inter. J. of Bioassays ISSN: 2278-778X

Bhat, S.A., P. D. Juyal and L. D. Singla (2012). Prevalence of cryptosporidiosis in neonatal buffalo calves in Ludhiana district of Punjab, India. Asian J. Anim. Vet. Adv. 7 (6):512-520.

Bielaszewska, M., A. W. Friedrich, T. Aldick, R. Schürk-Bulgrin and H. Karch (2006). Shiga toxin activatable by intestinal mucus in *Escherichia coli* isolated from humans: predictor for a severe clinical outcome. *Clin. Infect. Dis.* 43: 1160–1167. 10.1086/508195

Boileau, M. J and S. Kapil. (2010). Bovine coronavirus associated syndromes. Vet. Clin. North Am. Food Anim. Pract. 26:123–146.

Brandao, P. E., G. S. Gregor, R. M. Monteleoni, C. A. Soares, C. A. R. Rosales and J. A. Jerez (2003). Nested PCR assay for detection of Bovine Coronavirus S1 gene. Sao Paulo, Arq. Inst. Biol. 70: 1-3.

Brister J. R., D. Ako-adjei, Y. Bao and O. Blinkova (2015) NCBI Viral Genomes Resource Nucleic Acids Research.

Cebula, T. A., W. L. Payne and P. Feng (1995) Simultaneous identification of strains of *Escherichia coli* serotype O157: H7 and their Shiga-like toxin type by

mismatch amplification mutation assay- multiplex PCR. J. Clin. Microbiol. 33: 248-250.

Chapman, P. A., M. Ellin, R. Ashton and W. Shafique (2001). Comparison of culture, PCR and immunoassays for detecting *Escherichia coli* O157 following enrichment culture and immunomagnetic separation performed on naturally contaminated raw meat products. Int. J. Food Microbiol. 68(1-2):11-20.

Chinsangaram, J., C. E. Schore, W. Guterbock, L. D. Weaver and B. I. Osburn. (1995). Prevalence of group A and group B rotaviruses in the feces of neonatal dairy calves from California. Comp. Immunol. Microbiol. Infect. Dis. 18:93- 103.

Cho, Y.I., J. I. Han, C. Wang (2013). Case-control study of microbiological etiology associated with calf diarrhea. Vet. Microbiol. 166: 375–385.

Cho, K. O., M. Hasoksuz, P. R. Nielsen, K. O. Chang, S. Lathrop, and L. J. Saif (2001). Cross-protection studies between respiratory and calf diarrhea and winter dysentery coronavirus strains in calves and RT-PCR and nested PCR for their detection. Arch. Virol. 146:2401-19.

Clark, M.A (1993). Bovine coronavirus. Br. Vet. J. 149(1): 51-70.

Cowan, S. T and K. J. Steel (1975). Manual for the identification of medical bacteria, 2nd edition, Cambridge University Press, Cambridge

Cowan, S. T and K. J. Steel (1993) Manual for the identification of Medical Bacteria. The syndics of the Cambridge University Press. Bentlay House, 200 Euston Road, London, U.K.

Cruickshank, R., J. P. Duguid, B. P. Marmoin and R. H. A. Swain (1975) Med. Microbiol. 2 (12) Churchill Livingstone, Edinburgh, London and New York.

Dadawala, A. I (2008). Studies on prevalence of Rotavirus and Enterotoxigenic *Escherichia coli* infection in neonatal calf diarrhoea in Gujarat. M.V.Sc. Thesis, submitted to Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar.

Dash, S. K., A. Tewari, K. Kumar, A. Goel and A. K. Bhati (2011) Detection of Rotavirus from diarrhoeic cow calves in Mathura. Vet. World 4 (12):554-556

Das, S., M. Medhi, M. Khaound, P. Doley, M. Islam and D. P. Borah (2018) Detection of group A rotavirus infection in diarhoeic calves by electropherotyping and reverse transcriptase polymerase chain reaction, J. of Entomol. and Zool. Stud. 6 (3): 1071-1075

Decaro, N., V. Martella, G. Elia, M. Campolo, V. Mari, C. Desario, M. S. Lucente, A. Lorusso, G. Greco, M. Corrente, M. Tempesta, and C. Buonavoglia (2008). Biological and genetic analysis of a bovine-like coronavirus isolated from water buffalo (Bubalus bubalis) calves. Virology 370:213-22.

Desselberger, U. and M. K. Estes (2005). Rotaviruses. In B.W.J. Mahy and V.T. Meulen (eds). Topley and wilson's Microbiology and Microbial infections, ASM press, USA: 946-958.

Deswal, S., P. Minakshi, K. Ranjan, S. Dhillon, Singh, Y. S. Malik and G. Prasad (2015) Genetic characterization of an emerging G3P[3] rotavirus genotype in buffalo calves, India, As. J. of Ani. and Vet. Adv. 10 (8): 376-385

De Vries, A.A.F., M. C. Horzinek, P. J. M. Rottier and R. J. de Groot. (1997). The genome organization of the nidovirales: Similarities and differences between arteri-, toro- and coronaviruses. Seminars in Virology, 8: 33-47.

Dhama, K., R. S. Chauhan, M. Mahendran, and S. V. Malik (2009). Rotavirus diarrhea in bovines and other domestic animals. Vet. Res. Commun. 33:1-23.

Drancourt, M., B. Claude, C. Antoine, M. Rolland, G. Jean and R. Didier (2000) 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J. Clin. Microbiol. 38(10): 3623-3630.

Duckmanton, L., S. Carman, E. Nagy and M. Petric (1998) Detection of bovine torovirus in fecal specimens of calves with diarrhea from Ontario farms. J. Clin. Microbiol. 36:1266–1270.

Edgar, R.C (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32:1792–1797.

Edwards, R and W. N. Ewing (1972). Identification of Enterobacteriaceae. 3rd edn., Burgess Publishing Co., Minnesota.

El-Ella, G. A., A. M. Mohamed and A. A. Ahmed (2013). Prevalence of Enteropathogens Associated with Neonatal Calf Scour in Cattle and Buffalo Calves using (Fastest® Strips) Rapid Field Test Assiut Vet. Med. J. 59 (138)

Erdogan, H. M., A. Unver, V. Gunes and M. Citil (2003). Frequency of Rotavirus and Coronavirus in neonatal calves in Kars district. Kafkas Univ. Vet. Fak. Derg. 9 (1): 65-68.

Escherich (1885). Fortschr. Med. 3: 541-547. (c.f. Wilson and Miles, 1964).

Estes, M and A. Kapikan (2007) Rotavirus Fields Virology, 5th Edition, Kluwer Health/ Lippincott, Williams and Wilkins; Philadelphia. 5: 1917-1974.

Estes, M. K. and J. Cohen (1989). Rotavirus gene structure and function. Microbiol. Rev. 53 (4): 410-449.

Estes, M.K. (2001). Rotaviruses and their replication. In: D.M. Knipe, P.M. Howley, D.E. Griffin, R.A. Lamb, M.A. Martin, B. Rizman and E. Straus (eds.) Fields

Virology, 4th Edn. Lippincott Williams and Wilkins, Philadelphia, pp 1747-1785.

Falcone, E., Tarantino, M., Trani, L. D., Cordioli, P., Lavazza, A and Tollis, M (1999) Determination of G and P serotypes in Italy using PCR. J. of Clin. Microbiol. 37(12): 3879-3882.

Fenner (2011). Veterinary Virology. 4th ed. Acdemic Press, Burlington, MA: 274-280.

Foster, D. M., and G. W. Smith (2009). Pathophysiology of diarrhea in calves. Vet. Clin. North Am. Food Anim. Pract. 25:13-36.

Francis, D. H., S. D. Allen and R. D. White (1989). Influence of bovine intestinal fluid on the expression of K99 pili by Escherichia coli. Am. J. Vet. Res. 50: 822-6.

Francoz, D., S. Buczinski and A. M. Belanger (2015). Respiratory pathogens in Qu!ebec dairy calves and their relationship with clinical status, lung consolidation, and average daily gain. J. Vet. Intern. Med. 29: 381–387.

Fukushima, M., K. Kakinuma and R. Kawaguchi (2002). Phylogenetic analysis of *Salmonella, Shigella* and *Escherichia coli* strains on the basis of gyrB gene sequence. J. Clin. Microbiol. 40(8): 2779-2785.

Garcia A., J. A. Ruiz-Santa-Quiteria, J. A. Orden *et al*. (2000). Rotavirus and concurrent infections with other enteropathogens in neonatal diarrheic dairy calves in Spain. Comp. Immunol. Microbiol. Infect. Dis., (23):175–183.

Ghosh, S., V. Varghese, M. Sinha, N. Kobayashi, and T. N. Naik (2007). Evidence for interstate transmission and increase in prevalence of bovine group B rotavirus strains with a novel VP7 genotype among diarrhoeic calves in Eastern and Northern states of India. Epidemiol. Infect. 135:1324-30.

Gill, G. S., S. Kaur, P. N. Dwivedi and J. P. S. Gill (2017) Comparative prevalence and molecular characterization of Group A rotavirus in cow calves of Punjab, J. of Ani. Res. 7 (5): 927-933.

Gomez, D. E., L. G. Arroyo, Z. Poljak and J. S. Weese (2017) Detection of Bovine coronavirus in healthy and diarrhoeic dairy calves, J. Vet. Intern. Med. DOI: 10.1111/jvim.14811

Greenberg, H.B., V. McAuliffe, J. Valdesuso, R. Wyatt, J. Flores, A. Kalica, Y. Hoshino and N. Singh (1983). Serological analysis of the subgroup proteins of rotavirus using monoclonal antibodies. Infection and Immunity; 39: 91-99.

Gulati, B. R., R. Deepa, B. K. Singh, and C. D. Rao (2007). Diversity in Indian equine rotaviruses: identification of genotype G10, P6 [1] and G1 strains and a new VP7 genotype (G16) strain in specimens from diarrheic foals in India. J. Clin. Microbiol. 45:972-8.

Hammami, S., A. E. Castro and B. I. Osburn (1990). Comparison of polyacrylamide gel electrophoresis, an enzyme-linked-immunosorbent assay and an agglutination test for the direct identification of bovine rotavirus from feces and co-electrophoresis of viral RNAs. J. Vet. Diag. Inv. 2: 184–190.

Hansa, A., R. B. Rai, M. Y. Wani and K. Dhama (2012) ELISA and RT-PCR Based Detection of Bovine Coronavirus in Northern India, Asian J. of A. and Vet. Adv. 7 (11): 1120-1129

Hashem, S., S. A. Shoman and A. F. Mohamed (2012) Isolation and Molecular Genotyping of Group A Rotavirus Strains Circulating among Egyptian Infants and Children. Egyp. J. of Med. Microbiol. 21 (3): 11

Hasoksuz, M., A. E. Hoet and S. C. Loerch (2002). Detection of respiratory and enteric shedding of bovine coronaviruses in cattle in an Ohio feedlot. J. Vet. Diagn. Invest; 14: 308–313.

Hasoksuz, M., A. Kayar, T. Dodurka and A. Ilgaz (2005) Detection of respiratory and enteric shedding of bovine coronaviruses in cattle in Northwestern Turkey. Acta Vet. Hung. 53: 137–146.

Hasoksuz, M., K. Alekseev, A. Vlasova, X. Zhang, D. Spiro, R. Halpin, S. Wang, E. Ghedin, and L. J. Saif. (2007). Biologic, antigenic, and full-length genomic characterization of a bovine-like coronavirus isolated from a giraffe. J. Virol. 81:4981-90.

Hassan, H. A., Q. Kalefa, K. A. Haleem and M. Al-Qadisiya (2014) Detection of bovine rotavirus in diarrheic calves by using rapid test in some Mid-Euphrates provinces AL-Qadisiya J. of Vet. Med. Sci. 13 (2).

He, K.W., J.H. Lin, Z.D. Ding, J.H. He, J.Y. Jianj and J.B. Hou (2002). Serotyping and antigenic comprison of some animal rotavirus isolated in China. Zentralbl Vatrinarned. B. 39: 299-300.

Holland, R. E. (1990). Some Infectious Causes of Diarrhea in Young Farm-Animals. Clin. Microbiol. Reviews 3:345-375.

Hoshino, Y., R. G. Wyatt, H. B. Greenberg, J. Flores and A. Z. Kapikian (1984). Serotypic similarity and diversity of rotaviruses of mammalian and avian origin as studied by plaque-reduction neutralization. J. Infect. Dis. 149: 694-702.

Hossain, M. K., M. Rahman, A. Nahar, A. Khair and M. M. Alam (2013) Isolation And Identification of Diarrheagenic *Escherichia coli* Causing Colibacillosis in Calf in Selective Areas of Bangladesh. Bangl. J. Vet. Med. (2013).11(2): 145-149 ISSN: 1729-7893 (Print) 2308-0922 (Online)

Husain, M., P. Seth and S. Broor (1995). Detection of group A rotavirus by reverse transcriptase and polymerase chain reaction in feces from children with acute gastroenteritis. Arch. Virol. 140: 1225-33.

Hussain, I and G. K. Saikia (2000). Isolation and characterization of bacteria from diarrhoeic bovine calves. Ind. J. Comp. Microbiol. Immunol. Infect. Dis. 21: 125-127.

Irino, K., M. A. M. F. Kato, T. M. I. Vaz, I. I. Ramo, M. A. C. Souza, A. S. Cruz, T. A. T. Gomes, M. A. M. Vieira and B. E. C. Gath (2005). Serotypes and virulence markers of shiga toxin producing *E. coli* (STEC) isolated from dairy cattle in Sãu Paulo State, Brazil. *Vet. Microbiol*. 105: 29-36.

Isegawa, Y., O. Nakagomi, T. Nakagomi, S. Ishida, S. Uesugi and S. Ueda (1993) Determination of bovine rotavirus G and P serotypes by polymerase chain reaction, Molecular and Cellular Probes 7: 277-284.

Islam, K. M. A., M. Rahman, A. Nahar, A. Khair and M. M. Alam (2015). Investigation of pathogenic *Escherichia coli* from diarrheic calves in selective area of Bangladesh. *Bangl. J. of Vet. Med.* 13 (1): 45-51.

Izzo, M. M., P. D. Kirkland, X. Gu, Y. Lele, A. A. Gunna and J. K. Housea (2012) Comparison of three diagnostic techniques for detection of rotavirus and coronavirus in calf faeces in Australia, *Austr.Vet. J.* 90: 122-129

Jadhav, M. B (2009) Detection and molecular characterization of bovine and porcine rotaviruses from Nagpur region, M.V.Sc thesis submitted to MAFSU, Nagpur.

Jagannath, M. R., R. Robert, V. Reddy, S. Y. Raman and S. C. Durga Rao (2000) Characterization of human symptomatic rotavirus isolates MP409 and MP480 having long RNA electropherotype and subgroup I specificity, highly related to the P6 [1], G8 type bovine rotavirus A5, from Mysore, India. Arch. Virol. 145: 1339-1357

Jenny, B. F., G. E. Cramling and T. M. Glaze (1981). Management factors associated with calf mortality in South Carolina dairy herds. J. Dairy Sci. 64: 2284-2289.

Jindal, S. R., N. K. Maiti and M. S. Oberoi (2000) Genomic Diversity and Prevalence of Rotavirus in Cow and Buffalo Calves in Northern India. Revue Et Scientifique Technique. Office International Des Epizootics 19: 871-876.

Jones, T. C. and R. D. Hunt (1985).Veterinary Pathology, 5th ed. Lea and Febiger, Philadelphia, PA 19106, USA.

Kaper, J. B., J. P. Nataro, and H. L. Mobley (2004). Pathogenic *Escherichia coli*. Nat. Rev. Microbiol. 2:123-40.

Kapikan, A., Y. Hoshino and R. Chanock (2001). Rotavirus, D. Knipe, P.M. Howley, etal.(ed), Fields Virology, 4th ed. Lippincott Williams & willikins, Philadelphia: 1787-1834.

Khafagi, M. H., M. A. Mahnoud and A. R. Habashi (2010). Prevalence of rotavirus Kobayashi N, Lintag LC, Urasawa T, Taniguchi K, Saniel M. C, Urasawa S. Unusual human rotavirus strains having subgroup I specificity and “long” RNA electropherotype. Arch. Virol. 1989; 109:11-23.

Khalili, M., A. Morshedi, H. Keyvanfar and F. Hemmatazadeh (2006). Detection of bovine coronavirus by RT-PCR in a field study. Vet. Arhiv.76: 291-296.

Khamrin, P., N. Maneekarn, S. Peerakome, W. Chan-it, F. Yagyu, S. Okitsu, and H. Ushijima (2007). Novel porcine rotavirus of genotype P [27] shares new phylogenetic lineage with G2 porcine rotavirus strain. Virology 361:243-52.

Kin, N., F. Miszczak, L. Diancourt, V. Caro, F. Moutou, A. Vabret, and M. ArGouilh (2016). Comparative molecular epidemiology of two closely related coronaviruses, bovine coronavirus (BCoV) and human coronavirus OC43

(HCoV-OC43), reveals a different evolutionary pattern. Infect. Genet. Evol. 40:186 –191.

Klein, D., A. Kern, G. Lapan, V. Benetka, K. Mostl, A. Hassl and W. Baumgartner (2009) Evaluation of rapid assays for the detection of bovine coronavirus,rotavirus A and *Cryptosporidium parvum* in faecal samples of calves. *The Vet. J.*182: 484–486.

Krumperman, P. H (1983). Multiple antibiotic resistance indexing *Escherichia coli* to identify risk sources of faecal contamination of foods. Appl. Environ. Microbiol. 46: 165-170.

Kumar, S., G. Stecher and K. Tamura (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33:1870–1874.

Lai, M. M (1990). Coronavirus: organization, replication and expression of genome. Annu. Rev. Microbiol. 44: 303-33.

Lanz., F. Uhde, T. Kaufmann, H. Sager *et al.,* (2008). Prevalence of four enteropathogens in the faeces of young diarrhoeic dairy calves in Switzerland. Vet. Rec. 163:362– 366.

Lathrop, S. L., T. E. Wittum and S. Loerch (2000) Antibody titers against bovine coronavirus and shedding of the virus via the respiratory tract in feedlot cattle. Am. J. Vet. Res. 61: 1057–1061.

Lin, X. Q., O. e. KL, J. Storz, C. W. Purdy, and R. W. Loan (2000). Antibody responses to respiratory coronavirus infections of cattle during shipping fever pathogenesis. Arch. Virol. 145:2335-49.

Liu, L., S. Hagglund, M. Hakhverdyan, S. Alenius, L. E. Larsen and S. Belak (2006). Molecular epidemiology of bovine coronavirus on the basis of comparative analyses of the S gene. J. Clin. Microbiol. 44:957-60.

Lucchelli, A., S. E. Lance, P. B. Bartlett, G. Y. Miller and L. J. Saif (1992). Prevalence of bovine group A rotavirus shedding among dairy calves in Ohio. Am. J. Vet. Res. 53:169-74.

Ludwig, K., V. Sarkim, M. Bitzan, M. A. Karmali, C. Bobrowski and H. Ruder (2002) Shiga Toxin-Producing *Escherichia coli* infection and antibodies against Stx2 and Stx1 in household contacts of children with enteropathic hemolytic-uremic syndrome. J. Clin. Microbiol. 2002; 40: 12-17.

Malaviya, S. G (2012) Prevalence of rotavirus and enterotoxigenic *Escherichia coli* infection in neonatal calf diarrhoea in south Gujarat. MVSc Thesis submitted to College of Veterinary Science and Animal Husbandary, Navsari, Gujarat.

Malik, S., A. K. Verma, A. Kumar, M. K. Gupta and S. D. Sharma (2012). Incidence of calf diarrhea in cattle and buffalo calves in Uttar Pradesh, India. Asian J. Anim. Vet. Adv. 10:1049-1054.

Malik, Y. S., K. Sharma, N. Kumar, A. A. Haq and N. Vaid (2014) Determination of bovine rotavirus genotypes (G and P) circulating in Uttarakhand (2010-2012) by polymerase chain reaction, Ind. J. of Ani. Sci. 84 (2): 42

Malik, Y. S., N. Kumar, K. Sharma, R. Sharma, H. B. Kumar, K. Anupamlal, S. Kumari, S. Shukla and K. M. Chandrahekar (2013). Epidemiology and genetic diversity of rotavirus strains associated with acute gastroenteritis in bovine, porcine, poultry and human population of Madhya Pradesh, Central India, 2004–2008**.** Adv. Anim. Vet. Sci. 1 (4): 111 – 115.

Malik, Y. S., S. Sharma, K. Vaid, N. Batra, M. Prasad, I. Prasad, B. R. Gulati and K.N. Bhilegaonkar (2013) Designing and validation of new primers for

efficient genotyping of animal G3 rotaviruses. Veterinarsk Archives. 83 (6): 611-622

Martella, V., K. Banyai, J. Matthijnssens, C. Buonavoglia and M. Ciarlet (2010). Zoonotic aspects of rotaviruses. Vet. Microbiol. 140:246-55.

Martella, V. A., G. Pratelli, M. Greco, M. Tempesta, S. Ferrari, M. N. Losio, and C. Buonavoglia (2001) Genomic characterization of porcine rotavirues in Italy. Vet. Microbiol. 8:129-132.

Martella, V., M. Ciarlet, K. Banyai, E. Lorusso, S. Arista, A. Lavazza, G. Pezzotti, N. Decaro, A. Cavalli, M. S. Lucente, M. Corrente, G. Elia, M. Camero, M. Tempesta and C. Buonavoglia (2007). Identification of group A porcine rotavirus strains bearing a novel VP4 (P) Genotype in Italian swine herds. J. Clin. Microbiol. 45:577-80.

Mason, B.B., D.Y. Graham and M.K. Estes (1983). Biochemical mapping of the simian rotavirus SA11 genome. J. of Virol. 46: 413-23.

Merchant, I. A and R. A. Packer (1967). Veterinary Bacteriology and Virology 6th Edn. Iowa State University Press USA.

Molinari, B. L. D., A. F. Alfieri and A. A. Alfieri (2014) Genetic Variability of VP6, VP7, VP4, and NSP4 Genes of Porcine Rotavirus Group H Detected in Brazil, Virus Research, 197:48–53.

Mondal, A., K. Sharma, Y. P. S. Malik and S. N. Joardar (2013). Detection of Group A Rotavirus in Faeces of Diarrhoeic Bovine Porcine and Human Population from Eastern India by Reverse Transcriptase–Polymerase Chain Reaction. Adv. in Ani. and Vet. Sci. 1 (1S):18 – 19.

Moon, W. H (1974). Pathogenesis of enteric diseases caused by *Escherichia coli*. Adv Vet Sci Comp Med. 18: 179-211.

Mukhtar, N., T. Yaqub, A. Masood, H. Javed, J. Nazir, A. Aslam, A. Ali, M. Javed, A. Nadeem, T. Hussain, Z. Tahir and H. B. Aslam (2016) Molecular characterization of bovine rotaviruses in Pakistan, Jundishapur. J. Microbiol. 9 (12): e41001

Mulik, S. N (2006). Characterization of *E. coli* isolates from calves and poultry with reference to some of the virulence associated attributes. M.V.Sc. Thesis, submitted to Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar.

Murphy, F. A., E. P. J. Gibbs, M. C. Horzinek and M. J. Studdert (1999) Reoviridae and its classification. 3rd ed., Academic Press, USA, 391-404.

Nataro, J. P and J. B. Kaper (1998). Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. 11: 142-201.

Nayak, J. B., J. H. Chaudhary, M. N. Brahmbhatt, P. D. Rabari, S. A. Patel and D. M. Paghdar (2018) Group A rotavirus detection from diarrhoeal samples from buffalo calves and human beings by ELISA and RT-PCR. J. of Ani. Res. 8 (6): 1005-1008.

Odonkor, S. T and K. K. Addo (2018) Prevalence of Multidrug- Resistant *Escherichia coli* isolated from drinking water sources. Inter. J. Microbiol. 2018: 7204013, 7 pages

Oliveira, M. G., J. R. Brito, R. R. Carvalho, B. E. Guth, T. A. Gomes, M. A. Vieira, A. M. F. Maria, Kato, M. I. Kato Taˆnia, Vaz and Kinue Irinoet (2008). Water buffaloes (*Bubalus bubalis*) identified as an important reservoir of Shiga toxin-producing *Escherichia coli* in Brazil. App. and Environ. Microbiol. 73(18): 5945-5948

Osman, K.M., A. M. Mustafa, M. Elhariri and G. S. Abdelhamed (2013). The Distribution of *Escherichia coli* Serovars, Virulence Genes, Gene

Association and Combinations and Virulence Genes Encoding Serotypes in Pathogenic *E. coli* recovered from Diarrheic Calves, Sheep and Goat. J. Transbound. Emerg. Dis. 60: 69-78.

Paesi, S., C. E. Rosa, P. R. M. Dornelles, F. Luz, C. A. Schenkel and D. Zampieri (2012) Evaluation of a Latex Agglutination Kit for Detecting Rotavirus in Piglets. *Acta Scientiae Veterinariae*, 40(1): 1014.

Park, S. J., C. Jeong, S. S. Yoon, E. Hyoun, Choy, L.J. Saif, S. H. Park, Y. J. Kim, J. H. Jeong, S. I. Park, H. H. Kim, B. J. Lee, H. S. Cho, S. K. Kim, M. Kang and K. O. Cho (2006). Detection and Characterization of Bovine Coronaviruses in Fecal Specimens of adult Cattle with Diarrhea during the Warmer Seasons. J. Clin. Microbiol. 44: 3178-3188.

Park, S. J., G. Y. Kim, H. E. Choy, Y. J. Hong, L. J. Saif, J. H. Jeong, S. I. Park, H. H. Kim, S. K. Kim, S. S. Shin, M. I. Kang and K. O. Cho (2007). Dual enteric and respiratory tropisms of winter dysentery bovine coronavirus in calves. Arch. Virol. 152:1885-900.

Park, S. T (2010) Kim trends in next generation sequencing and a new era for whole genome sequencing. J. Biores. 5: 76-83

Patel, B. B (2009). Isolation and identification of *Escherichia coli* with special reference to colicinogeny, toxin detection and molecular characterization. M.V.Sc. Thesis, submitted to Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar.

Paul, S. K., M. S. R. Khan, M. A. Rashid, J. Hassan and S. M. S. Mahmud (2010) Isolation and Characterization of *Escherichia Coli* From Buffalo Calves in Some Selected Areas of Bangladesh Bangl. J. Vet. Med. 8(1): 23 – 26

Payne, H. R., J. Storz, and W. G. Henk (1990). Initial events in bovine coronavirus infection: analysis through immunogold probes and lysosomotropic inhibitors. Arch. Virol. 114:175-89.

Prasad, M., K. Ranjan, N. Pandey, S. Dahiya, S. Khurana, N. Bhardwaj, S. Bhardwaj, Gaya Prasad (2015) Predominance of G10 genotype of rotavirus in diarrhoeic buffalo calves: A potential threat for animal to human zoonotic transmission. Adv. in Ani. and Vet. Sci. (1):16

Rao, C. D., K. Gowda, and B. S. Reddy (2000). Sequence analysis of VP4 and VP7 genes of non-typeable strains identifies a new pair of outer capsid proteins representing novel P and G genotypes in bovine rotaviruses. Virology 276:104-13.

Saeki, K., N. Ohtsuka and F. Taguchi (1997). Identification of spike protein residues of murine coronavirus responsible for receptor-binding activity by use of soluble receptor-resistant mutants. J Virol. 71: 9024-31.

Saif, L.J (1990). A review of evidence of implicating bovine coronavirus in the etiology of winter dysentry in cows: an enigma resolved? Cornell Vet. 80: 303-311.

Saif, L.J (1993). Coronavirus immunogens. Vet. Microbiol. 37: 285-297.

Saif, L.J (2004). Bovine Coronavirus Infections. In: Coetzer, J.A.W., *et al.* (Eds.) Infectious Diseases of Livestock. Oxford, England: Oxford Univ. Press. 23: 643-660

Saif, L. J., K. V. Brock, D. R. Redman and E. M. Kohler (1991). Winter dysentery in dairy herds: Electron microscopic and serological evidence for an association with coronavirus infection. Vet Rec. 128: 447–449.

Salvadori, M. R., G. F. Valadares, D. S. Leite, J. Blanco and T. Yano (2003). Virulence factors of *Escherichia coli* isolated from calves with diarrhoea in Brazil. Braz. J. Microbiol. 34: 230- 235.

Sambrook, J and D. W. Russel (2001) *Molecular Cloning: A Laboratory Manual*, New York: Cold Spring Harbor Laboratory Press: 6.10– 6.11.

Santhosh Kumar, S., R. B. Rai, K. Dhama, G. J. Ranganath, M. Saminathan, M. Y. Wani and R. Saravanan (2013) Detection of bovine coronavirus in calf diarrheic samples by indirect antigencapture ELISA and RT-PCR: Research Opinions in Animal & Veterinary Sciences: 225-234.

Schultze, B., H. J. Gross, R. Brossmer and G. Herrler (1991). The S protein of bovine coronavirus is a hemagglutinin recognizing 9-O-acetylated sialic acid as a receptor determinant. J. Virol. 65: 6232-7.

Sepehriseresht, S., T. Z. Salehi, M. Sattari, H. Tadjbakhsh and M. M. Aslani (2009). Detection of shiga toxigenic *Escherichia coli* from faecal samples of calves and cattle by molecular and serological methods. Comp. Clin. Pathol. 18 : 53-57.

Sharma, K., Y. S. Malik, N. Kumar, A. A. Haq, A. Kumar and M. Prasad (2012) Sequence and phylogenetic analysis of bovine rotavirus isolates (G6 genotypes) from India. Adv. Anim. Vet. Sci. 1 (1): 41-43

Sharma, B. C and B. Rai (2012) Incidence of multi-drug resistance in *Escherichia coli* strains isolated from three lakes of tourist attraction (Mirik lake, Jorepokhari lake and Nakhapani lake) of Darjeeling hills, India. Ind. J. of Fund. and App. Life Sci. 2, 108–114.

Sharma, S. K (2013) Epidemiological, clinical and hematobiochemical characterization of calf diarrhea and evolution of therapeutic regimens. Ph.D

Thesis submitted to Rajastan University of Veterinary and Animal Sciences, Bikaner.

Singh T. R (2009). Electropherotyping, RT-PCR detection and G-typing of bovine rotavirus from diarrhoeic calves. M.V.Sc. Thesis submitted to the Gujarat Agricultural University, Anand Campus, Anand.

Singla, L.D., M. P. Gupta, H. Singh, S. T. Singh, P. Kaur and P. D. Juyal (2013). Antigen based diagnosis of *Cryptosporidium parvum* infection in cattle and buffalo faeces. Ind. J. Anim. Sci. 83: 37-39.

Smith, G. W (2009). Treatment of calf diarrhoea: oral fluid therapy. Vet. Clin. North Am. Food Anim. Pract. 25: 55-72.

Snodgrass, D. R., H. R. Terzolo and D. Sherwood (1986) Etiology of diarrhoea in young calves. Vet. Rec. 119:31–34.

Sravani, G. V. D., G. Kaur, M. Chandra, P. N. Dwivedi (2014) P and G genotyping of bovine rotavirus and its prevalence in neonatal calves, Vet. Arhiv. 84, 475-484

Srivani, M., Y. Narasimha Reddy, K. V. Subramanyam, M. Ramakoti Reddy and T. Srinivasa Rao (2016) Prevalence and antimicrobial resistance pattern of Shiga toxigenic *Escherichia coli* in diarrheic buffalo calves, Veterinary World, EISSN: 2231-0916

Stanton, A. L., D. F. Kelton and S. J. LeBlanc (2013) Effects of tulathromycin on incidence of various diseases and growth of young heifers. J. Am. Vet. Med. Assoc. 20; 243:267–276.

Steele, A., A. Geyer and G. Gerdes (2004). Rotavirus infections. In:coetzer, J.A.W. and Tustin, R.C(eds). Infectious diseases of Livestock, Oxford University Press, Southern Africa: 1256-1264.

Storz, J., C. W. Purdy and X. Lin (2000) Isolation of respiratory bovine coronavirus, other cytocidal viruses, and *Pasteurella* spp from cattle involved in two natural outbreaks of shipping fever. J. Am. Vet. Med. Assoc. 216: 1599–1604.

Sunabe, T and Y. Honma (1998). Relationship between O-serogroup and presence of pathogenic factor genes in *Escherichia coli*. Microbiol. Immunol. 42 : 845–849.

Tahamtan, Y., M. Hayati and M. M. Namavari (2010) Prevalence and distribution of the *stx1*, *stx2* genes in Shiga toxin producing *E. coli* (STEC) isolates from cattle Iran. J. Microbiol. 2 (1): 8-13

Takiuchi, E., T. S. Danilo, A. F. Alferi and A. A. Alferi (2005). Improved detection of bovine coronavirus N gene in feces of calves infected naturally by a semi-nested PCR assay and an interal control. J. of Virological Methods.131: 148-154.

Tambekar, D. H., S. J. Wankahde, S. D. Yadav and S. D. Tambekar (2008) Correlation of antibiotics resistance profiling of *E. coli* and source of faecal pollution in water, Pollution Research, 27, 507-510.

Thais, N. S., Medeiros, E. Lorenzetti, F. Alice, Alferi and A. Amauri (2011). Severe diarrhea outbreak in beef calves (Bos indicus) caused by G6P[11], an emergent genotype of bovine rotavirus group A, Pesq. Vet. Bras. 34(8): 717-722.

Tripathi, R. D and J. L. Soni (1984). Enteropathogenic *E. coli* in neonatal calf diarrhea in crossbred calves. Ind. Vet. J. 61: 4-8.

Tsunemitsu, H., B. Jiang and L. J. Saif (1992). Detection of group C rotavirus antigens and antibodies in animals and humans by enzyme-linked immunosorbent assays. J. Clin. Microbiol. 30:2129-34.

Tumlam, U. M (2018) Molecular detection of rotavirus from different species. Ph. D thesis, MAFSU, Nagpur.

Tumlam, U. M., V. C. Ingle, P. A. Tembhurne, N. V. Kurkure, S. P. Chaudhari, S. D. Chitambar and S. Bhoyar (2018) Detection of VP6 gene of Rotavirus in Feces of Diarrhoeic calves, kids, lambs, piglets, pups and human infants by Reverse Transcriptase–Polymerase Chain Reaction. The Ind. J. of Vet. Sci. & Biotech. 13 (4): 12-16.

Udaykar, A., R. Sharda, Y. S. Malik, V. A. Sharma, N. Shrivastava (2013) Occurrence of Group a Rotavirus in Diarrhoeic Buffalo and Cow Calves, Madhya Pradesh, India Adv.Anim.Vet.Sci.1 (4S): 51-53.

Van Regenmortel, M. H. V., C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle and R. B. Wickner (2000). Virus Taxonomy: Classification and Nomenclature of Viruses. Academic Press, San Diago, CA., USA., ISBN-13: 9780127141817, P: 1162.

Verdier, D. K., C. G. Ann Nyman and B. Björn B (2012). Antimicrobial resistance and virulence factors in *Escherichia coli* from Swedish dairy calves. *Acta Veterinaria Scandinavica*, 54: 2

Vu-Khac, H and N. A. Cornick (2008). Prevalence and genetic profiles of Shiga toxin- producing *Escherichia coli* strains isolated from buffaloes, cattle, and goats in central Vietnam. Vet. Microbiol. 126 (4): 356–363

Wani, S. A., M. A. Bhat, I. Samanta, Y. Nishikawa and A. S. Buchh (2003) Isolation and characterization of shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic *Escherichia coli* (EPEC) from calves and lambs with diarrhea in India. Lett. Appl. Microbiol. (37): 121-126.

Yilmaz, V (2016) Investigation of Rotavirus Infection in Calves with Diarrhea in Northeast Turkey. Ani. and Vet. Sci. 4 (1): 1-4. doi: 10.11648/ j.avs.20160401.11

Yoo, D. W., M. D. Parker and L. A. Babiuk (1991). The S2 subunit of the spike glycoprotein of bovine coronavirus mediates membrane fusion in insect cells. Virology 180:395-9.

Yousif, A. Y., J. Anderson, C. Bergstrom, A. Bustamante, M. Muenzenberger, K. Austin and S. Kapil (2001). Evolution of a latex agglutination kit (virogen Rotatest) for detection of bovine Rotavirus in faecal. Clin. Diag. Lab. Immunol. 8 (3)**:** 496-498.

Zweifel, C., S. Schumacker, M. Blanco, J. E. Blanco, T. Tasara, J. Blanco and R. Stephen (2005). Phenotypic and genotypic characteristics of non-O157 Shiga toxin-producing *E. coli* (STEC) from Swiss Cattle. Vet. Microbiol. 105: 37-45.

# APPENDIX-I

1. **Ethidium bromide (10 mg/ml)**

| | |
|---|---|
| Ethidium bromide | 10 mg |
| Distilled water | 1 ml |

2. **Tris borate EDTA (TBE) buffer (5x stock solution)**

| | |
|---|---|
| Tris base | 54 g |
| Boric acid | 27.5 g |
| 0.5 M EDTA (pH 8.0) | 20 ml |
| Distilled water | 1000 ml |

Store at room temperature

The 5x concentrated stock solution wsa diluted to 1x before use.

3. **Gel loading dye**

| | |
|---|---|
| Bromophenol blue | 0.25% w/v in DW |
| Sucrose in water | 40% w/v in DW |
| Solution was stored at | $4^0$C |

4. **Phosphate Buffered Saline (PBS)- 1000 ml (0.06 M, pH 7.3)**

| | |
|---|---|
| Sodium chloride (NaCl) | 8.0 g |
| Potassium chloride (KCl) | 0.2 g |
| Disodium hydrogen phosphate ($Na_2HPO_4$. $2H_2O$) | 1.15 g |
| Potassium hydrogen phosphate anhydrous ($KH_2PO_4$) | 0.24 g |

Ingredients were dissolved in 800 ml of double distilled water and pH was adjusted to 7.3 with concentrated HCl. The remaining amount of nuclease free water was added to make the final volume to 1000 ml. This was then autoclaved at 15 lbs pressure, $121^0$C temperature for 15 minutes. The prepared buffer was stored at $4^0$C.

## APPENDIX-II

1. **Eosin methylene Blue (EMB) Agar (Dehydrated, Himedia)**

| Ingredients | Grams/ liter |
|---|---|
| Peptone | 10.00 |
| Lactose | 10.00 |
| Dipotassium hydrogen phosphate | 2.00 |
| Eosin yellow | 4.00 |
| Methylene blue | 0.065 |
| Agar | 25.00 |
| Final pH (at $25^0$C) | 7.2±0.2 |

Suspended 36 g of dehydrated EMB in 1000 ml distilled water and sterilized by autoclaving at 15 lbs pressure, $121^0$ C temperature for 15 minutes. The molten medium was cooled to about $50^0$ C and poured into petri plates under sterilized conditions.

2. **Muller Hinton (MH) Agar (Dehydrated, Himedia)**

| Ingredients | Grams/liter |
|---|---|
| Casein acid hydrolysate | 17.50 |
| Beef heart infusion | 2.00 |
| Starch, soluble | 1.5 |
| Agar | 17.0 |
| Final pH (at $25^0$C) | 7.3±0.2 |

Suspended 38 g in 1000 ml distilled water. Sterilized by autoclaving at 15 lbs pressure, $121^0$ C temperature for 15 minutes. The molten medium was cooled to about $50^0$ C and poured into petri plates under sterilized conditions.

3. **Mac Conkey Agar (MCA) (Dehydrated, Himedia)**

| Ingredients | Grams/liter |
|---|---|
| Peptic digest of animal tissue | 20.00 |

| | |
|---|---|
| Lactose | 10.00 |
| Bile salt | 5.00 |
| Sodium chloride | 5.00 |
| Neutral red | 0.07 |
| Agar | 15.00 |
| Final pH (at $25^0$C) | 7.5±0.2 |

Suspended 55.07 g in 1000 ml distilled water. Sterilized by autoclaving at 15 lbs pressure, $121^0$ C temperature for 15 minutes. The molten medium was cooled to about $50^0$ C and poured into petri plates under sterilized conditions.

4. **Nutrient Agar**

| **Ingredients** | **Grams/liter** |
|---|---|
| Peptic digest of animal tissue | 5.00 |
| Beef extract | 1.00 |
| Yeast extract | 2.00 |
| Sodium chloride | 5.00 |
| Agar | 15.00 |
| Final pH (at $25^0$C) | 7.5±0.2 |

Suspended 28 g in 1000 ml distilled water. Sterilized by autoclaving at 15 lbs pressure, $121^0$ C temperature for 15 minutes. The molten medium was cooled to about $50^0$ C and poured into petri plates under sterilized conditions.

5. **Triple Sugar Iron Agar**

| **Ingredients** | **Grams/liter** |
|---|---|
| Peptic digest of animal tissue | 10.00 |
| Yeast extract | 3.00 |
| Beef extract | 3.00 |
| Lactose | 10.00 |
| Sucrose | 10.00 |
| Dextrose | 1.00 |

| | |
|---|---|
| Sodium chloride | 5.00 |
| Ferrous sulphate | 0.2 |
| Sodium thiosulphate | 0.3 |
| Phenol red | 0.024 |
| Agar | 12.00 |
| Final pH (at $25^0$C) | 7.4±0.2 |

Suspended 64.52 g in 1000 ml distilled water. Sterilized by autoclaving at 15 lbs pressure, $121^0$ C temperature for 15 minutes. The molten medium was cooled to about $50^0$ C and poured into test tubes under sterilized conditions in such a way that the medium was set in a slant form with a butt about 1 inch long.

6. **Brain Heart Infusion Broth (BHI) (Dehydrated, Himedia)**

| **Ingredients** | **Grams/liter** |
|---|---|
| Peptic digest of animal tissue | 10.00 |
| Calf brain infusion (solids) | 12.50 |
| Beef heart infusion (solids) | 5.00 |
| Sodium chloride | 5.00 |
| Dextrose | 2.00 |
| Disodium phosphate | 15.00 |
| Final pH (at $25^0$C) | 7.4±0.2 |

Suspended 37 g in 1000 ml distilled water and distributed in to test tubes. Sterilized by autoclaving at 15 lbs pressure, $121^0$C temperature for 15 minutes.

7. **Nutrient Broth**

| **Ingredients** | **Grams/liter** |
|---|---|
| Peptone | 5.0 |
| Sodium chloride | 5.0 |
| Yeast extract | 2.0 |
| Beef extract | 1.0 |

Suspended 13 g in 1000 ml distilled water and distributed in to test tubes. Sterilized by autoclaving at 15 lbs pressure, $121^0$C temperature for 15 minutes.

**8. Indole test**

a) Peptone water

| | |
|---|---|
| Peptone | 10 g |
| Sodium chloride | 5 g |
| Distilled water | 1000 ml |

Dissolve and adjust the pH to 7.5. Sterilize by autoclaving at 15 lbs pressure, $121^0$C temperature for 15 minutes.

b) Kovac's reagent

| | |
|---|---|
| Paradimethylaminobenzaldehyde | 50 g |
| Pure amyl or isoamyl alcohol | 75 ml |
| Concentrated pure hydrochloric acid | 25 ml |

Dissolve the aldehyde in alcohol by gentle warming in water bath, cool and add the acid. Protect it from light and store at $4^0$C

**9. Methyl Red test**

a) Medium (Glucose Phosphate Broth)

| | |
|---|---|
| Peptone | 5 g |
| Dipotassium hydrogen phosphate | 5 g |
| Distilled water | 1000 ml |
| 10% Glucose solution (sterilized separately) | 50 ml |

Dissolve the peptone and phosphate, adjust the pH to 7.6. Filter, dispense in 5 ml in test tube and sterilize at 15 lbs pressure, $121^0$C for 15 minutes. Sterilize the glucose solution by filtration and dispense 0.25 ml to each test tube (final concentration 0.5%)

b) Methyl Red Indicator Solution

| | |
|---|---|
| Methyl red | 0.1 g |
| Ethanol | 300 ml |
| Distilled water | 200 ml |

**10. Voges- Proskauer Test**

a) Glucose Phosphate Broth

| | |
|---|---|
| Peptone | 5 g |
| Dipotassium hydrogen phosphate | 5 g |
| Distilled water | 1000 ml |
| 10% Glucose solution (sterilized separately) | 50 ml |

Dissolve the peptone and phosphate, adjust the pH to 7.6. Filter, dispense in 5 ml in test tube and sterilize at 15 lbs pressure, $121^0$C for 15 minutes. Sterilize the glucose solution by filtration and dispense 0.25 ml to each test tube (final concentration 0.5%)

b) 40% Potassium Hydroxide solution

| | |
|---|---|
| Potassium Hydroxide | 40 g |
| Distilled water | 100 ml |

c) 5% α-Naphthol solution

| | |
|---|---|
| α-Naphthol | 5.0 g |
| Absolute alcohol | 100 ml |

**11. Citrate Utilization Test**

| **Ingredients** | **Grams/liter** |
|---|---|
| Sodium ammonium phosphate | 1.5 |
| Monopotassium phosphate | 1.0 |
| Magnesium sulphate | 0.2 |
| Sodium citrate | 3.0 |
| Final pH (at $25^0$C) | 6.7±0.2 |

Suspended 5.7 grams in 1000 ml distilled water. Dispensed into tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

**Gram's staining**

1. **Crystal violet**

   **Solution 1:**

   | | |
   |---|---|
   | Crystal violet | 2.0 g |
   | Ethyl alcohol (95%) | 20 ml |

   **Solution 2:**

   | | |
   |---|---|
   | Ammonium oxalate | 0.8 g |
   | Distilled water | 80 ml |

   Solution 1 and 2 were mixed well and then filtered.

2. **Gram's iodine**

   | | |
   |---|---|
   | Iodine | 1.0g |
   | Potassium iodide | 2.0 g |
   | Distilled water | 100 ml |

   The ingredients were dissolved and then filtered.

3. **Ethyl alcohol (95%)**
4. **Safranin (counter stain)**

   | | |
   |---|---|
   | Safranin (2.5% solution in 95% alcohol) | 10 ml |
   | Distilled water | 100 ml |

# VITA

The author Sruthy S was born on 19th February 1992 in Alleppey district of Kerala. She completed her S.S.L.C Examination in the year 2008 from V.S.S.H.S, Koippallikaranma, Kerala with 95% marks and Higher Secondary Examination in the year 2010 from G.G.H.S.S, Mavelikara, Kerala with 89% marks.

The author has successfully completed B.V.Sc and A.H degree from College of Veterinary and Animal Sciences, Mannuthy, Thrissur in the year 2017 with 82% marks.

She joined Nagpur Veterinary College, Nagpur for Post-Graduation (M.V.Sc) in the discipline of Veterinary Microbiology in the year 2017.

## Thesis Abstract

| | | | |
|---|---|---|---|
| a) | Title of thesis | : | **MOLECULAR DETECTION OF CAUSATIVE AGENTS ASSOCIATED WITH DIARRHOEA IN CALVES.** |
| b) | Full Name of Student | : | **SRUTHY S** |
| c) | Name & Address of Advisor/ Guide | : | **Dr. U. M. TUMLAM**<br>**Assistant Professor**<br>**Department of Veterinary Microbiology and Animal Biotechnology, T & R Cell, Nagpur Veterinary College, Nagpur** |
| d) | Degree to be awarded | : | **MASTER OF VETERINARY SCIENCE** |
| e) | Year of award of degree | : | **2019** |
| f) | Major subject | : | **VETERINARY MICROBIOLOGY** |
| g) | Total number of pages in the thesis | : | **65** |
| h) | Number of words in the thesis abstract | : | **282** |
| i) | Signature of student | : | |
| j) | Signature, Name & Address of forwarding authority | : | |

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## ABSTRACT

Calf diarrhea is a commonly reported disease of calves and is a major cause for neonatal mortality. Many agents are responsible for calf diarrhea associated with mortality. It includes viruses like bovine rotavirus and

coronavirus; protozoa like *Cryptosporidium* spp; bacteria like *E.coli*, *Salmonella*, etc. Bovine rotavirus is primarily and foremost, secondly bovine coronavirus, then *Escherichia coli* is important pathogenic organisms responsible for 50% cases of neonatal diarrhea.

In the present study, 54 fecal samples collected from diarrheic calves of 0-3 months of age were screened for the presence of bovine rotavirus and coronavirus by Latex Agglutination Test and Lateral Flow Test, respectively. Out of 54 samples 11 and 1 were positive for bovine rotavirus and coronavirus, respectively. All 11 positive samples were confirmed for the presence of bovine rotavirus whereas none of the samples were positive for bovine coronavirus by RT-PCR.

All the 54 samples were also processed for the isolation and identification of predominant bacteria. 41 out of 54 diarrhoeic samples were positive for *E.coli* and this was carried out by morphological, biochemical and cultural characterization. The *E.coli* isolates were highly sensitive to Gentamicin (82.92%) and Ampicillin-Sulbactam (78.08%), whereas complete resistance was observed with Metronidazole (100%).

The *E.coli* isolates were subjected to molecular characterization for the detection of *stx* 1 and *stx* 2 genes. Out of 41 *E.coli* isolates, 10 and 13 were positive for *stx* 1 and *stx* 2 genes respectively. Sequencing and phylogenetic analysis of VP6 RT-PCR positive samples showed 100% homology with already reported sequence from Maharashtra and 80% identity with human rotavirus VP6 protein. Similarly, sequencing of *stx* 2 gene showed analogy with already reported sequence like strain TS07/07 and c466-01B strain of world and Indian isolates.

## प्रबंध सारांश

| | | | |
|---|---|---|---|
| अ. | प्रबंधाचे शिर्षक | : | वासारांतील अतिसाराशी निगडीत प्रयोजक घटकाचे सुक्ष्मरेवणीय तपासणी |
| ब. | विद्यार्थाचे पुर्ण नांव | : | श्रुती. एस |
| क. | मार्गदर्शकाचे नांव व पत्ता | : | डॉ. यु. एम. तुमलाम<br>सहायक प्राध्यापक<br>पशुवैद्यकीय सुक्ष्मजीवशास्त्र आणि पशु जैव तंत्रज्ञान विभाग, टी आणि आर सेल, नागपूर पशुवैद्यकीय महाविद्यालय, नागपूर |
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| इ. | पदवी प्रदान करण्याचे वर्ष | : | 2019 |
| फ. | मुख्य विषय | : | पशुवैद्यकीय सुक्ष्मजीवशास्त्र |
| ग. | प्रबंधातील एकूण पृष्ठ | : | **६५** |
| ह. | सरांशातील एकूण शब्द | : | **२१३** |
| ई. | विद्यार्थाची सही | : | |
| ज. | अग्रेषित करणाऱ्या अधिकाऱ्याची सही, नांव आणि पत्ता | : | |

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## सारांश

जनावरांतील नवजात वासरांत मृत्युदर होण्यासाठी वासरातील अतिसारांशी निगडीत प्रयोजक घटक जबाबदार असतात व नवजात वासरातील अतिसार हा

मृत्युदराकरिता प्रमुख्याने कारणीभुत आहे. यात गाईचा शेटा विषाणु, कोराना विषाणु, क्रिप्टोस्पोरिडियम सारख्या प्रोटोझोआ, ई.कोली, साल्मोनेला इत्यादी सारखे जिवाणु यांचा समावेश आहे.

गाईचा रोटाविषाणु सर्वात महत्वाचा आणि प्रामुख्याचा असुन गाईचा कोरानाविषाणु, इश्लेस्चिया कोलाई हा जिवाणु सुद्धा प्रामुख्याने 50 टक्के नवजात वासरांमध्ये अतिसाराकरिता जबाबदार आहे.

सदर अभ्यासामध्ये 0-3 महिने या वयोगटातील अतिसार असलेल्या वासरांचे नमुने प्राथमिक चाचणीद्वारे लॅटेक्स अॅग्लुटिनेशन चाचणी (लॅट) व जलद प्रतिजन तपासणी चाचणी द्वारे 54 नमुण्यांपैकी 11 रोटाविषाणुकरिता व 1 कोरानाविषाणु करिता सकारात्मक आढळले.

तसेच 54 नमुन्यांपैकी 11 नमुने जनुकांच्या आरटीपीसीआर श्रृखंलेद्वारे रोटाविषाणुकरिता सकारात्मक असलेले असुन एकही नमुना कोरानाविषाणु करिता सकारात्मक आढळले नाही.

सर्व 54 नमुणे प्रमुख जिवाणुंच्या वैशिष्टये, आकारिकीय, जिवरसायन प्रक्रिया विलगीकरणाकरिता करण्यात आले. अतिसार असलेल्या 54 नमुन्यापैकी 41 नमुने ई.कोली करिता सकारात्मक आढळते. 54 नमुण्यांची जीवानुंची प्रतिजैविक संवेदन शिलता चाचणी केली असता सर्वात जास्त संवंदनशीलता जेनटामायसीन (82.92 टक्के) व एमपीसीलीन-सलबॅक्टम (78.08 टक्के) या प्रतिजैविकांना तर मेट्रोनीडेझोल ला (100 टक्के) प्रतिरोधक दर्शविला ई.कोली चे सुक्ष्मरेणीय तपासणी stx-1 व stx-2 जनुकेद्वारे केली असता 41 पैकी 10 नमुने stx-1 आणि 13 नमुने stx-2 जनुकेकरिता सकारात्मक आढळली.

व्हीपी 6 जनुकेच्या वंशावळी विश्लेषणाद्वारे महाराष्ट्रातील 100 टक्के आणि 80 टक्के माणसांची व्हीपी 6 जनुकेसोबत साम्यता दर्शविली. तसेच stx-2 जनुके भारतातील व जगभरातील स्ट्रेन TS071007 आणि C466-01B सोबत अनुरूप आढळली.