INVESTIGATIONS ON COMMON PATHOGENS OF NEONATAL DIARRHOEA AND ASSESSEMENT OF PASSIVE TRANSFER OF IMMUNITY IN BUFFALO CALVES

By
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CERTIFICATE - I

This is to certify that the dissertation entitled "Investigations on Common Pathogens of Neonatal Diaorrhea and assessement of Passive transfer of Immunity in Buffalo Calves" to the Lala Lajpat Rai University of Veterinary and Animal sciences, Hisar, is a bonafide research work carried out by Ismaila Alhaji Mairiga, under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE - II

This is to certify that the dissertation entitled"Investigations on Common Pathogens of Neonatal Diaorrhea and assessement of Passive transfer of Immunity in Buffalo Calves" submitted by Ismaila Alhaji Mairiga to the Lala Lajpat Rai University of Veterinary & Animal Sciences, Hisar, in partial fulfillment of the requirement for the degree of Ph.D. in Veterinary sciences in the subject of Veterinary Medicine has been approved by the Student's Advisory Committee after an oral examination of the same.

MAJOR ADVISOR **EXTERNAL EXAMINER**

HEAD OF THE DEPARTMENT

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ABBREVIATIONS

ACRONYMS		MEANING
"	-	Inches
#	-	Number
%	-	Percentage
@	-	At the rate of
<	-	Less than
>	-	Greater than
±	-	Plus minus
≤	-	Less than equal to
≥	-	Greater than equal to
°C	-	Degree Celsius
∞ toxin	-	Epsilon toxin
α	-	Alpha
α-toxin	-	Alpha toxin
A/E	-	Attaching and effacing lesion
A260	-	Absorbance at 260 nm wavelength
A280	-	Absorbance at 280 nm wavelength
AEEC	-	Attaching and effacing *Escherichia coli*
$AgNO_3$	-	Silver nitrate
Approx.	-	Approximately
APS	-	Ammonium per-sulphate
BCA	-	Bicinchoninic acid
BCV	-	Bovine Coronavirus
bp	-	Base pair
BSA	-	Bovine Serum Albumin
cAMP	-	cyclic Adenosine Mono Phosphate
CARD15	-	Carpass activation and recruitment domain 15
cDNA	-	Complementary DNA Chain Reaction
D	-	Diarrhoeic
DMEM	-	Dulbecco's Modified Eagle Medium
DNA	-	Deoxyribo Nucleic Acid
DNase	-	Deoxyribonuclease
dNTP	-	23'-dideoxyribonucleoside 5 '-triphosphate
dNTPs	-	deoxy Nucleoside TriPhosphates
E. coli	-	*Escherichia coli*
e.g.	-	For example (abbr. from *exempli gratia)*

eaeA	-	Attaching and effacing gene
EDTA	-	Ethylene diamine tetra acetic acid
EHEC	-	Enterohaemorrhagic *Escherichia coli*
EIEC	-	Enteroinvasive *Escherichia coli*
ELISA	-	Enzyme-linked immunosorbent assay
EPEC	-	Enteropathogenic *Escherichia coli*
et al.	-	And others (abbr. from *et alii)*
ETEC	-	Enterotoxigenic *Escherichia coli*
FBS	-	Fetal Bovine Serum
Fig	-	Figure
g	-	Gram
h(s)	-	Hour(s)
IgG	-	Immunoglobulin G
I. Toxin	-	Iota toxin
k	-	Kappa
KCl	-	Potassium Chloride
kDa	-	Kilo Dalton
L	-	Liter
LPS	-	Lipopolysaccharide
LRR	-	Leucine Rich Repeats
LT	-	Heat labile
LTA	-	Lipoteichoic acid
M	-	Molar
MAb	-	Monoclonal antibody
MAMP	-	Microbial Associated Molecular Patterns
MEM	-	Minimum essential medium
mg	-	Milligram
mg/ml	-	milligram per milliliter
MgCl2	-	Magnesium Chloride
MHCII	-	Major Histocompatibility Complex II
min	-	Minutes
Mix	-	Mixture
mM	-	Mili Molar
mol	-	Mole
mRNA	-	Messenger RNA
No	-	Number
NaCl	-	Sodium Chloride
NaOH	-	Sodium Hydroxide
NF-kβ	-	Nuclear factor-kappaβ

ng	-	Nanogram
NLR	-	NOD- like Receptor
NOD2	-	Nucleotide and oligomerization domain 2
O.D.	-	Optical density
OIE	-	World animal health organization (*Office International des*
PAGE	-	Polyacrylamide gel electrophoresis
PAMP	-	Pathogen Associated Molecular Patterns
PB	-	Phosphate buffer
PBS	-	Phosphate buffer saline
PCR	-	Polymerase Chain Reaction
PGN	-	Peptidoglycan
pH	-	Negative logarithm of hydrogen ion concentration
Pic	-	Picture
ρmol	-	Picomolar
PRRs	-	Pattern recognition receptors
qPCR	-	quantitative Reverse Transcription-Polymerase
RNA	-	Ribonucleic acid
rpm	-	revolutions per minute
rRNA	-	Ribosomal RNA
RT	-	Reverse Transcriptase
RT-PCR	-	Reverse Transcription-Polymerase Chain Reaction
SDS	-	Sodium dodecyl sulphate
SDS-PAGE	-	Sodium Dodecyl Sulfate-Polyacrylamide Gel
sec	-	seconds
sp	-	Species
ß toxin	-	Beta toxin
ST	-	Heat stable
STEC	-	Shiga toxin Producing *Escherichia coli*
Tab	-	Table
TBST	-	Tris-Base Saline Tween-20
TEMED	-	N, N, N', N'–tetramethylenediamine
TGDW	-	Triple glass distilled water
TLR	-	Toll-Like Receptor
TLR4	-	Toll-Like receptor 4
Tm	-	Melting Temperature
TNF	-	Tumor Necrosis Factor-alpha
U	-	Units
U.V.	-	Ultra violet

U/µL	-	Units per microliter
ug	-	Microgram
ul	-	microliter
uM	-	micromolar
USA	-	United States of America
UV	-	Ultraviolet
x g	-	Relative centrifugal force
β	-	Beta
γ	-	Gamma
µg	-	Microgram
µg/µl	-	Microgram per microliter
µl	-	Microlitre
µM	-	Micro Molar

CHAPTER-I

INTRODUCTION

The domestic water buffalo (*Bubalus bubalis*) contributes to agriculture economically in many developing countries in Asia, as it provides many benefits such as milk, meat and draught power. It is also used in some Mediterranean and Latin American countries as a source of milk and meat for specialized markets. Ninety six percent (96%) of the total buffalo population is present in Asia (Palta and Madan, 1996). In India, the water buffalo (*Bubalus bubalis*) contributes a major share (more than half) in the total milk production although their population is just 1/3rd of the total cattle population. The buffalo can adapt to harsh environments and can live on poor quality forage.

India is host to 47 distinct breeds of indigenous cattle and 7 of buffaloes, including the Murrah. India is ranked the first in milk production globally (102 MT, Economic Survey 2007-2008) and possessed 98 million out of the 171 millions of world buffalo population. Murrah is the best breed among buffaloes and produces 2000 liters of milk in 305 days. However, due to increased prevalence of infections, realization of their true genetic merit has been hampered. Among these diseases, calf diarrhoea caused by infections with *Escherichia coli,* S*almonella* spp, *Cryptosporidium* sp, Rota and Corona viruses is the most common, costly and devastating disease in dairy animals causing losses of crores of rupees annually.

Murrah is one of the most premium breeds of water buffalo found in Haryana State. Buffalo dam transmits not only genes to its calf but also environmental features in the form of protection through colostrum and maternally transmitted diseases. Dams also contribute nutritional benefits to calves in the form of milk. Cryopreservation of semen is an important contribution for conservation of variety of genotypes amongst the breeds so that the diversity of the breed in its tract of origin is maintained. The danger of losing diversity could be through indiscriminate breeding policy of artificial insemination which may result into 'Inbreeding Depression' and may also result into 'loss of resistance alleles in the otherwise outbreeding population of the breed in its tract of origin. This kind of loss of resistant genes or reduction in their frequency of occurrence could have direct consequences in reducing the potentials of innate and adpative immunity. This may become one of many important

factors leading to increase in the incidence of infectious diseases and syndromes such as respiratory diseases and diarrhoea (Svensson *et al*. 2006).

Diarrhoea is the most commonly reported calf disease and a major cause of calf morbidity and mortality worldwide (Gitau *et al*. 1994; Bendali *et al.* 1999; Bazeley 2003; Svensson *et al*. 2006; Millemann 2009; Marce *et al.* 2010). The average within-herd incidence of diarrhea in pre-weaning calves is around 20%, varying between 0 and 70% worldwide (Bendali *et al.* 1999). This wide gap of 0 to 70 percent in incidence may be attributed to the contributions of genotype attributes and the environmental factors associated with a calf. Genotype of a Calf has half the contribution of its genome from the mother (Dam) and half from father (Sire) where inheritance are in the form of genes of resistance (R/R), genes of tolerance (R/r) and genes of susceptibility (r/r).

However, the environment of a calf relates to optimisation of conditions for expression of the genes of resistance and a resultant optimum production by milch breeds of livestock. Other environmental factors are temperature, humidity and farm management practices which influence the expression of genes for zero percent mortality mostly attainable at an organised farm which is indicative of clear facts that calves carry best of the genes and get optimum management in the farm (Bendali *et al.* 1999).

Diarrhoea as a syndrome based on the pathogen perspective requires an interplay of multiple pathogens for a display of phenotype - genotype relationships whereas on the host perspective, this relationship is established by the role of protection provided by the immune system. The arm of innate immunity creates barriers based upon genetic potentials. If these barriers are crossed by the pathogens, adaptive immunity takes control. If adaptive immunity fails, that significe a strong display of the roles of multitude of pathogens (Svensson *et al*. 2006).

However, some species/breeds are less susceptible than others in acquisition of diseases and syndromes such as diarrhea. Resistance through genes is an attribute of innate immunity and its heritability index is about 0.3. On the contrary the heritability index of adaptive immunity is as low as less than 0.1. It is therefore ovious that a disease resistance breeding programme requires building on innate immunity for optimal results (Younis *et al.* 2009).

The most vulnerable age for animals of a breed or a species in a class Mammalia is neo-natal stage. It is in this group that innate immunity provides the

protection shield through attributes of maternal immunity and immunotherapy of colostrum.

Identification of a gene regulating resistance, tolerance and susceptibility of the host to diarrhoea caused by one or more pathogens will enable a selection of animal type for breeding purposes as resistant (RR), tolerant (Rr) or susceptible (rr).

The economic implications of calf diarrhea include calf losses, treatment costs, time costs and reduced liveweight gain (Lorenz *et al*. 2009) and, despite numerous studies worldwide, costs of calf diarrhoea remain high, compared to other diseases on-farm (Younis *et al.* 2009).

Newly born calves represent an important source of animal production as they serve as replacement heifers for meat, milk or breeding worldwide (Radostits *et al.*, 2007; Lorenz *et al.*, 2011; Özkan *et al*., 2011; Tajik *et al*., 2012).

Neonatal calf diarrhoea is a multifactorial disease, which despite decades of research in the topic the disease remains the most common cause of mortality in calves less than one month of age (Heinrichs and Radostits 2001 and Alfieri *et al*., 2006). Neonatal calf diarrhoae has a complex etiology, but bovine infections with *Escherichia coli,* S*almonella* species, *Cryptosporidium* species, Rotavirus and Coronaviruses have been found to be the most common causative agents (Gulliksen *et al.*, 2009; Izzo *et al*., 2011 and Ammar *et al*., 2014).

An association has been demonstrated between the presence of the intestinal pathogens and the presence of diarrhoea in calves, but these enteropathogens may not always necessarily cause diarrhoea (Bartels *et al*. 2010).

Mixed infections with Rotavirus and *Cryptosporidia* (Eschrig *et al.* 2004; Bartels *et al*. 2010), Rotavirus and *E.coli* (Younis *et al.* 2009) or Rotavirus and *Salmonella* are relatively common (Clark and Gill 2001). Most outbreaks of calf diarrhoea in dairy and dairy-beef operations in various studies involved multiple pathogens, with Rotavirus and *Cryptosporidium* most frequently identified (Izzo *et al*. 2011). Similarly, in Europe, the cause of diarrhoea is often complex and usually involves an interplay between enteropathogenic bacteria (Vermunt 2002). Multiple infections may also result in more severe diseases (McDougall and Cullum 1999).

Despite reliable recommendations to curb the occurrence of calf diarrhoea, the condition still remained a global threat to livestock industries. Diagnostic procedures needed innovation in reducing the painful and often, repulsive invasive procedures. Invasive procedures during sampling causes stress responses which generate animal welfare issues in veterinary practice and iconophobia in human patients and therefore

a strong quest for alternatives. Oral-fluid, urine and faeces based IgG antibody detection and quantification assays for most animal infectious disease are currently rare.

Important considerations in the design of the present study includes: Grouping of calves into 3 age categories, selection of murrah breed of buffalo and selection of buffalo dams up to 6^{th} lactation. This will enable proper investigations of phenotype/ genotype relationships as it will also help for a meaningful analysis of data (statistically and otherwise).

Although structure and function of nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) have been well studied in human and mice, little information exists on genetic composition and the role of NLRs in innate immune system of water buffalo, a species known for its exceptional disease resistance. Besides bacterial cell recognition by Toll like receptor 4 (TLR4), carspass activation and recruitment domain15/ nucleotide binding and oligomerization domain 2 (CARD15/NOD2) activation seems to play an important role in host cell activation by internalized bacterial pathogens. Downstream of NOD2 signal-transducing molecules might mediate NF-kB-dependent cell activation for inflammatory response. This knowledge of molecular interaction of bacterial pathogens with target cells may, however, pave the way to innovative therapeutic strategies.

The Present study was therefore undertaken with the following objectives:

1. To determine the common pathogens associated with calf diarrhea from meconium and faecal samples of buffalo calves and their dams.
2. To determine total immunoglobulin concentrations in the sera, oral fluid, urine and faeces of buffalo calves and from colostrums and milk of their respective dams for assessing the success of passive immunization in the calves.
3. To detect the presence of Toll-Like Receptor 4 (TLR4) and Caspase Activation and Recruitment Domain 15 (CARD15)/Nucleotide binding and Oligomerization Domain-2 (NOD2) as Pattern Recognition Receptor (PRR) for Lipopolysaccharide (LPS) and Peptidoglycans.

CHAPTER-II

REVIEW OF LITERATURE

2.1 Quantification of resistance/susceptibility to a disease/syndrome through tools of genetics and breeding

Maximum diversity of alleles for each gene locus (innate immunity) is required at tract of origin of aspecies or a breed which may be lost because of inbreeding caused by indiscriminate practices and inprovisioning of the bulls for mating as well as in arteficial insemination (AI). Breeding programs in developed countries do measure innate immunity and records of an animal for possession of resistance traits are obtained through measurement of heritability index for innate immunity. Achievement of optimum adaptive immunity is also done at these farms through 'full proof vaccination programs'. Species have evolved because of preponderance of resistant (RR) genes in their tracts of origin such as *Bos indicus* in India and this is an attribute of innate immunity."Origin of Species" is an indicative of its state of innate immune response (Younis *et al.* 2009).

The more resistant a species is the more is the chance of its survival for centuries as it was well stated in 'Origin of Species' by Charles Darwin. Natural selection of genes gets done if there are so many alleles for a single gene locus in naturally outbreeding population called random selection, otherwise, it could be 'man-made selection' which may not take care of 'Disease Resistance' as cross-breeding programmes of indigenous cattle species with *Bos taurus* germplasm may introduce alleles of susceptibility for an infectious disease (Younis *et al.* 2009).

2.2. Antecedents of *Escherichia coli*

Escherichia coli were first described by a Bavarian paediatrician, Theodor Escherich, in the late 19th century. In a series of pioneering studies of the intestinal flora of infants he described a normal microbial inhabitant of healthy individuals (Kaper, 2005).

2.2.1 Classification of *Escherichia coli*

E. coli has been classified as enteropathogenic (EPEC), enterotoxigenic (ETEC), attaching and effacing (AEEC), enteroinvasive (EIEC) and enterohaemorrhagic (EHEC) or shiga toxin producing *E. coli* (STEC). Each form of *E. coli* is associated with the production of specific enteric damage and physiological

alterations by different toxins. In calves the most common form is ETEC but STEC also play a role and are important for their impact on public health. *E. coli* strains for many years have been associated with intestinal disease in a variety of animal species (Lofstedt *et al*., 1999; Kaper, 2005).

2.2.2 Enterotoxigenic *Escherichia coli* (ETEC)

ETEC produce profuse watery diarrhoea. They are mainly a problem in calves up to 4 days old, although they can occasionally produce diarrhoea in older calves too (Naylor, 2002). *E. coli* adhere to the intestinal mucosa and produce enterotoxins. The osmotic diarrhea is due to secretory effect at the crypt cells and inhibition of absorption by villus tip cells. Several types of enterotoxins have been identified and a single ETEC, may be capable of producing one or more enterotoxins. Both heat-labile (LT I, LT n) and heat stable (STa, STb) enterotoxins have been identified in ETEC. In calves, ETEC producing the low molecular weight STa cause the majority of neonatal diarrhea problems (Naylor, 2002).

2.2.3 Incidence of *Escherichia coli* infection

Diarrhea in young calves is the main cause of economic losses through poor growth, morbidity and mortality (Constable 2004; Gaber 2004), and the role played by *E. coli* in producing diarrhea in calves has received a great attention by many researchers (Shahrani *et al*., 2014). Paul *et al*., (2010) reported that *E.coli* was isolated with an incidence of 50% of occurrence in a research conducted to determine the role of *E. coli* in calf diarrhoae. Anwarullah *et al*., (2014) isolated *E.coli* with an incidence of 14.6% during an investigation for detection of common pathogens associated with neonatal diarrhoae in Egypt. Higher incidence of 72.8% of *E.coli* infection was recorded by Majueeb *et al*., (2014) in a separate study that involved isolation of *E.coli* as one of the causes of calf diarrhoae.

The higher prevalence of *E.coli* isolation from diarrheagenic and non-diarrheagenic buffaloes calves was observed in young ages; one and two weeks. The higher prevalence of *E. coli* in these ages may be due to poor managemental practices and predisposing factors like overcrowding and malnutrition, which are supposed to be a primary cause of immunosuppression (Malik *et al*., 2012). Furthermore, *E. coli* is a commensal organism and is responsible for diarrhea in calves, particularly calves receiving less or no maternal antibodies through colostrum (Malik *et al*., 2012) especially in farms where milk is mainly used for commercial purposes.

Chang *et al*., (1986) and Kong *et al*., (1999) reported that *phoA* gene is a housekeeping gene present in all *E.coli* strains.

Hala, (2012) detected *eaeA* gene by 20% but Nguyen *et al*. (2011) reports the detection of *eaeA* gene by 9.8%. Mohamed *et al*., (2014) reported that all isolates of *Escherichia coli* detected in their study were *eaeA*-negative. Beraldo *et al*., (2014) reported that intimin gene of *Escherichia coli* is mainly linked to the EPEC pathotype and *eaeA*-positive strains are considered to be more virulent to human than the *eaeA*-negative ones. This indicates a possible participation of buffalo calves in the zoonotic transmission of pathogenic *E.coli*.

The *tsh* gene encodes a temperature-sensitive hemagglutinin of *E.coli*, first identified by Provence and Curtiss (1994). Also the *tsh* protein was the first identified member of an expanding subclass of the IgA protease family of autotransporters present in *Shigella* spp. and numerous pathotypes of *E. coli* (Stathopoulos *et al*., 1999). Janßen *et al*. (2001) and Saidenberg *et al*., (2013) detected the *tsh* genes in their separate studies and recorded 85.3% and 78.3% respectively of these genes. Mohamed *et al*., (2014), Delicato *et al*., (2003) and Ewers *et al*., (2004), reported the detection of *Escherichia coli* positive samples with *tsh* genes of 28%, 39.5% and 53.3%, respectively. However, these authors detected the *tsh* genes from the APEC isolated from poultry.

Antibiotics are widely used in the treatment and prevention of disease in the veterinary practice as they are also used as growth enhancers in animals. To date, there are many reports regarding *E. coli* resistance in many countries and regions (Johns *et al*., 2012, Szmolka and Nagy, 2013).

Shahrani, *et al*. (2014) recorded 98.09% *E. coli* isolates as resistant strains to tetracyclines. Balasubramaniam *et a*l., (2014) also reported a detection of tetracycline resistant genes in *E. coli* isolated in a study conducted in India as 88%. Nizza *et al*., (2010) in a related studies found that of the *E. coli* isolates detected 34% harvouring tetracycline resistant genes. Similarly a detection of *E. coli* isolate with high resistance to Sulfamethoxazole-trimethoprime was recorded as 90.31% in separate studies by Shahrani *et al*., (2014). This is quite important as Sulfamethoxazole-trimethoprime and tetracycline are commonly used in veterinary and human practices. Similar, percentage was reported by Nelson *et al*. (2014) who detected the *sul1* gene in 73% of samples examined. On the contrary, Hilbert ., (2011), Momtaz *et al*.,

(2013), Dehkordi *et al*., (2014) and Shahrani *et al*.(2014), respectively detected the *sul1* genes in 39.5%, 82.78%, 18% and 90.31%.

2.3 *Salmonella* Infection

Salmonella infections occur worldwide in all species of animals as well as in man. It is a gram-negative, non-spore-forming facultative anaerobe, usually motile bacteria. Infections are usually limited to the digestive tract, although the musculoskeletal and nervous systems are occasionally affected too. In bovines *Salmonella* infections comprised of the second most economically important bacterial disease affecting the gastrointestinal system following *E. coli* infections. Of the 2200 or more known serotypes, the majority of bovine isolates are the following four serotypes: *S. typhimurium, S. dublin, S. muenchen* and *S. copenhagen* (Ekperigin and Nagaraja, 1998).

The majority of *Salmonella* strains found in bovines are not host-adapted. If infection does not progress into Salmonellosis, *Salmonella* organisms remain in the gastrointestinal tract as part of the host's commensal flora and may be shed in faeces. All food animals, except aquatic species in their usual habitats, are susceptible to natural infection with *Salmonella sp.* An animal infected with *Salmonella sp.* may or may not develop salmonellosis, the disease (Ekperigin and Nagaraja, 1998).

Calves with acute, chronic or subclinical intestinal infections shed varying levels of bacteria in their faeces; this serves as the major source of infection to naive herd mates via faecal-oral transmission. Calves with per-acute or acute disease often are septicemic and may shed organisms from other secretions such as saliva.

2.3.1 Prevalence of *Salmonella* infection

Fahmy *et al*, (2017) reported a 7% occurrence of *Salmonella* infections after examining faeces of calves from feedlot farms in Egypt. El-Shehedi *et al.* (2015) found 6.1% faecal samples of diarrhoeic calves to be positive for *Salmonella.* Similarly, detection of *Salmonella* isolates was reported by various workers. These includes Haggag and Khaliel (2002) who recorded 4% prevalence; Younis *et al*., (2009) with a record of 4.09%; Garcia *et al*., (2000) with a record of 1.8%; Achá *et al*., (2004) recorded 2% and Osama *et al.*, (2011) recorded 1.56% prevalence. Studies by others also recorded variable detection status; El-Seedy *et al*., (2016) with 18% prevalence; Youssef and El-Haig (2012) with 18.66% prevalence; Seleim *et al.*, (2004) with 17% prevalence and Riad *et al.*, (1998) with the record of 18.2% prevalence.

The variations in prevalence of *Salmonella* among apparently healthy and diarrhoeic calves reported in different countries reflect the effect of wide range of different management risk factors as reported by (Alam *et al*., 2009 ; Vanselow e*t al*., 2007 ; Jones, 2011).

An entirely contrary view of total lack of detection of any *Salmonella* genes or isolates were presented by the findings of Wani *et al*.,(2013) who after examining faecal samples of buffalo calves for detection *Escherichia coli* and *Salmonella* only reported a positive presence of the *E. coli* from Kashmir, in India.

A further report from other parts of India and from Mozambique revealed negative detections of Salmonella species or their genes as respectively reported by Hussain and Saikia (2000) and Acha *et al*., (2004).

Most investigations relating to calf diarrhoea are concerned with records of infections from calves only and this is because calves are at greater risk of infection than adults due to their naive immune system and the presence of concurrent infection with multiple enteric pathogens (*Escherichia coli, Cryptosporidium*) as reported by Divers and Peek (2008).

An outcome of positive detection of *Salmonella* by serotyping made by array of researchers across the globe revealed that the predominance of *S. enteritidis* serovar among diarrheic calves was recorded especially by Yousef and El-Haig, (2012); Younis *et al*., (2009); Seleim *et al*., (2004); Moussa *et al*., (2012) all from Egypt. This report was further substantiated by the findings of researchers across Europe as presented by Murray (1994) and Smith-Palmer *et al* (2003). *InvA* target gene is located on *Salmonella*, pathogenicity island 1 (SPI1) which is essential for the invasion of epithelial cells by *Salmonella*. This gene is highly conserved in almost all *Salmonella* species (serotypes) and has been used as a potential target for *Salmonella* detection (Jeong *et al.*, 2011).

Conventional PCR can contribute to meeting the need of fast identification and detection methods in disease monitoring and control. However, despite its specificity and sensitivity, thorough investigations should incorporate the use of other conventional methods such as bacterial culture (Smith-Palmer *et al*., 2003).

2.4 *Cryptosporidium* infection

2.4.1 Antecedents of *Cryptosporidium*

Cryptosporidiosis is the disease caused by a protozoan parasite of the genus *Cryptosporidium,* which was discovered in 1910 by Edward Ernst Tyzzer in the gastric

glands of mice. Tyzzer noticed that this parasite did not contain sporocysts within the oocysts and it sporulates while still attached to the host wall. For this reason Tyzzer named the genus *Cryptosporidium*, which was from the Greek word kruptos meaning 'hidden', and the parasite that he identified in the gastric glands was called *Cryptosporidium muris.* Three years later Tyzzer identified another species of *Cryptosporidium* which was not infective in the gastric glands of mice but only in the small intestine (Tyzzer, 1913). This second species produced oocysts which were smaller in size (4-5 μm) compared with *C. muris* oocysts (6-8 μm), Tyzzer named this species *Cryptosporidium parvum* (*parvum* comes from the Latin word and stands for little). It was originally believed that there were only two different species of *Cryptosporidium* but there are now over 27 described species and many more genotypes (Chalmers and Katzer, 2013).

2.4.2 Prevalence of *Cryptosporidium* infection

Although cryptosporidiosis in animals and birds has been reported from Egypt, Brazil, USA, Czech Republic, Malaysia, Tanzania, Spain, Morocco, France, UK, Canada, Japan, Oman, Poland, Iran and China, only a few published reports of cryptosporidiosis in animals are available from India (Kumar *et al*., 2005).

In India the disease was reported for the first time in Uttar Pradesh (Dubey *et al*., 1992) and later in Calcutta (Chattopadhyay *et al*., 2000; Das *et al*., 2003) Pondicherry (Kumar *et al*., 2004), Andra Pradesh (Shobhamani, 2005), UP (Jayabal and Ray, 2005) ,West Bengal (Roy *et al*., 2006), Punjab (Singh *et al*., 2006). The prevalence varied depending upon the age of the animal and other geographical and management practices.

It was observed that the majority of the animals between 1-6 months of age were found to have Cryptosporidiosis caused by *Cryptosporidium parvum*, compared to those above six months and one year of age. These observations were made by Ongerth and Stibbs (1989), Shobhamani (2005), Jayabal and Ray (2005), Roy *et al*. (2006), and Mehdiazami (2007) who reported higher rates of infection among calves less than 6 months of age. The study indicated that younger animals are highly susceptible to infection with cryptosporidiosis compared to adult animals.

A cross-sectional study conducted by Khair *et al.*(2014) to determine the prevalence of bovine cryptosporidiosis which used 110 fecal samples of crossbred diarrhoeic calves from two different areas (Muktagacha, Mymensingh and Shajadpur, Sirajgonj) in Bangladesh during April 2012 to September 2014 revealed an overall

prevalence of cryptosporidiosis in crossbred calves as 28.18% (31/110) by ELISA rapid detection kit. Higher prevalence of cryptosporidiosis was found in the calves from Shajadpur (29.76%) than the calves from Muktagacha (23.08%). The prevalence of cryptosporidiosis was significantly ($p<0.001$) higher in calves between 1-2 months (70%) age group than less than one month age group (24.49%). Cryptosporidiosis was not observed in calves over two months of age. The prevalence of cryptosporidiosis was reported as higher in males (34.75%) than females (24.64%) although not statistically significant. Khair *et al*, (2014) reported that the prevalence of cryptosporidiosis in bovine in areas examined in Bangledash was under diagnosed and the clinical status of infection was probably potentially high.

A similar result was obtained by Mallinah *et al.,* (2009) who studied the prevalence of cryptosporidiosis by screening 455 bovine faecal samples collected from five different organized dairy farms and veterinary hospitals located in and around Bangalore, South India. Faecal samples were examined by Sheather's sugar flotation method for detection of oocysts and recorded 5.71 percent positive for cryptosporidiosis. Sevinc *et al.,* (2009) has a species wise identification as *Cryptosporidium parvum* and *Cryptosporidium andersoni* based on the morphology and micrometry of the oocysts by modified Ziehl-Nelsen staining, Kinyoun'ning method and Safranin methylene blue staining methods. The prevalence and intensity of cryptosporidiosis was found more in calves of less than one month of age compared to adults and more frequently seen in diarrhoeic than in non-diarrhoeic sampled animals. The sex wise prevalence of cryptosporidiosis was observed more in females compared to males. A statistically significant difference was found between sexes and age prevalence of cryptosporidiosis in the calves. Three hundred (300) faecal samples from diarrhoeic and non - diarrhoeic calves were screened for the presence of *Cryptosporidium* infections. The prevalence of *Cryptosporidium parvum* was determined by using acid-fast staining method (Ziehl Neelsen) and ELISA kit. Calves were grouped according to their age as follows: 1-10, 10-20, 20-30, 30-45 and >45 days. The prevalence of infection in diarrhoeic and non diarrhoeic calves was 63.92% and 9.85%, respectively. *Cryptosporidium* infection was respectively detected according to the age groups of calves as 50.75%, 35.71%, 25.45%, 14.71% and 13.24%.

2.5 Rotavirus Infection

Rota- and Coronaviruses are the most common identified viral causes of diarrhoea of neonatal food animals. These viruses have also been associated with

diarrhoea in adult animals, but their disease incidence in adults is comparably low. However, clinically and subclinically infected adults shed the virus and are a source of infection for young animals (Garcia *et al*., 2000).

Viral infections alter cellular function and although the integrity of the epithelial cell layers are initially maintained, infected cells are desquamated into the intestinal lumen within a very short period. Functional alterations of the epithelial cells due to viral infection are thought to be responsible for abnormal absorption and secretion resulting in an imbalance with accumulation within a very short period of fluid in the lumen of the intestine, which contributes to diarrhoea (Garcia *et al*., 2000).

Other viruses that have been associated with diarrhoea in young farm animals includes; Togavirus, Bovine viral diarrhea virus (BVDV), Parvovirus, Calicivirus, Adenovirus, Bredavirus and Astrovirus (Garcia *et al*., 2000).

2.5.1 Prevalence of Rotavirus infection

Rotavirus represents one of the major causes of neonatal mortality in dairy buffaloes in India as reported by series of researchers. Sagar (2008) reported 20% and 2.7% incidence of bovine group A rotavirus in bovine calves in India. However, in a study of rotavirus in Kolkata, Nataraju *et al*. (2009) showed 10.52% (10/95) samples with characteristic of group A rotavirus-like and long-type electropherotype (e-type) pattern and 4.21% (4/95) samples with the characteristic of group B rotavirus long-type of electropherotype pattern in buffalo calves in Kolkata, eastern India. Similarly, Niture *et al*. (2011) detected rotavirus in 7.22% buffalo calves, 7.40% in poultry and 19.75% in human faecal samples in western India. Chitambar *et al.* (2011) detected group A Rotavirus in 2.8% apparently healthy and 14.3% diarrhoeic animals in Pune, western India. The differences in the incidence of rotavirus were probably due to season and climatic factors such as rainfall, temperature and relative humidity. Dhama *et al*. (2009) however, attributed temperature variations or chilling during winter in farms as enhancer to severity of rotaviral infection.

Bov9Com5 and Bov9Com3 primer pairs used in various studies was attributed to specificity for bovine rotavirus in revealing the expected product of 1,013 bp for VP7 gene. Such amplicon size of 1,013 bp of VP7 gene amplicon was obtained by Mondal *et al*. (2011).

Dash *et al*. (2011) detected rotavirus by RNA PAGE in 16.83% diarrhoeic calves from Mathura province of India. Ghosh *et al*. (2007) detected rotavirus in 22% diarrhoeic bovine samples from West Bengal. It was an established fact from earlier

studies that group A rotaviruses are the major cause of diarrhoea in calves all over the world (Okada and Matsumoto 2002; Saravanan *et al*., 2006).

Malik *et al*. (2012) performed an RT-PCR for the identification of G genotype and recorded an incidence of 52.9% of G3 and 47% of mixed G types in the samples. Beg *et al*. (2010) reported the occurrence of 9.67% of G8 from Srinagar. In a similar study conducted by Fukai *et al*. (1999) a prevalence of 4.7% of G8 genotype of the bovine group A rotavirus was reported.

2.6 Coronavirua infection

The *Coronaviridae* is a large family of enveloped, single stranded positive sense RNA viruses with 27-32 kb genome which imparts a high degree of genome plasticity and in part, adaptability and diversity to CoV. Spherical coronavirions (120-160 nm across) appear as peculiar crowns due to the presence of spike glycoproteins (Masters, 2006). They have a distinct replication mode that by use of template switching mechanism of transcription produces a set of sub-genomic mRNAs (Masters, 2006).

From human disease view scope, coronavirus studies were initially regarded as, virology backwater (Cavanagh, 2005) and were more of veterinarians´ interest due to fatal diseases in animals associated mostly with meat and dairy supplies or domestics purposes. Human CoV infections form HCoV-229E and HCoV-OC43 only caused mild cough and fever. With the severe acquired respiratory syndrome (SARS) epidemic in 2003, the CoV gathered spotlights from research communities hailing from almost all realms of disease biology (Masters, 2006). Following SARS, two more human CoV (HCoV) namely HCoV-HKU1 and HCoV-NL63 were identified (Woo *et al*., 2005) (van der Hoek *et al.*, 2004). The current scenario projects CoV as important pathogens of animals (including humans) that cause enteric, respiratory, neuronal and/or hepatic diseases incurring heavy economic losses (Perlman, 1998, Weiss and Navas-Martin, 2005, Masters, 2006, Enjuanes *et al.*, 2008), and high mortalities, as has been seen during SARS outbreaks a decade earlier (Perlman and Netland, 2009) and recently from the middle east respiratory syndrome (MERS) (De Groot *et al*., 2013).

2.6.1 Prevalence of Coronavirus infection

Although series of researchers (Schroeder *et al.* 1985; McDougall and Cullum 1999; Vermunt 2002; Svensson *et al.* 2003; Parkinson *et al*. 2010; Izzo *et al*. 2011), have identified Rotavirus as the major causal pathogen for infectious diarrhoea in

calves, pathogens and species of pathogen vary in their virulence (Howe *et al*. 2008), both within and between-countries as relates to calf diarrhoea. Although the presence of coronavirus infection was reported in New Zealand it appears to be of little consequence in calf diarrhea because of low detection of the virus (Vermunt 2002). The same, minimally pathogenic relationship between coronavirus and calves has also been noted in the Netherlands (Bartels *et al*. 2010). Similarly a Dutch study related to calf diarrhea showed that the prevalence of coronavirus infection was low (only affecting one or two calves) when compared with *Clostridium perfringens* bacteria (Bartels *et al.* 2010). A combined study on the incidence of Rotavirus and Coronavirus in faecal samples collected from different localities covering Menofiya governorate, Egypt during the period from November 2014 to March 2015 using direct sandwich ELISA for antigen detection of virus revealed an occurrence of highest rates of diarrhea in 1st group, followed by 2nd group, then 3rd group of animals sampled. Similar observation was reported by other workers [Lorino *et al.*, (2005) ; El-Naker *et al*., (2007) and Lorenz *et al*., (2011)], who recorded the occurence of diarrhea during neonatal period as high in the first days of calves' age. Coronavirus are ubiquitous and as a result, most of the animals, including pregnant cows coming from intensive livestock farms, have specific antibodies against these pathogens. The antibodies produced by cows in response to natural immunization or vaccination are transmitted to the calf at birth via the colostrum (Radostits *et al*., 2007 and Morshedi *et al*., 2010), so the diagnosis of Corona virus infection has been based primarily on the detection of virus or viral antigen in the faeces. There are a variety of diagnostic methods available for the detection of coronavirus including PCR, ELISA, Electron microscope and Immune electron microscope (Cho *et al*., 2010 and Jakobsson 2013).

ELISA is one of the essential methods in the determination of viral antigens and has the good qualities of being fast and having the capability to handle a big number of samples at the same time (Duman and Aycan 2010 and Jakobsson 2013). It is widely used for viral antigen detection from the faeces of diarrhoeic calves (Ali *et al.*, 2008; Dhama *et al*., 2009; Badiei *et al*., 2010 and El-Bagoury *et al*., 2014). Coronavirus infection is most often transmited through a faecal-oral route and calves are most often infected by contact with other calves, primarily or secondarily through objects, feeds and water. Calves can also be infected by virus shed by the dam at birth. The infected calves shed virus through the faeces from the second day of infection and the shedding may last for 7-8 days. (Malik *et al*., 2005; Dhama *et al*.,

2009; Suresh *et al*., 2013 and Collins *et al*., 2014).Examinationof 200 faecal samples revealved the detection of 51(25.5%) positive faecal samples using direct sandwich ELISA kit for detection of Coronavirus antigen. This result may be related to virus shedding in outbreaks in non vaccinated populations of calves (Brandão *et al*., 2007; Oliveira Filho *et al*., 2007 and Gay *et al*., 2012).

Depending on the age of the calf, some pathogens are more likely to be the cause of diarrhoea; Coronavirus mostly affect calves aged 5-20 days old, although can affect calves up to several months of age (Reidy *et al*., 2006; Dash *et al*., 2011 and Gay *et al.,* 2012).

2.7 Immunity

Disease/Syndrome (Diarrhoea) management in animals can be done in two ways. One is chemotherapeutic approach that involves the hefty cost of treatment and veterinary care. The other is breeding for enhanced disease resistance because resistance to infectious disease in animals has a genetic basis and additive genetic variation exists among animals in their response to various infectious challenges (Taylor, 2004). In response to pathogen infiltration, the vertebrate immune system has evolved multiple defense systems, which can be broadly classified into innate and adaptive immunity, to repel and kill the invasive microbe. The innate immune response exists in all multicellular organisms and it is mediated by the physical barriers, immunocytes such as macrophages, neutrophils, natural killer (NK) cells, and certain soluble factors (Sordillo and Streicher, 2002). The innate immune response is the first line of defense against invading organisms and is directly related to infections with pathogens associated with calf diarrhea (Moyes *et al*., 2009). The specific or acquired immune system is activated when a pathogen circumvents or not eliminated by innate immunity (Sordillo and Streicher, 2002).

The immunocytes have receptors called pattern recognition receptors (PRRs), which recognize specific pathogen associated molecular patterns (PAMPs). The interaction between PRR and PAMP stimulates extra cellular complement pathway as well as intracellular signaling pathways culminating in inflammatory responses. The major PRRs involved in intracellular signaling pathways are Toll-like receptors (TLRs) and nucleotide oligomerization domains (NODs)/carspass activation and recruitment domains 15 (CARD15) (Sordillo and Streicher, 2002).

2.8 Immunoglobulin G (IgG) in colostrum

Saving time and money are always important considerations in the agricultural sector and in dairy farming calves' management plays a central role. One of the most important aspects of rearing calves is the colostrum-feeding routines and several studies have come to the same conclusion that the volume fed, quality of milk fed and when the calves are fed after parturition, are the three most important aspects. Therefore the timely feeding of an adequate volume of high quality colostrum immediately after birth is one of the key factors influencing the health and survival of the neonatal dairy calf (Quigley *et al.* 2013).

The placenta of the bovine dam is cotyledonary, in which 100-140 focal villous aggregations (cotyledons) develop and attach to the maternal caruncles to form placentomes that function as the main exchange for oxygen, carbon dioxide, nutrients and fetal metabolic products (Haeger *et al*., 2016). However, the bovine placenta is also epitheliochorial, meaning that the uterine epithelium and the maternal blood vessels remain intact throughout gestation due to the non-invasive nature of the trophoblast (Pereira *et al*., 2013). This phenomena results in complete separation of the maternal and fetal vascular systems and thus there is no passive transfer of antibodies to the calf in utero (Wooding, 1992; Davis and Drackley, 1998; Weaver *et al.,* 2000; McGuirk and Collins, 2004)**.** The consequence of this is that the calf is born agammaglobulinemic, which means that the calf is born with very low levels of antibodies, rendering it immune deficient and susceptible to diseases during neonatal period. Therefore, the calf relies on immunoglobulin-rich colostrum to provide it with passive immunity for protection against pathogens it may encounter during early life (Vasseur *et al*., 2010). In regard to this condition, the calves are depending on the absorption of Ig (antibodies) from the colostrum they are fed after birth and the colostrum provides the calf with Ig from the mother. Passive transfer, which means the absorption of Ig from the colostrum through the calves' small intestine the first 24 h after birth, is protecting the calf from several diseases until the calf's own immune system is working (Weaver *et al.,* 2000). For optimal Immunoglobulin (Ig) transfer through the gut epithelium, the calf should be fed within 4 hours postpartum. After 12 hours postpartum the efficiency of Ig absorption is gradually decreasing (Weaver *et al.,* 2000*).* At least 100 grams of IgG during the first feeding is sufficient as passive transfer of Ig essential for a good management (Davis and Drackley 1998). Others

recommends for at least 123 grams of colostral IgG 2 hours after parturition and 164-226 grams of colostral IgG if fed 6 hours after parturition (Chigerwe *et al., 2008).*

Calf need to absorb a satisfactory amount of these molecules into their circulation (Godden, 2008). Success of absorption of Ig molecules into the circulation defense on how quickly the calf is fed the first colostrum after birth before cessation of macromolecular transport (closure) (Godden, 2008; Quigley and Wolfe 2003).

With an adequate passive transfer of Ig there is a lot to gain such as; a lower risk of pre-weaning morbidity and mortality, lower mortality in the post-weaning period, higher feed efficiency, lower age at first calving, enhanced milk production for both 1st and 2nd lactation and also a decreased risk of culling in the 1st lactation (DeNise *et al*., 1989; Wells *et al.,* 1996; Godden, 2008).

Among all immunoglobulin classes in colostrum, IgG is present at the highest concentration and colostrum containing 50 g of IgG per L or greater is considered good quality (Godden, 2008). However, the IgG content of colostrum can widely vary. An analysis of more than 150 colostrum samples from 7 dairy farms in the U.S. revealed that the concentration of IgG ranges from 7.1 to 159 g/L, with16% of samples containing less than 50 g/L (Quigley *et al.* 2013). Given this high variation, accurate measurement of colostral IgG concentrations before feeding it to calves is essential for proper management. Unfortunately, analysis of colostrum to determine IgG concentrations is not easily done and is only evaluated by 13% of producers, with 56% of those estimating the quality solely based on visual inspection (NAHMS, 2007). Typical on-farm tools to determine colostrum quality include the colostrometer, which measures the specific gravity of the colostrum, and the Brix refractometer that approximates the percentage of total solids (Fleenor and Stott, 1980; Quigley *et al*., 2013).

2.9 Immunoglobulin G (IgG) in Milk

The amount of immunoglobulins in milk varies and all factors influencing their concentration are not yet discovered (Korhonen *et al*., 2000; Krol *et al*., 2012). Different authors have indicated that concentration of immunoglobulins G in the cow milk varies depending not only on the degree of udder infection but also is considerably affected by the cow age, lactation period, keeping conditions, and feeding (McFadden *et al*., 1997; Korcina *et al*., 2012). The mean levels obtained for IgG in the whole population studied are 0.29±0.14 mg/ml, a value close to the range referred to as normal for mature milk, which is 0.3-0.5 mg/ml (Collin *et al*., 2002).

Similar IgG values were obtained by other researchers as well: 0.30–0.60 (Pakkanen, 1997; Krol *et al*., 2012). In the analysis of bovine IgG in milk, Grapper *et al*. (2007) reported a higher IgG value of 0.72 mg/ml. The highest concentration of the immunoglobulin G in milk as 2.05±0.83 mg/ml was indicated by Latvian researchers (Korcina *et al*., 2012).

The age of the cow and the number of lactations are considered as two relevant factors, which determine IgG concentration in milk. The poorest source of IgG proved to be the milk obtained from the 1st and 2nd lactations cows (0.26 and 0.15 mg/ml). Older cows, in the 5th and 6^{th} lactations, produced milk with higher concentration of IgG compared to younger ones (0.41• and 0.11 mg/ml). These findings are similar with the findings of Krol *et al*. (2010; 2012) research where primiparous cows was reported to produce significantly less IgG as compared to cows at 2 to 4 lactations ($P \leq 0.05$) and older ($P \leq 0.01$). The lowest level of IgG was found in the 1st lactation (0.454• and 0.16 mg/ml) and in subsequent lactations IgG compounds increased gradually (Krol *et al*., 2012).

2.10 Immunoglobulin G (IgG) in oral fluid

Prevalence of immunity to vaccine-preventable infections such as measles, rubella and Hepatitis-B (HBc) were determined by screening paired blood and oral-fluid samples from 853 individuals of all ages from a rural Ethiopian community.Enhanced IgG antibody capture (GAC) enzyme-linked immunosorbent assays (ELISAs), and anti-HBc antibodies with a prototype GACELISA for measles- and rubella-specific antibodies and anti-HBc antibodies were respectively used.The results of 98% and 87% respectively for sensitivity and specificitysuggested that oral fluid have the potential to replace serum in IgG antibody prevalence surveys. Earlier workers reported that the non-invasiveness in sample collection will serve as a remedy to the problems of Iconophobia in human medicine and also as same to the menance of cruelty in Veterinary practice (James *et al*., 2001).

The potential of oral fluid as a replacement for serum in antibody prevalence surveys should also be viewed in the context of the major advantages of oral-fluid sampling over blood collection: it is non-invasive, more acceptable to subjects of all ages (reflecting absence of pain and low or no perceived risk of contamination), easier to collect without the need for medically trained personnel, and safer for the collectors (Nokes, 1998). Nevertheless, wider application of this methodology requires further research and developments in a number of areas.

Further work is required to quantify and improve assay performance. Estimates of assay specificity for rubella- and measles-specific IgG were unreliable for older age groups because these groups had low numbers of seronegative individuals and studies with larger sample sizes are therefore needed.

The sensitivity of oral-fluid assays for specific rubella IgG declines with age (Nokes, 1998), and is associated with an age-related decrease in rubella-specific antibody levels in serum and in oral fluid (Nokes, 1998). These effects could be associated with the time lapsed since primary infection or an age-related decrease in boosting from re-exposure (Vyse, 1999; Nokes, 1998), although such effects do not influence the sensitivity of the measles assays. Assay performance is influenced by the quality of the oral-fluid sample, although there is some debate over what provides a good measure of quality (Nigatu, 1999, Eckstein, 1996; Vyse, 1999). For example, a sub-sample of 160 oral fluids collected in this study all had detectable levels of total IgG, and although the range in concentration was wide (1.1 mg/ml to >60 mg/ml (Nigatu, 1999), assay sensitivity to measles-specific IgG was only marginally lower in samples with lower total IgG. Furthermore, the nature of the relationship between Virus (rubella) specific IgG and total IgG in oral-fluid samples is a function of the type of device used to collect the specimen (Nokes, 1998). Previous studies suggest that the dental status of infants does not unduly affect the transudated serum IgG antibody component of oral-fluid samples (Bagg, 1991). Nonetheless, further data would be worth collecting. The thermal stability of IgG in oral fluid samples is a concern, particularly in developing countries that may be experiencing difficulties in the cold chain. It has been reported that there was no loss in performance with an HIV antibody assay carried out on saliva samples stored for one month at ambient temperature in a tropical country (Thwe, 1999).

2.11 Immunoglobulin G (IgG) in faeces

Selective immunoglobulin A (IgA) deficiency is the most common primary immunodeficiency in humans and may be associated with chronic gastrointestinal disease. This observation has led to the suggestion that the high susceptibility of German shepherd dogs (GSD) to chronic enteropathies is related to a deficiency in mucosal IgA production.Relative deficiencies of IgA has been reported in the serum, saliva, tears, and faeces of GSD both with and without alimentary disease; however, the findings of different studies are not consistent. The aim of the study was to confirm whether a relative deficiency of IgA exists in the faeces of GSD. Faeces were

collected from healthy GSD (*no.* 209), Labrador retrievers (*no.* 96), beagles (*no.*19), and miniature schnauzers (*no.* 32). Faecal IgA, IgM, and IgG were measured by capture enzyme-linked immunosorbent assays. Faecal IgG concentrations in the four breed groups were not significantly different. These findings do not support the hypothesis that GSD have a relative deficiency in faecal IgA. The differences in immunoglobulin concentrations measured from a single defecation, between individuals of the same breed and between breeds, as well as the lack of an internal control molecule, make the determination of a normal reference range for all dogs impossible. Therefore, the usefulness of faecal immunoglobulin quantification for the assessment of intestinal immunoglobulin secretion in dogs is limited. Measurement of IgG levels in stool requires an extensive preparation of the stool to obtain a clear globulin-containing fraction and the preparation losses may be considerable so that quantitative levels quoted can only be considered to be estimates (Peters *et al*., 2004).

2.12 Pattern Recognition Receptors (PRR)

Pathogen Associated Molecular Patterns (PAMPS) are genetic components of pathogens. The immunocytes have receptors called Pattern Recognition Receptors (PRRs). These are counterpart components of the host (Chuang *et al*., 2000). PRR specifically recognize PAMPs and the interaction between PRR and PAMP stimulates inflammatory responses. The major PRRs involved in intracellular signaling pathways are Toll-like receptors (TLRs) and Nucleotide Oligomerization Domains (NODs).

2.12.1 Toll-like receptors (TLR)

The toll-like receptors (TLRs) are a family of cell-surface signaling molecules that play a fundamental role in the immune response to recognize pathogens that bind to specific pathogen-associated molecular patterns (PAMPs) (Takeda *et al*., 2003). There are at least 10 members of the TLR family in mammals that recognize specific components conserved among microorganisms (Rock *et al*., 1998; Chuang *et al*., 2000). In the case of TLR4 they have been implicated as receptors, mediating cellular activation in response to bacterial lipopolysaccharide (LPS), a cell-wall component of gram-negative bacteria, which is the principal PAMP of TLR4 (McGuire *et al*., 2005). LPS-induced apoptosis and nuclear factor-Kappa B (NF-kB) activation can occur independently, with the pathways diverging downstream signaling molecules, including the adaptor molecule MyD88, IL-1R-associated protein kinases (IRAKs) and the Tumor Necrosis Factor Receptor Associated Factor 6 (TRAF6) (Bannerman

et al., 2002; Akira, 2003), which can induce the over-expression of several pro-inflammatory cytokines and chemokines.

2.12.1.1 TLR4 gene expression

TLR4 plays an important role in recognizing the conserved patterns unique to microbial surfaces (PAMPs), and LPS was reported to stimulate the expression of the bovine antibacterial peptide-encoding gene via the activation of NF-kB and induces inflammation that contributes to an immune response. It was proven that the TLR4 gene affects several diseases, including sepsis, immune-deficiencies, atherosclerosis and asthma (Bannerman *et al*., 2002; Akira, 2003). A research work permitting the alignments of the bovine TLR4 coding sequence (CDS) and human TLR4 CDS reveal a high similarity of 85%, and their corresponding proteins are 76% similar (Bannerman *et al*., 2002; Akira, 2003). The results of cDNA sequence analysis and protein domain of TLR4 inferred that bovine TLR4 might induce immune response for disease resistance of pathogen infection by the signal transduction pathway. Expressions of TLR4 mRNA using semi-quantitative RT-PCR with trinitrobenzene sulfuric acid (TNBS)-treated rat colon where different regions of the affected colonic tissue were separated into mucosal and muscular regions to establish any difference in mRNA expressions between them. TLR4 as the representative PAMP receptors involved in mediating inflammatory responses, show a higher expression of TLRs in the mucosal layer compared to that in the muscle layer.

2.12.2 Nucleotide Oligomerization Domains (NODs)

Nucleotide Oligomerization Domains (NODs) are a family of cytosolic proteins in innate recognition of bacteria. There are up to 12 different NODs in mammals. NOD2, also called Carspase Activation and Recruitment Domain 15 (CARD15) was identified in human as IBD1 gene (Inflammatory Bowel Disease1). Later it was named as NOD2 (Nucleotide Oligomerization Domain 2) and finally re-named as CARD15 (Caspase Activating Recruitment Domain15). CARD15 is a cytosolic protein initiating inflammation following PAMP recognition (Pant *et al*., 2007; Taylor, 2004). Other functions includes antibacterial (Ferwerda *et al*., 2005; Opitz *et al.*, 2004; Kobayashi *et al*., 2005 ; Ferwerda *et al.*, 2007; Kapetanovic *et al*., 2007), antifungal (Zhang *et al*., 2008) and apoptosis (Ogura *et al*., 2001a). NOD2 was later confirmed to be an intracellular receptor for muramyl dipeptide (MDP), a component of peptidoglycan (PGN) (Girardin *et al*., 2003; Inohara *et al.*, 2003). MDP is present in practically all Gram-positive and Gram-negative bacteria (Schleifer and

Kandler, 1972). NOD2 therefore acts as a general sensor of bacteria because MDP is part of the PGN structure that is conserved and common among nearly all bacteria (Girardin *et al.*, 2003; Inohara *et al*., 2005). Besides antibacterial effects, it recognizes Chlamydia (Derbigny *et al.*, 2005) and is upregulated against fungus, *Aspergillus fumigatus* (Zhang *et al.*, 2008). Initial studies indicated that CARD15 are expressed in immune cells only. However, now a number of reports are emerging which indicate a broader range of expressions.

2.12.2.1 NOD2 modulates the TLR system

Rakoff-Nahoum (2004) reported that TLR recognition triggers the innate immune system leading to an inflammatory response which serves as a protective role of TLR activation by intestinal commensal bacteria. Under steady state conditions activation of TLRs by commensal microflora was critical for tissue repair and protection against intestinal injury and associated mortality. Furthermore in vivo it is likely that innate immune pattern recognition receptors are not triggered by single ligands but rather complex activation of multiple receptors takes place concurrently due to the many TLR and NOD ligand motifs present on a microbial pathogen. (Sansonetti 2004; Philpott and Girardin 2004).The later further postulated that dual signalling via both TLR and NOD pathways may be necessary for efficient innate immune responses and that in the presence of abnormal NOD2 this process is compromised resulting in an abnormal initial defense against commensal and pathogenic bacteria or an abnormal tolerance mechanism which is critical in maintaining controlled activation of the immune system in the intestine.

TLR4 have been associated with host recognition of bacterial pathogen (Koedel *et al*., 2003; Malley *et al*., 2003; Schroder *et al*., 2003; Yoshimura *et al.*, 1999). Whereas these TLRs are likely to serve as the first line receptors for pathogens, the Nod proteins might play a major role in a subsequent phase of infection. Since TLRs mediate NF-kB activation and NF-kB binding sites have been identified in the Nod2 promoter (Gutierrez *et al*., 2002; Rosenstiel *et al*., 2003), recognition of bacterial pathogens by the TLRs might cause the up-regulation of Nod2 and thereby facilitate the immune response of the host against this pathogen. In line with this hypothesis, the penetration of epithelial and endothelial cells by bacteria is initiated during the first hours after infection, and it is most pronounced after 4–6 h. Mesenger RNA (mRNA) levels of Nod2 increased within a similar time frame, suggesting that

Nod-mediated NF-kB activation might play an important role in this subsequent phase of host responses against these pathogens.

The NOD-dependent NF-kB activation by intact or inactive bacterial pathogen is most likely due to cell wall peptidoglycan. NOD2 has been found to mediate cell activation by a muramyldipeptide conserved in basically all kinds of peptidoglycans (Girardin *et al.*, 2003; Inohara *et al*., 2003),

Thus, the Nod2-deficient mice as well as the recently generated Nod1 knockout mice will be of invaluable help to further elucidate the precise role of these proteins in host defense (Chamaillard *et al.*, 2003; Girardin *et al*., 2003; Pauleau and Murray *et al* ., 2003).

In the overall, besides bacterial cell recognition by TLRs, NOD2 activation seems to play an important role in host cell activation by internalized bacterial pathogens. Downstream of NOD2 and RIP2, signal-transducing molecules like IRAK, IRAK2, TRAF6, NIK, TAB2, and TAK1 might mediate NF-kB-dependent cell activation. Knowledge about the molecular interaction of bacterial pathogens with target cells may pave the way to innovative therapeutic strategies.

CHAPTER-III

MATERIALS AND METHODS

OBJECTIVE NUMBER 1

To determine the common pathogens associated with calf diarrhea from meconium and faecal samples of buffalo calves and their dams.

Experiment 1

3.1 Samples and sample collection

3.1.1 Blood sample/ Ethics Statement

Blood samples were collected from buffalo calves and their dams from the farm of the department of livestock production management (LPM), College of Veterinary Sciences, Lala lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana state, India, with permission for research use from Institutional Animals Ethics Committee (IAEC). The samples were collected by skilled technicians after proper restraining of animals under the supervision of a veterinary officer present at the cattle yard, LPM. Blood samples were collected for RNA extraction, PBMC isolation and for extraction of serum. No animal was specifically slaughtered for this research.

Experiment 2

3.2 Detection of *Escherichia coli*

3.2.1 Faecal samples collection

A total of 78 faecal samples (including meconium) from 38 buffalo calves and 40 buffalo dams with and without diarrhoea were collected from buffalo farm in the Department of Livestock Production and Management, LUVAS, Hisar.

Animal grouping was based on ages as shown in Table 3.1 to 3.14 below:

1- Day-1 to 2-days,

2-Day-3 to 3- months

3-Month-4 to Month-6

Samples were collected in sterile, screw-capped vials and transported to the laboratory on ice and stored at - 20^0 C for further use.

Group I

Table 3.1: Sampling in calves of Day 1 to 2 Days of age and their dams on 2nd July, 2017

Sample Collection Date - 2nd July 2017							
Sr. No.	**Calf No.**	**Sex**	**D.O.B**	**Dam No.**	**Parity**	**Diarrheoa Status**	
						Calf	**Dam**
1.	BC1369	M	01:07:17	BD799	4TH Lactation	D	ND

Key: Sr. No. = Serial Number
D.O.B=Date of Birth
M=Male
F=Female
D=Diarrhea
ND=No Diarheoa

Table 3.2: Sampling in calves of Day 1 to 2 Days of age and their dams on 20th July, 2017

Sample Collection Date – 20th July 2017							
Sr. No.	**Calf No.**	**Sex**	**D.O.B**	**Dam No.**	**Parity**	**Diarrheoa Status**	
						Calf	**Dam**
1.	BC1370	F	19:07:17	BD967	2nd Lactation	ND	D

Table 3.3: Sampling in calves of Day 1 to 2 Days of age and their dams on 22nd July 2017

Sample Collection Date - 2nd July 2017							
Sr. No.	**Calf No.**	**Sex**	**D.O.B**	**Dam No.**	**Parity**	**Diarrheoa Status**	
						Calf	**Dam**
1.	BC1371	F	21:07:17	BD791	4th Lactation	D	D

Table 3.4: Sampling in calves of Day 1 to 2 Days of age and their dams on 23rd July 2017

Sample Collection Date-23rd July 2017							
Sr. No.	**Calf No.**	**Sex**	**D.O.B**	**Dam No.**	**Parity**	**Diarrheoa Status**	
						Calf	**Dam**
1.	LPM79	F	22:07:17	LPM38	2nd Lactation	D	ND

Table 3.5: Sampling in calves of Day 1 to 2 Days of age and their dams on 24th July 2017

Sample Collection Date - 24th July 2017							
Sr. No.	**Calf No.**	**Sex**	**D.O.B**	**Dam No.**	**Parity**	**Diarrheoa Status**	
						Calf	**Dam**
1.	BC1370	M	23:07:17	BD1025	1st Lactation	ND	D
2.	LPM0080	M	23:07:17	LPM 16	5th Lactation	D	ND

Table 3.6: Sampling in calves of Day 1 to 2 Days of age and their dams on 1st August 2017

Sample Collection Date-1st August 2017							
Sr. No.	**Calf No.**	**Sex**	**D.O.B**	**Dam No.**	**Parity**	**Diarrheoa Status**	
						Calf	**Dam**
1.	BC1373	F	31:07:17	BD1046	1st Lactation	ND	ND
2.	BC1374	F	31:07:17	BD1038	1st Lactation	D	D

Table 3.7: Sampling in calves of Day 1 to 2 Days of age and their dams on 9th August 2017

Sample Collection Date - 9th August 2017							
Sr. No.	**Calf No.**	**Sex**	**D.O.B**	**Dam No.**	**Parity**	**Diarrheoa Status**	
						Calf	**Dam**
1.	LPM0082	M	08:08:17	LPM10	6th Lactation	ND	ND

Table 3.8: Sampling in calves of Day 1 to 2 Days of age and their dams on 10th August 2017

Sample Collection Date - 10th August 2017							
Sr. No.	**Calf No.**	**Sex**	**D.O.B**	**Dam No.**	**Parity**	**Diarrheoa Status**	
						Calf	**Dam**
1.	LPM0083	F	09:08:17	LPM185	4th Lactation	ND	D
2.	BC0069	M	09:08:17	BD1068	3rd Lactation	D	ND
3.	BC1265	M	09:08:17	BD181	4th Lactation	D	ND
4.	BC1268	M	09:08:17	BD173	4th Lactation	D	D
5.	BC1317	M	09:08:17	BD19	2nd Lactation	D	D
6.	-	-	-	BD190	2nd Lactation	-	D
7.	-	-	-	BD0015	2nd Lactation	-	ND

Table 3.9: Sampling in calves of Day 1 to 2 Days of age and their dams on 15th August 2017

Sample Collection Date - 15th August 2017							
Sr. No.	**Calf No.**	**Sex**	**D.O.B**	**Dam No.**	**Parity**	**Diarrheoa Status**	
						Calf	**Dam**
1.	BC1375	F	14:08:17	BD909	2nd Lactation	ND	D
2.	BC81	M	14:08:17	BD49	1st Lactation	D	D

Group II

Table 3.10: Sampling in calves of Day 3 to 3 months of age and their Dams on 13th July 2017

Sample Collection Date - 13th July 2017							
Sr. No.	**Calf No.**	**Sex**	**D.O.B**	**Dam No.**	**Parity**	**Diarrheoa Status**	
						Calf	**Dam**
1.	BC1364	F	05:06:17	BD912	2nd Lactation	ND	ND
2.	BC1365	M	07:06:17	BD935	2nd Lactation	D	D
3.	BC1366	M	11:06:17	BD1010	1st Lactation	D	D

Key: **D.O.B**=Date of birth, **M**=Male, **F**=Female

Table 3.11: Sampling in calves of Day 3 to 3 months of age and their Dams on 14th July 2017

Sample Collection Date - 14th July 2017							
Sr. No	**Calf No.**	**Sex**	**D.O.B**	**Dam No.**	**Parity**	**Diarrheoa Status**	
						Calf	**Dam**
1.	BC1358	F	01:05:17	BD1068	1st Lactation	ND	ND
2.	BC1359	M	15:05:17	BD1003	1st Lactation	ND	ND
3.	BC1360	F	20:05:17	LPM847	3rd Lactation	D	D
4.	BC77	M	21:05:17	BD183	3rd Lactation	ND	ND
5.	BC1361	M	24:05:17	BD998	2nd Lactation	ND	D
6.	BC1363	M	30:05:17	BD848	2nd Lactation	D	D
7.	BC1367	M	14:06:17	BD902	4th Lactation	D	D
8.	BC1368	F	18:06:17	BD675	3rd Lactation	ND	ND

Table 3.12: Sampling in calves of Day 3 to 3 months and their Dams on 20th July 2017

Samples Collection Date - 20th July 2017							
Sr. No.	**Calf No.**	**Sex**	**D.O.B**	**Dam No.**	**Parity**	**Diarrheoa Status**	
						Calf	**Dam**
1.	BC22D	F	22 Days	_	_	D	ND
2.	BC2M	F	2 Months	_	_	D	ND
3.	BC2.5M	F	2.5 Months	_	_	D	ND

Group-III

Table 3.13: Sampling in calves of 4-6 months of age and their Dams on 1st August 2017

Sample Collection Date - 1st August 2017							
Sr. No.	**Calf No.**	**Sex**	**D.O.B**	**Dam No.**	**Parity**	**Diarrheoa Status**	
						Calf	**Dam**
1.	BC1306	M	01:11:16	BD182	3rd Lactation	D	ND
2.	BC1317	F	22:11:16	BD190	2nd Lactation	ND	D
3.	BC1324	M	01:12:16	BD170	4th Lactation	ND	ND
4.	BC0075	M	27:12:16	BD24	3rd Lactation	ND	ND
5.	BC1344	M	23:01:17	BD176	4th Lactation	ND	ND

Table 3.14: Sampling in calves of Day 4-6 months of age and their Dams on 27th February 2017

Sample Collection Date - 27th February 2017							
Sr. No.	**Calf No.**	**Sex**	**D.O.B**	**Dam No.**	**Parity**	**Diarrheoa Status**	
						Calf	**Dam**
1.	BC1279	M	03:09:16	BD178	4th Lactation	D	D
2.	BC72	F	20:09:16	BD20	4th Lactation	ND	ND
3.	BC1377	F	07:10:16	BD19	2ndLactation	ND	D

Experiment 2.1

3.2.3 Protocol for genomic DNA extraction using PureLink DNA exraction kit for detection of *Escherichia coli*

To the faecal sample PBS was added, stired, vortexed and kept at room temperature. The supernatant was separated from the debri. The supernatant was centrifuged and the pellet was collected for lysate production. The pellet was re-suspended in 200µl PBS and was vortexed . Tweenty microliters (20µl) of proteinase

K, was added and vortexed.Twenty microliters (20µl) of RNase A was added, vortexed and incubated at room temperature for 2 minutes. Two hundred microliters (200µl) of Genomic Lysis/Binding Buffer was added and mixed well. Vortexing was done and incubated at 55^0C for 10 minutes to promote protein digestion. Two hundred microliters (200µl) of 96%-100% ethanol was added to the lysate and vortexed for 5 seconds. The resultant supernatant was added to a column and centrifuged at 12,000 rpm for two minutes at room temperature. Five hundred microliters (500 µl) of wash buffer 1 was added centrifuged at 12,000 rpm at RT and discarded the collection tube. Two hundred microliters (200 µl) of wash buffer 2 was added and centrifuged at 12,000 rpm and flow through was discarded. The empty column was centrifuged at 12,000 rpm for 2 minutes and placed in a sterile 1.5 ml microcentrifuge tube. The column was incubated at RT for 1minute and centrifuged again at 12,000 rpm for 1 minute at RT. The tube contained purified DNA. The column was discarded and the purified DNA was stored at -20^0C or used for downstream application.

Figure 3.1: Protocol for genomic DNA extraction using PureLink DNa exraction kit

Table 3.15: List of *Escherichia coli* primers for amplification of gene transcripts

Sr. No.	Primer Name	Sequence of Primer	References
1.	phoA - f	5'- CGATTCTGGAAATGGCAAAAG-3'	Hu *et al*., 2011
	phoA - r	5'- CGTGATCAGCGGTGACTATGAC-3'	
2.	*eaeA* - f	5'- ATG CTT AGT GCT GGT TTA GG-3'	Bisi-Johnson *et al*., 2011
	eaeA - r	5'- GCC TTC ATC ATT TCG CTT TC-3'	
3.	tsh - f	5'- GGT GGT GCA CTG GAG TGG -3'	Delicato *et al*., 2003
	tsh - r	5'- AGT CCA GCG TGA TAG TGG - 3'	
4.	tet *A*- f	5'- GGTTCACTCGAACGACGTCA -3'	Randall *et al*.,2004
	tet *A*-r	5'- CTGTCCGACAAGTTGCATGA -3'	
5.	Sul1- f	5'- CGG CGT GGG CTA CCT GAA CG-3'	Ibekwe *et al*., 2011
	Sul1- r	5'- GCC GAT CGC GTG AAG TTC CG -3'	
6.	LT- f	5'- AGCAGGTTTCCCACCGGATCACCA-3'	Wani *et al*., 2013
	LT-r	5'- GTGCTCAGATTCTGGGTCTC-3'	
7.	ST-f	5'-TTTATTTCTGTATTGTCTTT-3'	Wani *et al*., 2013
	ST-r	5'- ATTACAACACAGTTCACAG -3'	
8.	EAEC-f	5'- CTGGCGAAAGACTGTATCAT-3'	Wani *et al*., 2013
	EAEC-r	5'- CAATGTATAGAAATCCGCTGTT-3'	
9.	afa1	5'- GCTGGGCAGCAAACTGATAACTCTC-3'	Wani *et al*., 2013
	afa2	5'- CATCAAGCTGTTTGTTCGTCCGCCG -3'	

Table 3.16: Annealing temperatures and respective amplicon sizes for different *E coli* gene primers after gradient PCR

Primer No.	Name of Primer	T_a (^{0}C)/30sec	Amplicon Size
Primer 1	PhoA (Alkaline phosphotase)	45.9	720bp
Primer 2	eaeA (Virulence gene)	60.8	248bp
Primer 3	tsh (Virulence gene)	56.0	620bp
Primer 4	tetA (Tetracycline) (Antibiotic resistance)	45.9	576bp
Primer 5	Sul (Sulfonamide) (Antibiotic resistance)	45.9	433bp
Primer 6	LT(Heat labile) (Virulence gene)	47.4	132bp
Primer 7	ST (Heat stable) (Virulence gene)	40.7	177bp
Primer 8	EAEC((Virulence gene)	52.6	630bp
Primer 9	Afa (afimbrial adhesive sheath) (Virulence gene)	66.8	750bp

Table 3.17: Ingredients for standardized PCR reactions for *Escherichia coli* genes

Sr. No.	Reagents	Volume/25µl
1	NFW	16.0 µl
2	5×PCR Buffer	5.0 µl
3	dNT ps Mix, 10 mM stock	0.5 µl
4	Spp. specific primers	
i)	Forward	0.6 µl
ii)	Reverse	0.6 µl
5	Taq DNA polymerase	0.3 µl
6	Template DNA	2.0 µl

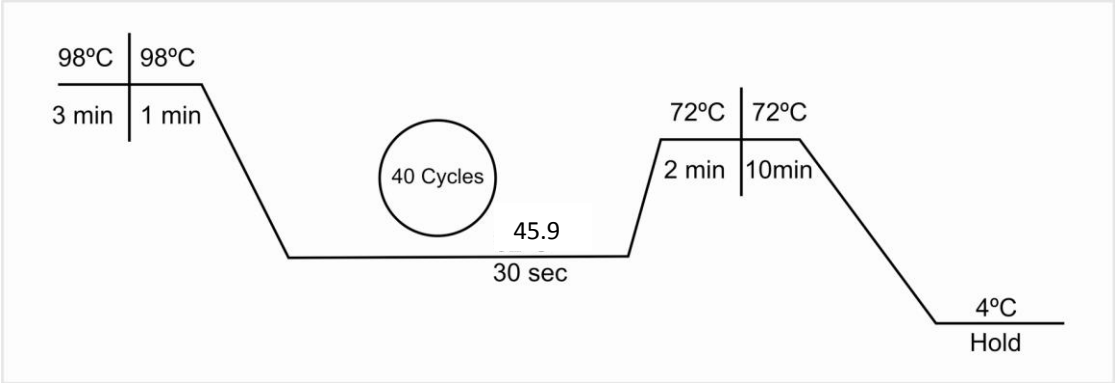


Figure 3.2: PCR profile for detection of *Escherichia coli* genes

Table 3.18: Solutions for casting the polyacrylamide gel (Sambrook and Russell, 2001)

Sr. No.	Stock Solution(Appendix)	8% Resolving gel
1	30% Acrylamide/ Bis-acrylamide	3.35 ml
2	1.5 M Tris HCl, pH 6.8	3.15 ml
3	0.5 M Tris HCl, pH 6.8	-
4	TEMED	10.0 ul
5	Ammonium persulphate (10%)	150 ul
6	Glass distilled water	5.75 ml

Experiment 3

3.3 Detection of *Salmonellae*

3.3.1 Faecal Samples

A total of 78 faecal samples from 38 buffalo calves and 40 buffalo dams with and without diarrhoea were collected from buffalo farm in Lala Laj Pat Rai, University of Veterinary and Animal Sciences (LUVAS), Hisar. The age grouping of the animals was based

on the age of buffalo calves as Day-1 to 2-days, Day-3 to 3- months and 4 to 6 months. Samples were collected in sterile, screw-capped vials and transported to the laboratory on ice.

Experiment 3

3.3.2 *Salmonellae* DNA extraction

Salmonellae were detected from the faecal samples through DNA extraction and PCR. One gram of each faecal sample was processed for extraction of DNA to detect various genes in PCR assay. The procedure for extracting bacterial DNA was as described previously under *Escherichia coli*.

3.3.3 Polymerase chain reaction

Table 3.19: List of *Salmonella* primers for amplification of gene transcripts

Sr. No.	Primer Name	Primer Sequences
1.	**Sal-f**	**5'- TGTTGTGGTTAATAACCGCA-3'**
	Sal-r	**5'- CACAAATCCATCTCTGGA-3'**
2.	**Ent-f**	**5'TGTGTTTTATCTGATGCAAGAGG-3'**
	Ent-r	**5'- TGAACTACGTTCGTTCTTCTGG-3'**
3.	**Typh-f**	**5'- TTGTTCACTTTTTACCCCTGAA-3'**
	Typh-r	**5'- CCCTGACAGCCGTTAGATATT-3'**
4.	**Inv-f**	**5'- TTGTTACGGCTATTTTGACCA-3'**
	Inv-r	**5'- CTGACTGCTACCTTGCTGATG-3'**
5.	**Sef-f**	**5'- GCAGCGGTTACTATTGCAGC-3'**
	Sef-r	**5'- TGTGACAGGGACATTTAGCG-3'**
6.	**Stn-f**	**5'- TTGTGTCGCTATCACTGGCAACC-3'**
	Stn-r	**5'- ATT CGT AAC CCG CTC TCG TCC-3'**

Table 3.20: Annealing temperatures and respective amplicon sizes for different *Salmonella* gene primers

Primer No.	Name of Primer	T_a (^{0}C)/45sec	Amplicon Size
Primer 1	Sal(18SrRNA)	51.4	544bp
Primer 2	Ent.(*Sal.enteritidis*)	57.5	304bp
Primer 3	Typhi (Sal.typhi)	54.5	401bp
Primer 4	Inv (Invasion gene)	58.5	521bp
Primer 5	Sef (enteritidis fimbriae)	59.5	330bp
Primer 6	Stn (enterotoxin of salmonella)	58.5	617bp

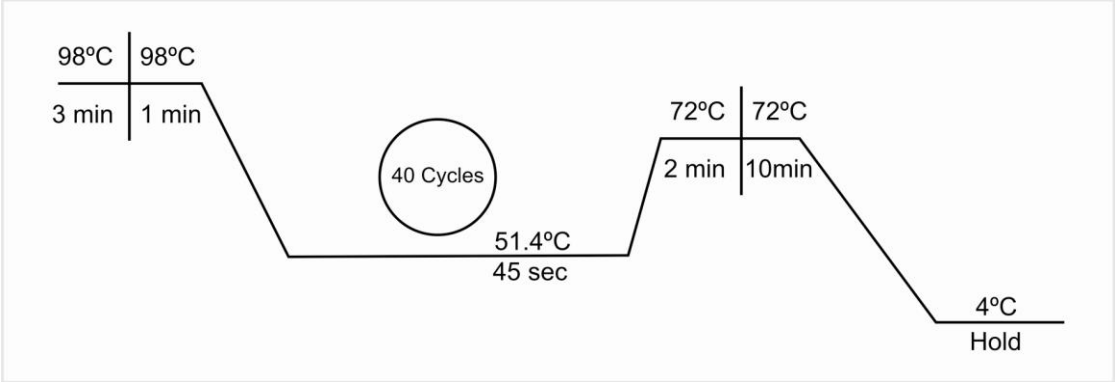


Figure 3.3: PCR profile for detection of Salmonella gene (Sal 18SrRNA)

The PCR assays were carried out in 25 µl reaction volumes containing 1 U of Taq polymerase, 200 µmol of each dNTP and 2.5 µl of 10× PCR buffer.

Reactions were performed in a GeneAmp^{R} PCR System 2400 Thermal Cycler (Applied Biosystems, Foster City, California) and a FlexiGene^{R} thermal cycler (Techne Inc., Princeton, New Jersey). Oligonucleotide primers were obtained from Thermo ^{R} (USA), and details of the primer sequences are given in Table 3.3.3. The DNA extracted from 18SrRNA as genus specific, served as positive controls for screening other genes. Sterile distilled water was used as negative control.

Experiment 4

3.4: Detection of Cryptosporidium

3.4.1 Faecal samples

For the study of the prevalence of cryptosporidiosis, faecal samples of buffalo calves and dams were collected from the buffalo farm in the department of livestock production management, LUVAS, Hisar.The faecal samples were collected directly from the rectum in a plastic container with a detailed history about age group, diarrhoea status and the sex and the particulars of individual animals were labelled on the container. Each sample was assessed macroscopically to establish its consistency as liquid, soft or solid, and the presence of mucus or blood was noted. The samples were then stored at 4 °C in a refrigerator until examination as stipulated by Garcia *et al.*, (1983).

3.4.2: DNA extraction for detection of *Cryptosporidium*

Cryptosporidium were detected from the faecal samples through DNA extraction and PCR. One gram of each faecal sample was processed for extraction of DNA to detect various genes in PCR assay. The procedure for extracting bacterial DNA was as described previously.

Table 3.21:List of Cryptosporidium primers for amplification of gene transcripts (Laberge *et al.*, 1996)

Sr. No.	Primer Name	Primer Sequences
1.	Forward primer 1	5'-GCC CAC CTG GAT ATA CAC TTT C -3'
	Reverse primer 1	5'-TCC CCC TCT CTA GTA CCA ACA GGA - 3'
2.	Forward primer 2	5'-CCG AGT TTG ATC CAA AAA GTT ACG AA -3'
	Reverse primer 2	5'- TAG CTC CTC ATA TGC CTT ATT GAG TA -3'
3.	SSU-F2:	5'- TTCTAGAGCTAATACATGCG -3'
	SSU-R2:	5'-CCCATTTCCTTCGAAACAGGA -3'

Table 3.22: Annealing temperatures and respective amplicon sizes for different *Cryptosporidium gene* primers

Primer No.	Name of Primer	T_a (^{0}C)/2min	Amplicon Size
Primer 1	Cryptosporidium(F1/R1)	46.3	1325bp
Primer 2	SSU-F2R2 (SSU16SrRNA)	52.0	525bp
Primer 3	Cryptosporidium(F2/R2)	54.0	525bp

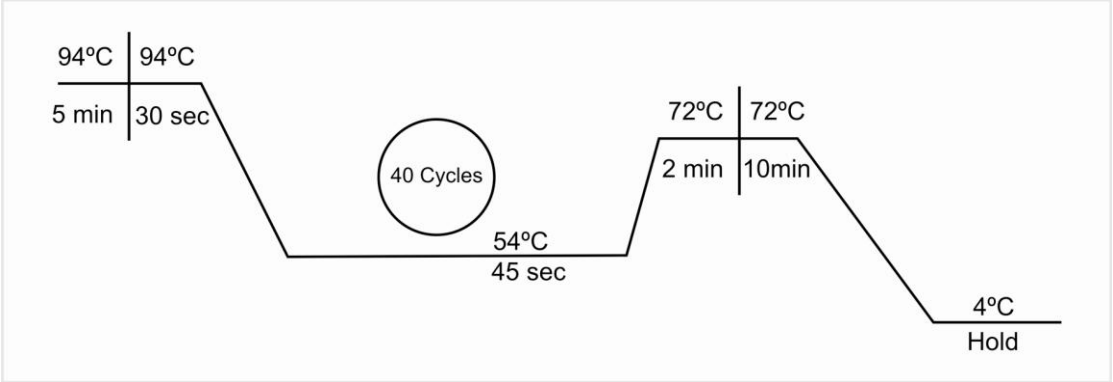


Figure 3.4: PCR profile for detection of Cryptosporidium genes

Experiment 5

3.5 Detection of Rotavirus in faecal samples

3.5.1 Faecal samples collection

A total of 78 faecal samples comprising 38 samples from buffalo calves and 40 samples from buffalo dams with diarrhoea and without diarrhea of less than 6 months of age were collected from the farm in the Department of Livestock Production Management (LPM), LUVAS, Hisar. The samples were stored at -20°C for extraction of viral RNA. A 10% suspension of each faecal sample was prepared in lysis buffer. The suspension was vortexed for 10 min followed by centrifugation at 10,000×g (10,000 rpm) for 15 min at 4°C to remove coarse particles and cellular

debris. The clarified supernatant was transferred into sterilized vial and stored at −20°C or processed for RNA extraction.

3.5.2 Isolation of RNA by TRIZOL^{R} method

An aliquot of 1 ml of Trizol (Invitrogen) was added to the DNase and RNase treated faecal sample pellet, vortexed immediately for one minute until the pellet was dissolved. The suspension was transferred to 1.5 ml eppendorf tube. A volume of 200 µl of chloroform was added to the suspension, vortexed until the pink cloudy solution was formed and then incubated on ice for 10 minutes. The solution was centrifuged for 10 minutes at 12000 rpm.

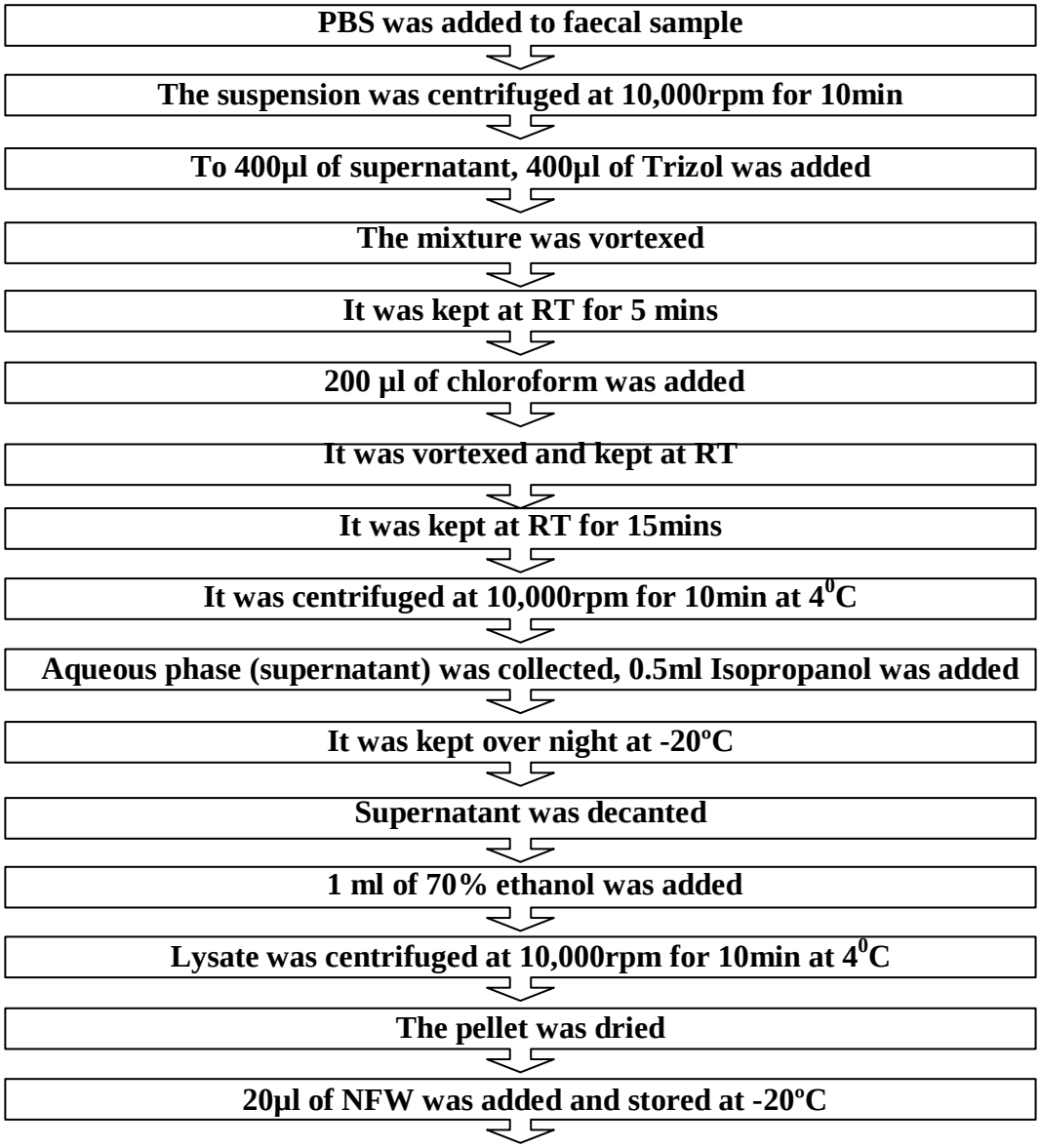


Figure 3.5: Flow chart showing RNA extraction from faecal samples

After the sample was separated into three distinct layers, the clear top layer (approximately 400-500 µl) was transferred to a labelled eppendorf tube using a

pipette. The remaining supernatant containing Trizol was discarded in accordance with health and safety guidelines.

Using a pipette tip, a volume of 500 µl isopropanol was added to all the eppendorf tube and vortexed to mix. The sample was incubated at -20°C, over-night. Next day the sample was centrifuged for 30 minutes at 12000 rpm. The supernatant was discarded into disinfectant leaving behind the visible pellet. Washing of the RNA pellet was done using 70% solution of ethanol. The viral RNA was re-pelleted by centrifugation at 12000 rpm for 10 minutes. Again the supernatant was discarded and a pipette was used to remove any excess ethanol. The resultant pellet was air dried and then dissolved in 20 µl RNase free water.

3.5 .4 RNA-PAGE

The segmented dsRNA genome of the virus was analyzed by RNA-PAGE using the discontinuous buffer system of Laemmli (1970) without SDS. Eight percent resolving gel and five percent stacking gel was prepared by adding the reagents sequentially. The resolving gel solution was poured in the gel casting plates assembled in the gel caster. One ml of glass distilled water (GDW) was overlaid on the top of the gel to prevent surface drying.

After polymerization of the resolving gel, water layer was removed and the stacking gel solution was overlaid on to the resolving gel. Subsequently the comb was put in the stacking gel solution and was left undisturbed till the gel solidifies. The samples were loaded after removing the comb.

Table 3.23: Stock solutions for casting the polyacrylamide gel (Sambrook *et al.*, 2001)

Sr. No.	Stock solution (Appendix II)	8% Resolving gel	5% Stacking gel
1.	30% Acrylamide/Bisacrylamide	6.7 ml	1.0 ml
2.	1.5 M Tris HCl pH 8.8	6.3 ml	-
3.	0.5 M Tris HCl pH 6.8	-	0.75ml
4.	TEMED 15 µl 6.0 µl	15 µl	6.0 µl
5.	APS (10%) 250 µl 60 µl	250 ul	60 ul
6.	Glass distilled water 11.5 ml 4.1 ml	11.5 ml	4.1 ml

The viral dsRNA extracted by Trizol method was dissolved in 2X RNA-PAGE sample buffer by heating at 56^0C for 5-10 min and the samples were loaded into the wells. The electrophoresis was carried out at a constant voltage of 100 V/cm in 1X Tris glycine buffer till the dye came out of the gel.

3.5.1 Silver staining of the gel

The gel was stained by silver nitrate following the method of Svensson *et al.* (1986).

The gel was removed from the plates after the run and marked at the lower left corner for identification of the lanes. Subsequently, the gel was placed in the fixative solution (Appendix II) for 30 min at room temperature (25^0 C) with gentle shaking on the shaker. The fixative was removed and the gel was stained (Appendix II) for 30 min on gentle shaking platform. The staining solution was drained off and the gel was quickly and thoroughly rinsed with GDW twice to remove the excess silver nitrate to eliminate the chances of background staining. RNA bands were visualized in developer solution (Appendix II) by manual shaking. The reaction was stopped by the stop solution (Appendix II) in which gel was kept for 15 min and then stored in 10 % ethanol.

3.6.1 RT-PCR

The cDNA of the sample was in turn used as template for group specific PCR. The reaction conditions for reverse transcription and PCR, such as concentration of MgCl2, dNTPs, Taq DNA polymerase (Fermentas), dimethylsulfoxide (DMSO), denaturation, annealing and extension temperatures and number of cycles were used as per the standardized protocol, to get the desired specific product. The extracted RNA (2 µl) was added to 0.2-ml thin-walled PCR tubes containing 1.5 µl of dimethyl sulfoxide, 30 pmol of primers Bov9com5 and Bov9com3 and 7.9 µl of DEPC-treated water. The RNAwas denatured at 65°C for 5 min and snap chill on ice. To the denatured RNA, 8 µl of reaction mixture containing 4 µl of 5× reaction buffer, 1 µl of RNAse inhibitor (20 U/µl), 2 µl of 10 mM dNTP mix and 1 µl of M-MuLV reverse transcriptase were added. The mixture was incubated at 25°C for 10 min for annealing. Incubation temperature was raised to 42°C for reverse transcription for 60 min in thermal cycler. M-MuLV reverse transcriptase was heat inactivated by increasing the incubation temperature to 70°C for 10 min. The cDNAwas stored at −20°C tillfurther use.

Table 3.24: Ingredients for standardized cDNA synthesis reaction

Sr. No.	Reagents	Volume/20µl
1	Oligo dT	1.0 µl
2	NFW	8.0 µl
3	Template RNA	3.0 µl
4	5×Reaction buffer	4.0 µl
5	Rileolock RNase Inhibitor	1.0 µl
6	10mM dNTPs	2.0 µl
7	Revert Aid M muLVRT	1.0 µl

3.6.2 Protocol for cDNA synthesis

Reverse transcription was carried out in a 20 µl reaction mixture using following protocol.Viral dsRNA was 3.0 µl (Approx. 1.2 µg / µl), DMSO was 1.5 µl, Random primers was 0.5 µl (30 pmol), Nuclease free water (NFW) was 5.0 µl in atotal Volume of 10 µl. The mixture was heated at 99^0C for 5 min in thermalcycler (Biorad i-cycler and eppendorf master cycler gradient TM, Germany), snap chilled on ice and then the following reagents were added: Mo-MuLV-RT 1.0 µl (200 units / µl), 5X RT buffer, 5.0 µl, 100 mM dNTPs, 0.5 µl and 3.5 µl NFW in a total Volume of 10 µl. After allowing the primers to anneal at 25ºC for 5 min, reverse transcription was carried out at 40ºC for 60 min in thermal cycler. The reverse transcriptase was heat inactivated at 70ºC for 5 min.

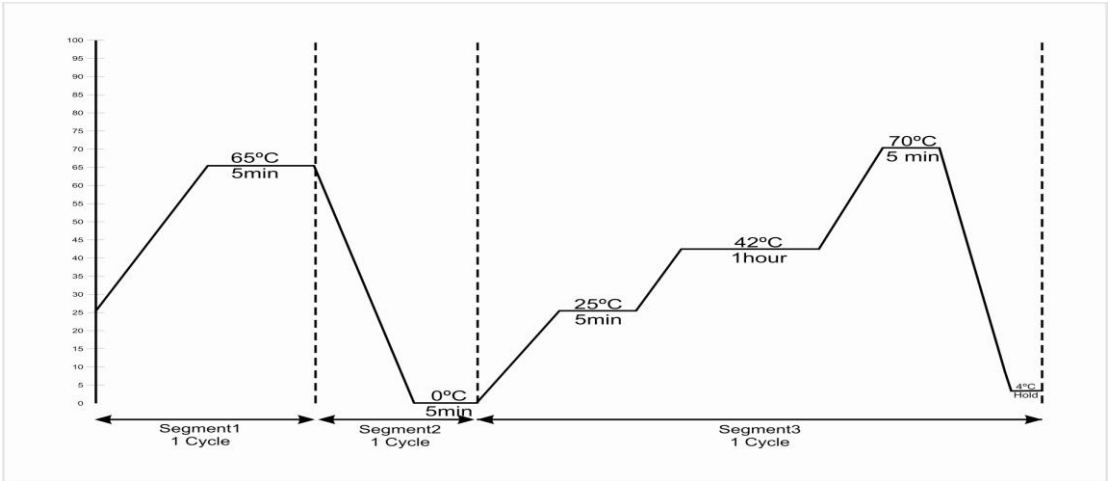


Fig. 3.6: Cyclic conditions for cDNA synthesis

3.6.3 Polymerase chain reaction (PCR)

The genome segment 9 (VP7 gene) specific primer sequences were used for RT-PCR amplification (Table 3.25). In this study, Bov9Com5 and Bov9Com3 primer

pairs were used, because these were specific for amplification of bovine rotavirus and the expected product 1,013 bp for VP7 gene.

Table 3.25: List of Rotavirus primers for amplification of gene transcripts

Sr. No.	Primer Name	Primer Sequences
1.	Bov9com5 Forward primer	5'- TGTATG GTATTG AATATA CCA C-3'
2.	Bov9com3 Reverse primer	5'-TCA CAT CATACA ACT CTA ATC T -3'
3.	G6	5'-CTA GTT CCT GTG TAG AAT C-3'
4.	G8	5'-CGG TTC CGG ATTAGA CAC-3'
5.	G10	5'-TTC AGC CGT TGC GAC TTC-3'

3.7 Agarose gel electrophoresis (AGE) for PCR product of Rotavirus gene

The PCR products were resolved in 1.0 % agarose (LifeTech) gel containing 0.5 µg ethidium bromide (Sigma) per ml in tris-acetate-EDTA (TAE) buffer (Appendix III) along with 1kbp DNA ladder (MBI Fermentas). The 3 µl of PCR product was mixed with 1 µl of 6X loading dye and was loaded in the wells. The electrophoresis was carried out at 12 V/cm of gel in 1X TAE running buffer in horizontal electrophoresis unit (Biometra, USA) and power supply (Pharmacia) till the indicator 6X loading dye reached last third of the gel. The gels were visualised under UV transilluminator (Biovis) and photographed. The expected size of PCR products were estimated by comparison with that of standard DNA ladder.

Experiment 6

3.6 Detection of Coronavirus

3.6.1 Faecal sample collection

A total of 78 faecal samples comprising 38 samples from buffalo calves and 40 samples from buffalo dams with diarrhoea and without diarrhea of less than 6 months of age were collected from the farm in the Department of Livestock Production Management (LPM), LUVAS, Hisar. The samples were stored at -20°C for extraction of viral RNA. A 10% suspension of each faecal sample was prepared in lysis buffer. The suspension was vortexed for 10 min followed by centrifugation at 10,000×g (10,000 rpm) for 15 min at 4°C to remove coarse particles and cellular debris. The clarified supernatant was transferred into sterilized vial and stored at −20°C or processed for RNA extraction.

3.6.2 RNA extraction for detection of Coronavirus

To faecal sample add PBS to make a suspension and centrifuge at 10,000rpm for 10mins. Collect 400µl of supernatant and add 400µl of Trizol^{R}. Vortex and Keep it for 5 minutes. Add 200µl of chloroform and vortex. Keep it for 15minutes and

centrifuge afterwards at 10,000rpm for 10minute at 4^0C. Collect agueous phase (supernatant), add isopropanol (0.5 ml) and keep over right at -20^0C. Decant the supernatant and add 1 ml of 70% ethanol. Centrifuge at 10,000rpm for 10min at 4^0C. Dry the pellet and add 20ul of NFW and store at - 20^0C.

3.6.2.1 Isolation of RNA by Trizol^R method

An aliquot of 1 ml of Trizol (Invitrogen) was added to the DNase and RNase treated faecal sample pellet, vortexed immediately for one minute until the pellet was dissolved. The suspension was transferred to 1.5 ml eppendorf tube. A volume of 200 µl of chloroform was added to the suspension, vortexed until the pink cloudy solution was formed and then incubated on ice for 10 minutes. The solution was centrifuged for 10 minutes at 12000 rpm.

After the sample was separated into three distinct layers, the clear top layer (approximately 400-500 µl) was transferred to a labelled eppendorf tube using a pipette. The remaining supernatant containing Trizol was discarded in accordance with health and safety guidelines.

Using a pipette tip, a volume of 500 µl isopropanol was added to all the eppendorf tube and vortexed to mix. The sample was incubated at -20°C, over-night. Next day the sample was centrifuged for 30 minutes at 12000 rpm. The supernatant was discarded into disinfectant leaving behind the visible pellet. Washing of the RNA pellet was done using 70% solution of ethanol. The viral RNA was re-pelleted by centrifugation at 12000 rpm for 10 minutes. Again the supernatant was discarded and a pipette was used to remove any excess ethanol. The resultant pellet was air dried and then dissolved in 20 µl RNase free water.

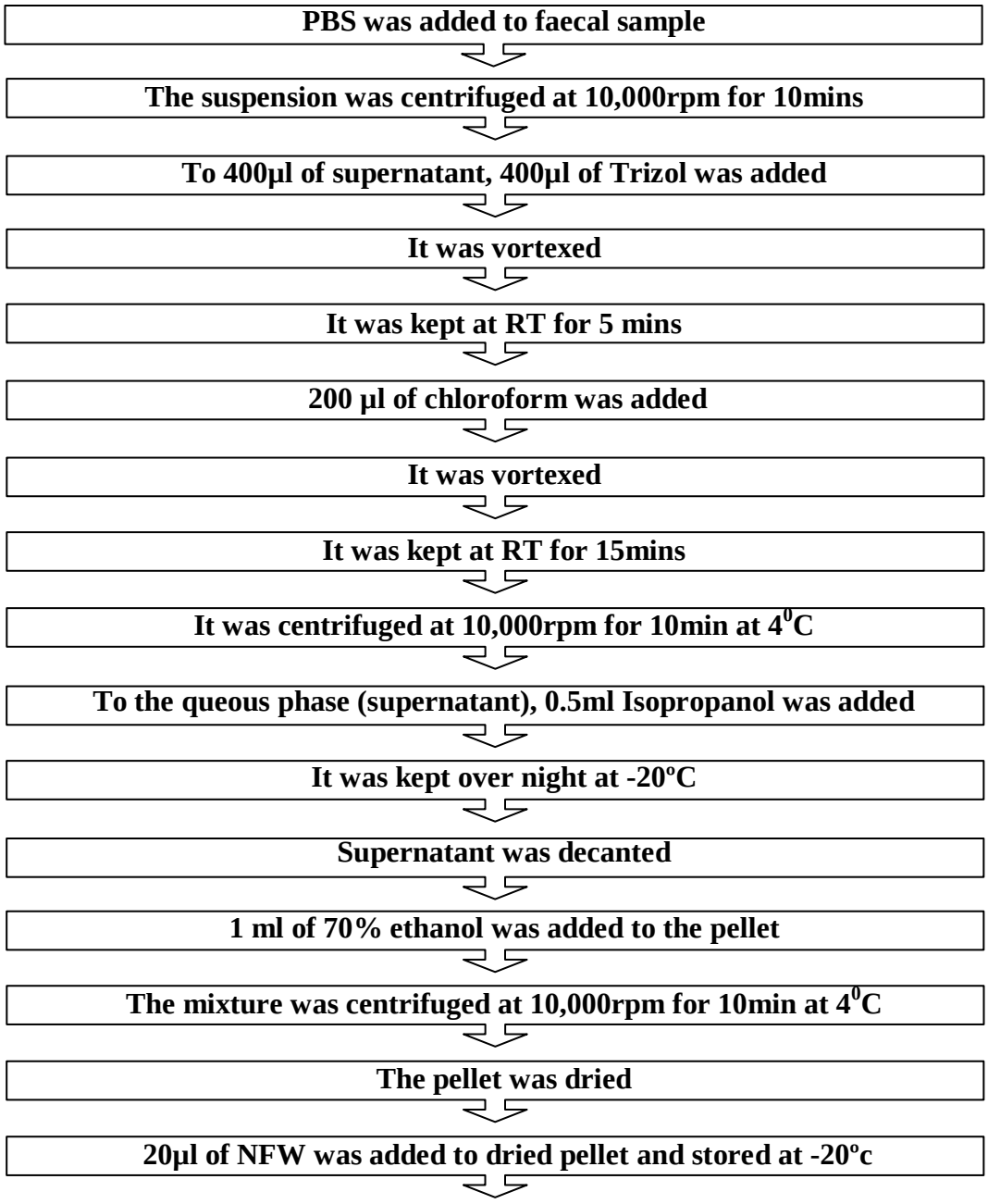


Figure3.7: Flow chart showing RNA extraction from faecal samples

Table 3.26: List of Corona virus primers for amplification of gene transcripts

Sr. No.	Primer Name	Primer Sequences
1.	BCoVs[2]	5'- GCA ATC CAG TAG TAG AGC GT-3'
2.	BCoVas[2]	5'- CTT AGT GGC ATC CTT GCC AA -3'
3.	Con2[3]	5'- ATT TCG GAC CAT TTA TAA CC -3'
4.	Con3[3]	5'- TGG CTT CGC TCA TTT ATA GAC A - 3'

3.6 .3 RNA-PAGE

The segmented dsRNA genome of the virus was analyzed by RNA-PAGE using the discontinuous buffer system of Laemmli (1970) without SDS. The gel was stained with silver nitrate as described by Svensson *et al.* (1986). Eight percent resolving gel and five percent stacking gel was prepared by adding the reagents sequentially as listed in the Table 3.1. The resolving gel solution was poured in the gel casting plates assembled in the gel caster. One ml of glass distilled water (GDW) was overlaid on the top of the gel to prevent surface drying.

After polymerization of the resolving gel, water layer was removed and the stacking gel solution was overlaid on to the resolving gel. Subsequently the comb was put in the stacking gel solution and was left undisturbed till the gel solidifies. The samples were loaded after removing the comb.

Table 3.27: Stock solutions for casting the polyacrylamide gel (Sambrook *et al.*, 2001)

Sr. No.	Stock solution (Appendix II)	8% Resolving gel	5% Stacking gel
1.	30% Acrylamide/Bisacrylamide	6.7 ml	1.0 ml
2.	1.5 M Tris HCl pH 8.8	6.3 ml	-
3.	0.5 M Tris HCl pH 6.8	-	0.75ml
4.	TEMED 15 µl 6.0 µl	15 µl	6.0 µl
5.	APS (10%) 250 µl 60 µl	250 ul	60 ul
6.	Glass distilled water 11.5 ml 4.1 ml	11.5 ml	4.1 ml

The viral dsRNA extracted by Trizol method was dissolved in 2X RNA-PAGE sample buffer (Appendix II) by heating at 56^0C for 5-10 min and the samples were loaded into the wells. The electrophoresis was carried out at a constant voltage of 100 V/cm in 1X Trisglycine buffer (Appendix II) till the dye came out of the gel.

3.5.1 Silver staining of the gel

The gel was stained by silver nitrate following the method of Svensson *et al.* (1986).

The gel was removed from the plates after the run and marked at the lower left corner for identification of the lanes. Subsequently, the gel was placed in the fixative solution (Appendix II) for 30 min at room temperature (25^0 C) with gentle shaking on the shaker. The fixative was removed and the gel was stained (Appendix II) for 30 min on gentle shaking platform. The staining solution was drained off and the gel was quickly and thoroughly rinsed with GDW twice to remove the excess silver nitrate to

eliminate the chances of background staining. RNA bands were visualized in developer solution (Appendix II) by manual shaking. The reaction was stopped by the stop solution (Appendix II) in which gel was kept for 15 min and then stored in 10 % ethanol.

3.6.4 RT-PCR

The cDNA of the sample was in turn used as template for group specific PCR. The reaction conditions for reverse transcription and PCR, such as concentration of MgCl2, dNTPs, Taq DNA polymerase (Fermentas), dimethylsulfoxide (DMSO), denaturation, annealing and extension temperatures and number of cycles were used as per the standardized protocol, to get the desired specific product. The extracted RNA (2 µl) was added to 0.2-ml thin-walled PCR tubes containing 1.5 µl of dimethyl sulfoxide, 30 pmol of primers and 7.9 µl of NFW. The RNAwas denatured at 65°C for 5 min and snap chill on ice. To the denatured RNA, 8 µl of reaction mixture containing 4 µl of 5× reaction buffer, 1 µl of RNAse inhibitor (20 U/µl), 2 µl of 10 mM dNTP mix and 1 µl of M-MuLV reverse transcriptase were added. The mixture was incubated at 25°C for 10 min for annealing. Incubation temperature was raised to 42°C for reverse transcription for 60 min in thermal cycler. M-MuLV reverse transcriptase was heat inactivated by increasing the incubation temperature to 70°C for 10 min. The cDNAwas stored at −20°C till further use.

Table 3.28: Ingredients for standardized cDNA synthesis reaction

Sr. No.	Reagents	Volume/20µl
1	Oligodt	1.0 µl
2	NFW	8.0 µl
3	Template RNA	3.0 µl
4	5×Reaction buffer	4.0 µl
5	Rileolock RNase Inhibitor	1.0 µl
6	10mM dNTPs	2.0 µl
7	Revert Aid M muLVRT	1.0 µl

3.6.5 cDNA synthesis

Reverse transcription was carried out in a 20 µl reaction mixture using following protocol.Viral dsRNA- 3.0 µl (Approx. 1.2 µg / µl), DMSO -1.5 µl, Random primers - 0.5 µl (30 pmol), Nuclease free water (NFW)-5.0 µl, Total Volume-10 µl. The mixture was heated at 99^0C for 5 min in thermalcycler (Biorad i-

cycler and eppendorf master cycler gradient TM, Germany), snap chilled on ice and then the following reagents were added. Mo-MuLV-RT-1.0 µl (200 units / µl), 5X RT buffer - 5.0 µl, 100 mM dNTPs , 0.5 µl, NFW -3.5 µl in total volume of 10 µl. After allowing the primers to anneal at 25ºC for 5 min, reverse transcription was carried out at 40ºC for 60 min in thermal cycler. The reverse transcriptase was heat inactivated at 70ºC for 5 min.

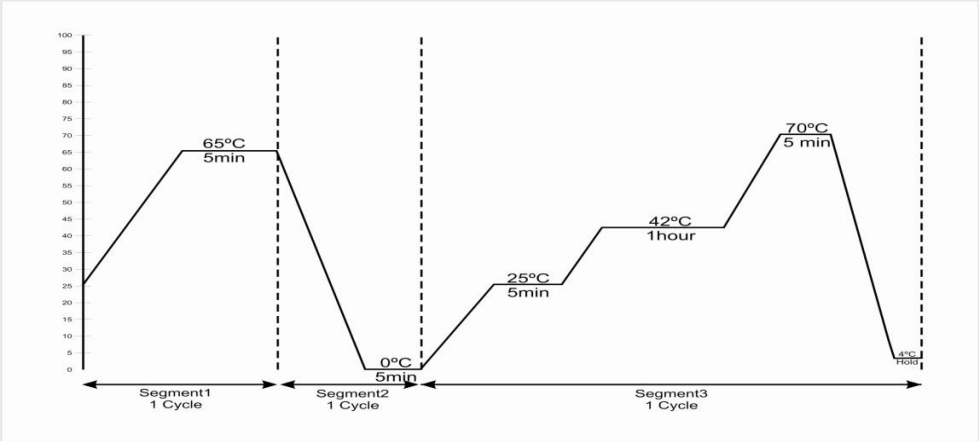


Fig. 3.8: Cyclic conditions for cDNA synthesis

OBJECTIVE NUMBER 2

To determine total immunoglobulin concentrations from the sera, oral fluid, urine and faeces of buffalo calves and from the colostrum and milk of their respective dams for assessing the success of passive immunization in the calves.

Experiment 1

3.7 Colostrum/milk sample collection

An overall of 40 buffalo dams were tested for detection of IgG in various samples. Buffalo dams enrolled in the study were from the 1st to 6th lactations of production. Colostrum and milk samples collected were from the Murrah breed of the buffaloes maintained in the farm of the department of Livestock production management. Colostrum samples were collected alongside the collection of other samples in the dams, including sampling of calves within the first 2 days of calving. Milk samples were collected during control evening milking time. After cleaning and disinfection of the teats, 15 ml of colostrum and milk were aseptically collected in sterile plastic tubes. Samples were kept under refrigeration until arrival to laboratory facilities and were tested within afterwards.

3.8 Processing of colostrum for precipitation of IgG

Colostrum and milk samples were centrifuged at 4000 rpm for 60 min at 4°C and the fat layer that appears on top with spatula was removed. Pipette tip was used to punch layer for decanting colostrum samples after centrifugation. The pH of colostrum was lowered to 4.6 with 1ml of 0.1 N HCl to remove caseins and the sample was incubated at 37 ^{0} C for 30 minutes. The sample was centrifuged again at 4000 rpm at 4°C for 30 minutes to spin out the precipitate. The whey colostrum and milk were collected in separate tubes. The pH was of the supernatants (colostrum) was adjusted to neutral (7.4) by adding 1 ml 0.1N NaOH.

Expreiment 2

3.9 Processing of milk for precipitation of IgG

Milk sample was centrifuged at 4000 rpm for 60 min at 4°C and a fat layer that appears on top was removed with spatula. Pipette tip was used to punch the fat layer for decanting the milk sample. The pH of the milk was lowered to 4.6 with 1ml of 0.1 N HCl to remove caseins and afterwards the sample was incubated at 37 ^{0} C for 30 minutes. The sample was centrifuged again at 4000 rpm at 4°C for 30 minutes to spin out the precipitate. The whey milk was collected in separate tubes. The pH of the supernatants (Milk) was re-adjusted to neutral (7.4) by adding 1 ml 0.1N NaOH.

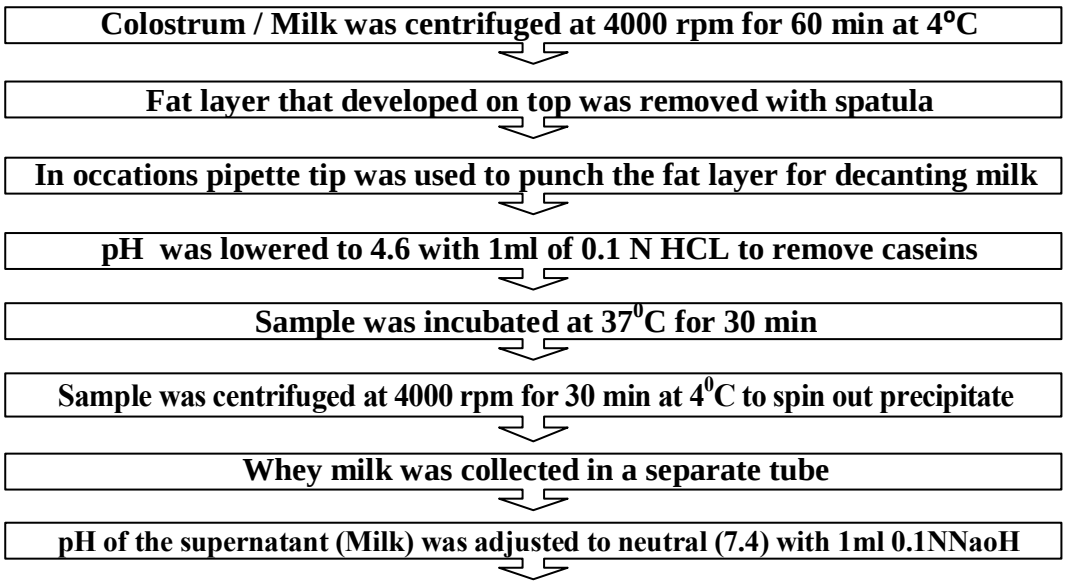


Figure 3.9: Flow chart showing processing of colostrums/ milk samples for precipitation of bovine IgG

Experiment 3

3.11 Extraction of serum from blood sample

Blood sample was collected from buffalo calves and dams. The sample was placed into a vacutainer tube without an anticoagulant.The tube was positioned in a slanting position for 1 hour to allow clothing of blood. Separated serum was collected from cloted blood in a fresh tube and the clot was centrifuged again at 3,000rpm for 10 minutes for further serum extraction. Collected serum was stored at -20ºC.

Experiment 4

3.12 Processing of faecal samples for precipitaction of Bovine IgG

One gram (1g) (wet/weight) of faeces was collected in sample scoop and extraction buffer (0.01 M PBS, PH 7.4), 0.5 % tween 20 and 0.05% sodium azide was added at the ratio of 10 ml of buffer to 1g of faeces. Feacal sample was centrifuged at 1,500 xg for 20 minutes at 5°C. Two milliliter (2 ml) of supernatant was transferred to a sterile eppendorf tube containing 20 µl of proteinase inhibitor cocktail (Sigma-Aldrich). The tube containing sample was briefly vortexed and centrifuged at 10,000x g for 10 min at 5°C. The supernatant was transferred to clean eppendorf tube and stored at -20°C till use.

Experiment 5

3.13 Method of precipitation and concentration of bovine IgG from Urine samples

Ten milliliter (10ml) of urine was collected in a test tube, centrifuged at 10,000 rpm for 10 minutes at 4°C. Seven milliliter (7.0 ml) of supernatantwas colleted in fresh tube and 3 ml of 20% polyethelene glycol-6000 (PEG-6000) was added to a final concentration of 6%. Mixture was stired properly and incubated at 4°C for 1-2 hours. Milkiness appeared in urine as a sign of Immunoglobulin – G (IgG) precipitation. The sample was further centrifuged at 10,000 rpm for 10 minutes at 4° C and the supernatant was re-suspended in 1.0 ml of phosphate buffered saline (PBS), PH 7.0. The solution was transferred to Eppendorf tube and store at -20° C until use (Garland, A. J. M (1974).

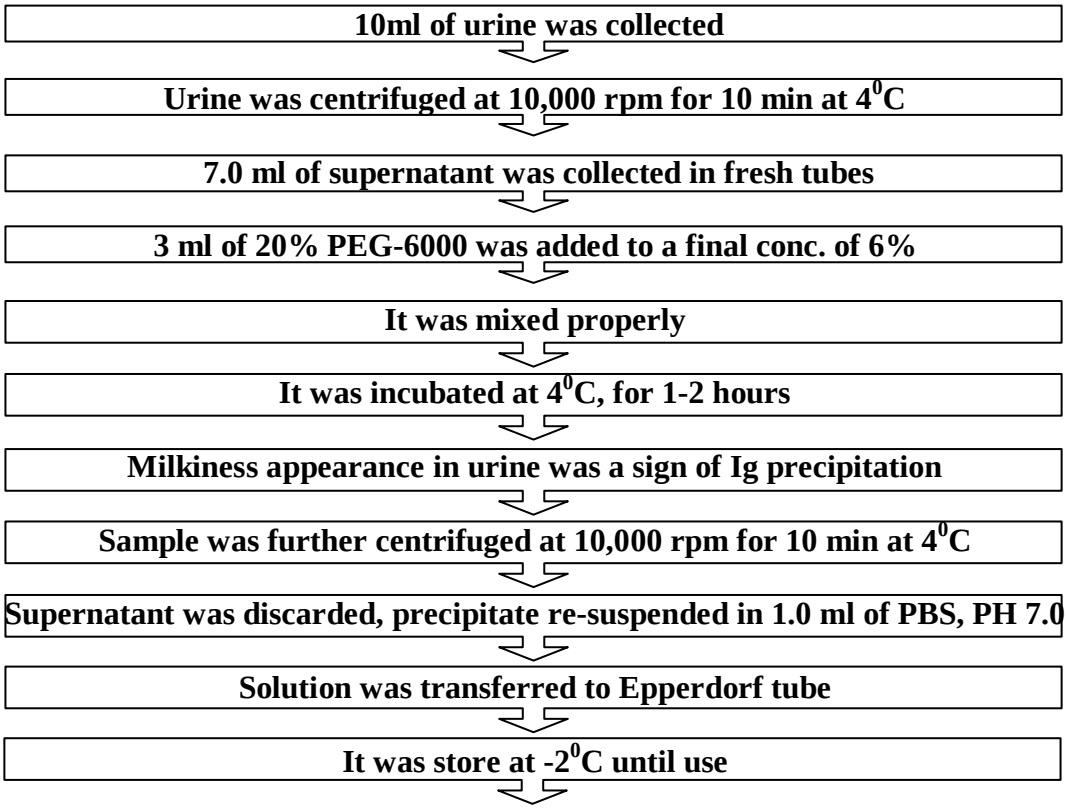


Figure 3.10: Flow chart showing precipitation and concentration of urine

Experiment 6

3.14 Methods of precipitation and concentration of bovine Ig G from oral fluid samples

Cotton swab containing oral fluids stored in phosphate buffered saline (PBS) was squeeded and the extracted volume was centrifuged at 10000 rpm for 10 min at 4°C. To seven milliliters (7.0ml) of supernatant in fresh tubes 3ml of 20% polyethelene glycol-6000 (peg-6000) was added to make a final concentration of 6%. Mixture was properly stirred and incubated at 4^{0}C for 1-2 hours. Milkiness appeared in oral fluid as a sign of IgG precipitation. The sample was centrifuged at 10,000rpm for 10 min at 4^{0}C. Discard The supernatant was discarded and precipitate was re-suspended in 1.0ml of PBS, pH 7.0. The solution was transferred to eppendorf tube and stored at -20°c until use (Garland, 1974).

Experiment 7

3.15 Determination of Concentration of bovine IgG by Bicinchoninic (BCA) Acid protocol

Bicinchoninic acid (BCA) working solution was prepared by mixing reagent A and B in 1:50 ratio. For the comparison, a standard solution of 2% BSA was taken and then serially diluted in the ELISA plate. Then the 25µl of serially diluted BSA were

transferred to the other wells in duplicates. Also, the IgG elutes were put in the wells in duplicates. To this, the BCA working solution was added 200µl.

The ELISA plate was incubated for one hour at 37ºC. Afterwards, the reading was taken on the spectrophotometer at A_{562} (BMG Labtech SPECTRO star). The graph was plotted forthe BSA standards, and the equation of the graph was obtained. The OD of the samples was substituted as follows (described formula), and the concentration of protein was determined.

Experiment 8

3.15 Estimation of protein concentration in pure bovine immunoglobulins (IgGs)

Two hundred milliliters (200ul/well) of freshly prepared Bicinconinic acid (BCA) regent was added into a 96 well plate. Also 25ul/well of protein/BSA suspension was added and mixed properly and incubated at 37^0C for colour development. The plate was cooled to room temperature (RT) for 30 minutes and reading was taken at A_{562} nm for optical density (O.D.). Standard curve was drawned in excel and determine protein concentration was determined.

Experiment 9

3.10 Sandwich ELISA for detection of Bovine IgG

The enzyme-linked immunosorbent assay (ELISA) used to determine IgG concentration in bovine biological fluids was carried out as follows. After antigen sensitizing, the wells were washed once with 200 µl of PBS containing 0.1% Tween 20 (PBS-T) and the reactive sites in the polystyrene wells were blocked with 100 µl of PBS-T containing 0.5% gelatin. After incubation for 1 hour at room temperature (RT) and washing thrice with PBS-T, 100 µL of urine, oral fluid, feaces, milk, clostrum and serum samples diluted at various log dilutions in PBS-T was added to the wells. Standards (100µl) in duplicate were added into each well and the plate was incubated at room temperature (RT) for 30 min. The wells were emptied by inverting the plate and tapping firmly onto absorbent tissue. The plate was washed in with 200 µl of diluted wash buffer per well, making three cycles of washing. After removing the excess liquid as described above, 100 µl of the diluted horseradish peroxidase (HRP) - antibody conjugate solution was added into each well and incubated at RT for 30 min. After this second incubation, the plate was washed as previously described and 100µl of the substrate OPD/H_2O_2 solution was added into each well. In order to allow the colour development, the plate was incubated at RT for 10 min. Finally, 50µl of the stop solution were added into each well. The concentration of antibodies in a sample was indicated by the yellow colour appearing during the reaction and turning brown after suppression of reaction with acid. The test

results were estimated by measuring the optical density (OD) of samples at wave length λ=492 nm using spectrophotometer Thermo Scientific Multiskan EX (Thermo electron corporation, China, 2005). In order to calculate the IgG concentration of each sample, a graphic representation was made by plotting the concentrations of the standards (y axis) versus the mean values of the corresponding absorbances (x axis) for each plate. The IgG concentration of the samples was determined by interpolating the corresponding absorbances in the standard curve, which was adjusted to a second-order polynomial equation.

Table 3.29: Optimized assay conditions of indirect (Sandwich) ELISA for detection of Bovine IgG from colostrum, milk, serum, saliva, faecal and urine samples

Step no.	Steps/reagents	Diluent/ buffer used	Volume/ Well (µl)	Time of incubation	Incubation Temperature
1	Capture Ab (Rabbit anti-bovine IgG ; Sigma (2.0µg/ml conc.)	1X PB (coating buffer)	100	O/N	4°C
2	Washing	PBST	200	3x3 min.	RT
3	Blocking	PBST-0.5% Gelatin	150	1 hrs.	RT
4	Washing	PBST	200	3x3 min.	RT
5	Test Samples/Controls (12mg/ml in positive control)	PBST	100	1 hr.	RT
6	Washing	PBST	200	3x3 min.	RT
9	Secondary (Rabbit) anti-bovine IgG-HRP conjugate conjugate) - Sigma	PBST	100	1 hr.	RT
10	Washing	PBST & Citrate phosphate buffer, pH 5.0 (CPB)	200 200	4x3 min. 1x3 min	RT
11	Substrate reaction /colour development (OPD/H_2O_2)	OPD (5 mg/10 ml CPB)/ H_2O_2 (2 µl)	100	10-15 min.	RT
12	Stop solution	4 N H_2SO_4	50	-	-

OBJECTIVE NUMBER 3

Experiment 1

To detect the presence of Toll-Like Receptor 4 (TLR4) and Caspase Activation and Recruitment Domain 15 (CARD15)/Nucleotide binding and Oligomerization Domain-2 (NOD2) as Pattern Recognition Receptor (PRR) for Lipopolysaccharide (LPS) and Peptidoglycan (PDG).

3.17 Isolation and maintenance of cells

Blood samples from both diarrheic and non-diarrheic calves of Day-1 to 2-days of age from buffalo farm in the department of livestock production management, LUVAS, Hisar, were collected in heparin coated vacutainers. The buffy coat was isolated by centrifugation (300 g for 8 min.) and diluted with Dulbecco phosphate buffer saline (DPBS). Lymphocyte-enriched peripheral blood mononuclear cells (PBMCs) from buffy coats were isolated by density gradient centrifugation using Histopaque- 1077 (Sigma-Aldrich, MO, USA). The interphase fraction was collected and washed twice with DPBS. The pellet was re-suspended in RNase later solution for protections against RNase before the procedure of RNA extraction.

Experiment 2

3.18 Extraction of peripheral blood mononuclear cells (PBMC's) from blood sample in anti-coagulant

Peripheral blood mononuclear cells (PBMC) were prepared from the buffy coat after the centrifugation of peripheral blood anticoagulated with EDTA.Three milliliters (3 ml) of histopaque in a 15ml sterile centrifuge tube was taken and kept in a stand. Three milliliters (3ml) of PBS diluted blood was gently overlaid to avoid mixing with the lower layer as histopaque is denser than blood. The mixture was entrifuged at 1,2000rpm for 30 minutes at 4^0 C. After centrifugation, the tubes were kept undisturbed in a stand. RBC's settled at the bottom after replacing histopaque. Histopaque and granulocytes were on top of RBC's, then plasma layer. PBMC's were seen as white layer suspended at interphase of histopaque and plasma. Up to 1 ml of this white layer was colleced with pipette tip and the collected cell volume was diluted to 12 ml with PBS in each tube.It was centrifuged at 3000rpm for 15 minutes at 4^0C. The supernatant was discarded and PBMC's were recovered. PBMCs' were re-suspended in 1 ml of RNA later solution and cell supernatants were collected and stored at -20°C till further use.

Experiment 3

3.19 RNA extraction from PBMC's

To 400µl of PBMC's in RNA later solution, 400ul of Trizol^{R} solution was added. The sample was vortexed and kept for 5 minutes. To the sample 200µl of chloroform was added and vortexed. It was kept for 15 minutes and centrifuged at 10,000rpm for 10 minutes at 4ºC. The aqueous phase (supernatant) was collected and 0.5ml of isopropanol was added. It was kept over night at -20ºC. The supernatant was decanted and 1 ml of 70% ethanol was added. It was centrifuged at 10,000rpm for 10 minutes at 4^0C. The pellet was dried and 20µl of NFW was added and store at -20^0C till use.

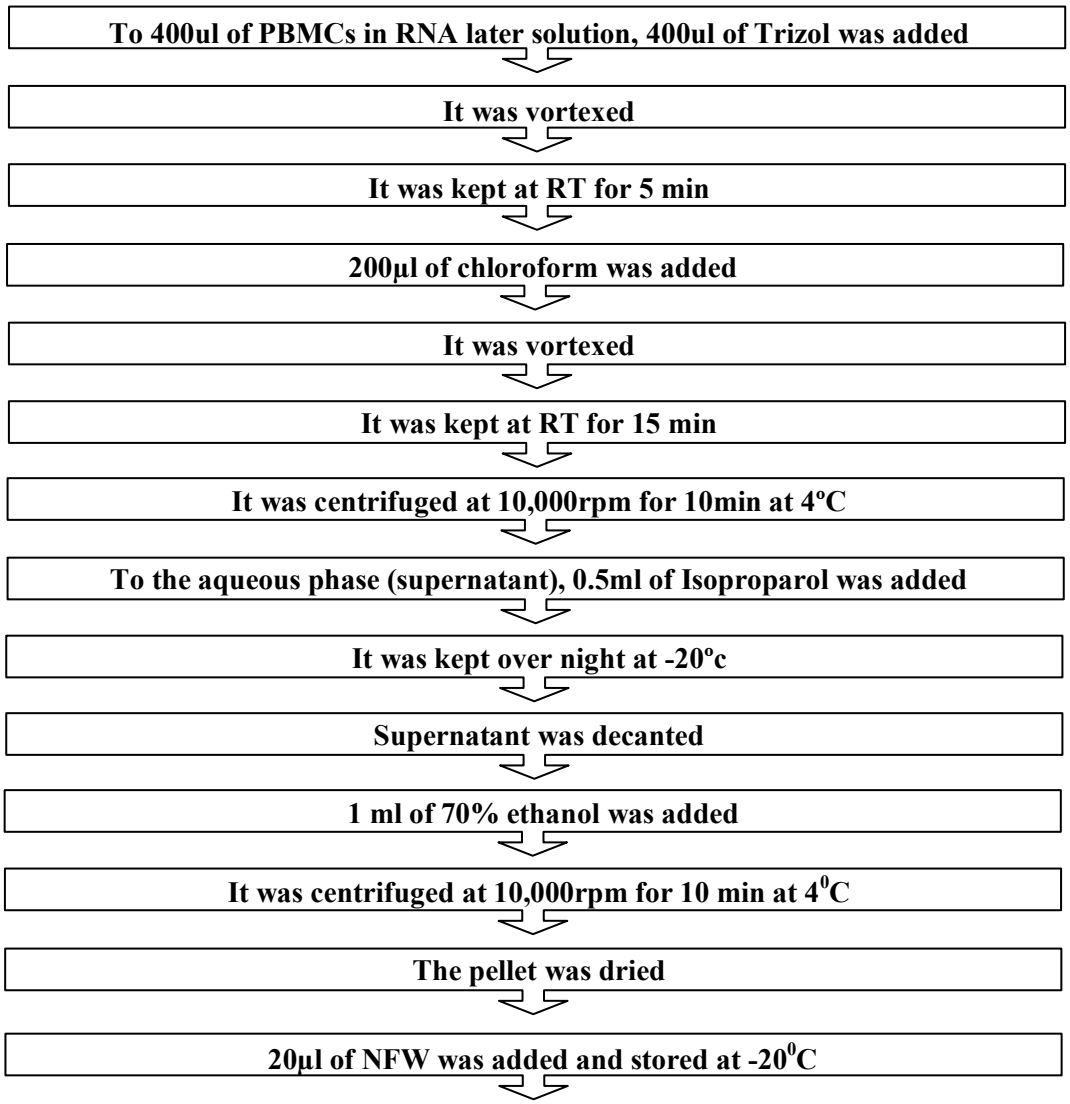


Figure 3.11: Flow chart showing RNA extraction from PBMC'S

Experiment 4

3.20 Extraction of colostral leucocytes from colostrum sample

Colostrum sample was centrifuged at 3000 rpm for 10 min at 4^0C. The supernatant was discarded and the pellet was collected. Pellet was re-suspended in 5 m Dulbeco's phosphate buffered saline (DPBS).Pellet was transfered to a fresh tube and volume was made up to 12 ml with PBS. Centrifuge at 3000 rpm for 10 min at 4^0C. Discard supernatant and re-suspend pellet of cells in 1ml of RNA later. Store at-20^0C.

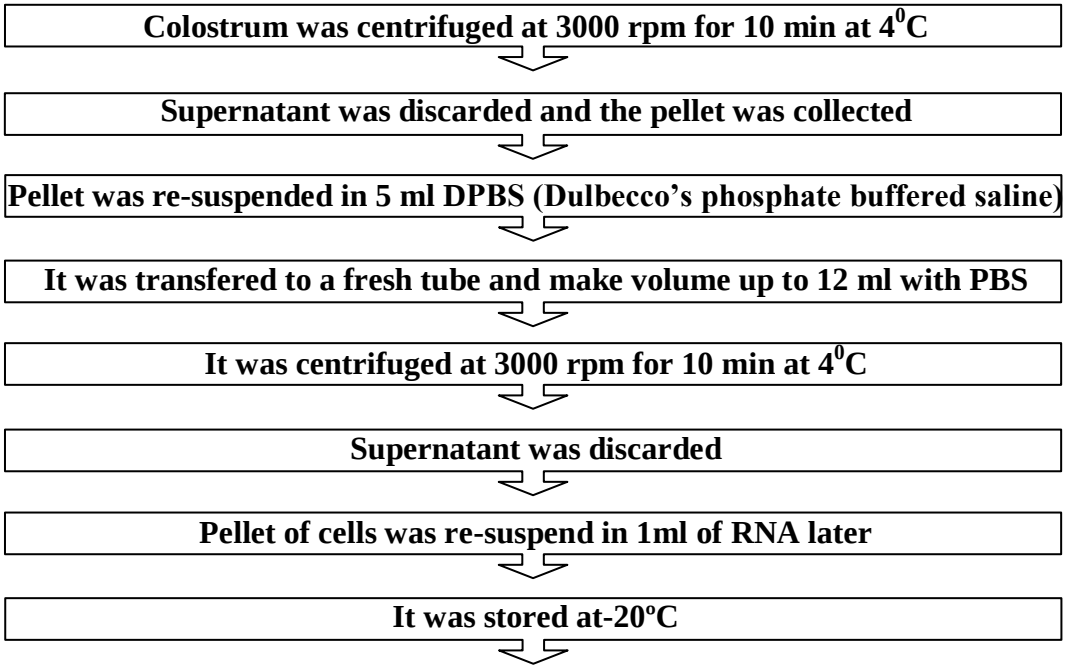


Figure 3.12: Flow chart showing extraction of colostral leukocytes from colostrum

Experiment 5

3.21 RNA extraction from colostral leucocytes

To 400µl of colostral leucocytes, 400µl of Trizol ^{R} solution was added. The mixture was vortexed and kept at RT for 5 minutes. Afterwards 200µl of chloroform was added and vortexed. It was kept at RT for 15 minutes and centrifuged at 10,000rpm for 10 minutes at 4ºC. To the aqueous phase (supernatant) 0.5 ml of isopropanol was added.It was kept over night at -20^0C. The supernatant was decanted and 1ml of 70% ethanol was added. It was centrifuged at 10,000rpm for 10 minutes at 4^0C. The pellet was dried and 20µl of NFW was added and store at -20^0C.

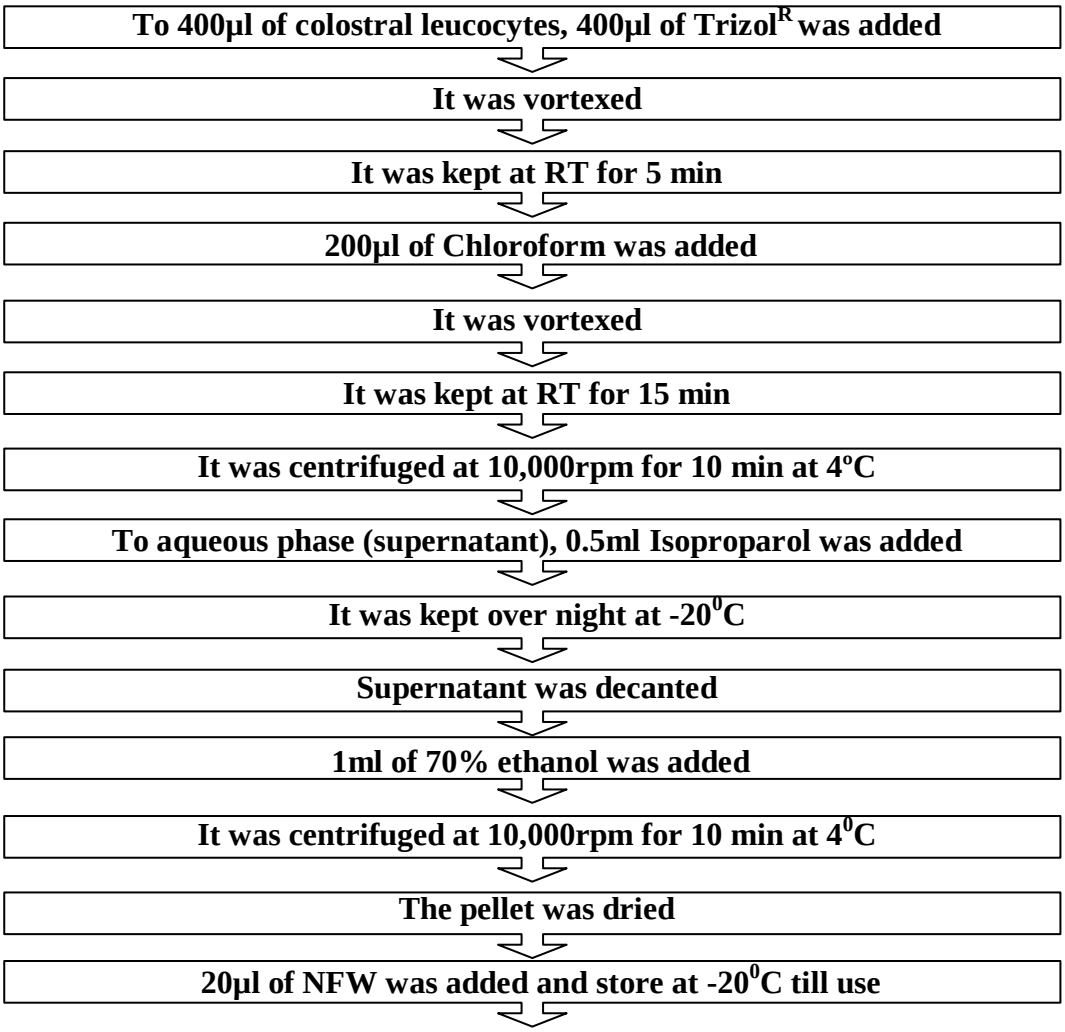


Figure 3.13: Flow chart showing RNA extraction from Colostral leucocytes

Table 3.30: Primers for TLR4

Sr. No.	Forward Primer	Reverse Primer
1	5' AGGCAGCCATAACTTCTCCA 3’	5’ GGTTGAGTAGGGGCATTTGA 3’
2	5' CAAGAAGCGACAACCTCCACCT 3'	5'CAGGCAGGAGAGGATGGCCGTGG3'
3	5' ATGCTTTCACAGAGCCACT 3'	5' GGTTGTCCCAAAATCAGTGT 3
4	5' AATGGATTGACTCTGCGAAG 3'	5' GGTCTGGGCAATCTCATACT 3'
5	5' CACTGTGCTCCTGGTGTCTGT 3'	5' GCGTACCACTGAATCACCA 3'

Table 3.31: Annealing temperatures and amplicon sizes for TLR4 gene primers

Primer No.	Name of Primer	T_a (^{0}C)/45 min	Amplicon Size
primer 5	TLR4-Forward/Reverse	54.0	800bp

Table 3.32: Primers for CARD15/NOD2

Sr. No.	Forward Primer	Reverse Primer
1	5' AGGCAGCCATAACTTCTCCA 3'	5'AAAGGCAGCCAACCCATTCG CCT TCAC 3'
2	5'ATTGTGAAATGTGCGCACAAGA TGCTTTTCAG3'	5'CGGCAGCTAAATGGGAAGAC GA AGAG 3'
3	5' GCAGACACTGTGCTGGTGGTG GG 3'	5'CTGTGATCTGGAGGTTGTGC GGC TC 3'
4	5'CTGCATTCTACCTCGCCCTCAG TGC 3'	5' GGAACATCAGAGCAAGAGT CTG GTATCC 3'
5	5' CTGCATTCTACCTCGCCCTCAG TGC3'	A5'GGAAACATCAGAGTCAA GAGTCGTCTGGTATCC3'

Table 3.33: Annealing temperatures and amplicon sizes for NOD 2/ CARD 15 gene primers

Primer No.	Name of Primer	T_a (^{0}C)/ 45sec	Amplicon Size
primer 1	NOD2/CARD 15 Forward/Reverse	58.0	200bp

Table 3.34: Ingredients for standardized NOD2/CARD 15 and TLR4 genes PCR reactions

Sr. No.	Reagents	Volume/24µl
1	NFW	15.0 µl
2	5×PCR Buffalo	5.0 µl
3	dNTPS mix, 10mM stock	0.5 µl
4	Gene specific primers	
i)	Forward	0.6 µl
ii)	Reverse	0.6 µl
5	Taq DNA polytmerase	0.3 µl
6	Template cDNA	2.0 µl

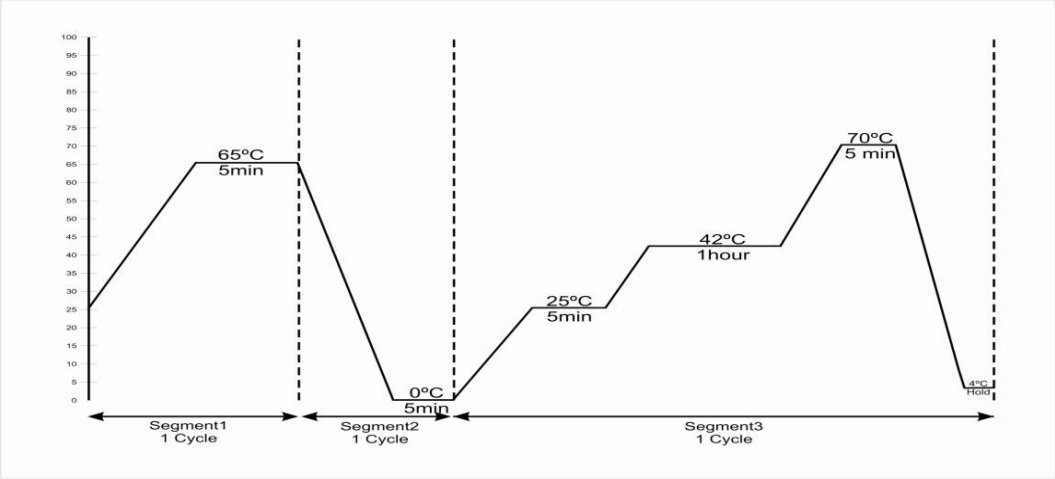


Fig. 3.14: Cyclic conditions for cDNA synthesis

Experiment 3.1 and 4.1

3.23 PCR amplification

Primers for full length gene of buffalo were obtained from published sequences. Total RNA from cells were isolated by Trizol method by following manufacturer's instructions.

About 1µg of RNA was used for cDNA preparation (Superscript III cDNA synthesis kit; Invitrogen, USA). All PCR amplifications were performed in 25 µl reaction volume. Each reaction contained 2.5 µl 10× buffer, 200 µM of dNTPs, 0.5 µl of each primers (10 pmol), 0.5 units of Taq DNA polymerase and nuclease free water to bring the total volume to 25 µl. Around 100 ng of cDNA was used as template. Thermal cycling parameters were optimized for different fragments/gene. The PCR products were resolved on a 1.5% agarose gel.

Experiment 3.2 and 4.2

3.23.1 Reverse transcription and PCR analysis

The total RNA quantity and quality were assessed by using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA quality was verified by agarose gel electrophoresis, and RNA integrity was analyzed using the Agilent Bioanalyzer system (Agilent Technologies, Inc., Santa Clara, CA). Total RNA (1 µg) was then reverse transcribed to cDNA: 1 ug of RNA was incubated with 1 µg of random primers (Promega, Madison, WI) for 10 min at 65°C and then for 5 min on ice in a final volume of 10 µl. Reverse transcription (RT) was carried out by adding avian myeloblastosis virus (AMV) reverse transcriptase buffer (Promega), 4 mM deoxynucleoside triphosphate (dNTP) (Promega), 15 U of AMV reverse transcriptase (Promega), and 40 U of RNase (Promega) to the mixture. The mixture

was incubated for 1.5 h at 42°C and 5 min at 95°C. Diluted cDNA samples were stored at 4°C until use.

PCR was performed using specific primers for TLR4 and NOD2 (CARD15), (Table 30.32 and 30.34). Each amplification began with a 2-min denaturation step at 94°C followed by 30 cycles of denaturation at 94°C for 30 s, annealing at the gene-specific temperature (Table 30.33) for 30 s, extension at 72°C for 30 s, and a final cycle at 94°C for 10 min. Amplification was performed with a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, Foster City, CA). PCR products (20 µl) were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide and analyzed using Fluorchem 8900.

CHAPTER-IV

RESULTS

OBJECTIVE NUMBER 1

4.1 Detection of *Escherichia coli* from faeces of buffalo calves and their dams

Examination of 78 faecal samples by PCR assay revealed a positive detection of 23 samples from buffalo calves and 15 from buffalo dams after examining of 38 and 40 samples respectively as showed in Table 4.1.

Table 4.1: Detection of *Escherichia coli* positive samples from faeces of buffalo calves and their dams using polymerase chain reaction assay

Animal No. during sample processing	No. of Animal Sampled	Sex / Parity	D.O.B.	Diarrhoea Status	
				Yes	No
3D	BC81	M	03-08-2017	D	-
4D	BC1374	F	31-07-2017	D	-
13D	BD959	2^{ND}	ADULT	-	ND
14D	BD183	3^{RD}	ADULT	-	ND
16D	BD177	4^{TH}	ADULT	-	ND
17D	BD847	3^{RD}	ADULT	D	-
18D	BC1366	M	11-06-2017	D	-
20D	BC69	M	03-08-2016	D	-
21D	BD909	2^{ND}	ADULT	D	-
22D	BD935	2^{ND}	ADULT	D	-
25D	BC1367	M	14-06-2017	D	-
26D	BC1265	M	13-08-2016	D	-
28D	BC73	F	07-10-2016	-	ND
29D	BC1364	F	12-08-2016	-	ND
30D	BC1369	M	01-07-2018	D	-
34D	BD10	6^{TH}	ADULT	-	ND
35D	BC1371	M	11-11-2016	D	-
36D	BC1360	F	20-05-2017	D	-
37D	BD967	2^{ND}	ADULT	D	-
38D	BC80	M	23-07-2017	D	-

39D	BD1025	1ST	ADULT	D	-
40D	BD185	4TH	ADULT	-	ND
41D	LPM79	F	22-07-2017	D	-
42D	BD1068	1st	ADULT	-	ND
43D	BC2M	F	2 MONTHS	D	-
47D	BD1038	1ST	ADULT	D	-
48D	BD190	2ND	ADULT	D	-
49D	BD967	2ND	ADULT	D	-
55D	BD19	2ND	ADULT	D	-
56D	BD196	2ND	ADULT	-	ND
57D	BD20	4TH	ADULT	-	ND
59D	BC1268	M	19-08-2016	D	-
61D	BC2.5M	F	2.5MONTHS	D	-
62D	BC22D	F	22 DAYS	D	-
63D	BC1373	F	31-07-2017	D	-
66D	BC1365	M	07-06-2017	D	-
67D	BC1363	M	30-05-2017	D	-
42D	BC1358	F	01:05:2017	D	-

4.2 Detection of *Escherichia coli* from faeces of buffalo calves

Examination of 38 faecal samples from diarrhoeic and a non-diarrrhoeic buffalo calves conducted using PCR during the study period was done to identify *E. coli* associated with calf diarrhoea, and 23 samples were positive for *E .coli* was showed in Table 4.2.

Table 4.2: Detection of *Escherichia coli* positive samples from faeces of buffalo calves using polymerase chain reaction assay

Animal No. during sample processing	No. of Animal sampled	Sex	Date of Birth	Diarrhoea Status	
				Yes	No
3D	BC0081	M	03-08-2017	D	-
4D	BC1374	F	31-07-2017	D	-
18D	BC1366	M	11-06-2017	D	-
20D	BC0069	M	03-06-2017	D	-
25D	BC1367	M	14-06-2017	D	-
26D	BC1265	M	13-08-2016	D	-
28D	BC1377	F	07-10-2016	-	ND
29D	BC1364	F	12-08-2016	D	-
30D	BC1369	M	01-07-2018	D	-
35D	BC1371	M	11-11-2016	D	-
36D	BC1360	F	20-05-2017	D	-
38D	BC0080	M	23-07-2017	D	-
41D	LPM079	F	22-07-2017	D	-
43D	BC002M	F	2 MONTH	D	--
59D	BC1268	M	19-08-2016	D	-
61D	BC2.5M	F	2.5 MONTHS	D	-
62D	BC22D	F	22 DAYS	D	-
63D	BC1373	F	31-07-2017	-	ND
66D	BC1365	M	07-06-2017	D	-
67D	BC1363	M	30-05-2017	D	-
54D	BC1311	F	11:11:2017	D	-
02D	BC1358	F	01:05:2017	D	-
45D	BC1372	M	32:07:2017	-	ND

4.3 Detection of *Escherichia coli* from faeces of buffalo dams

Examination of 40 faecal samples from diarrhoeic and non-diarrrhoeic buffalo dams conducted using PCR during the study on calf diarrhoea was done to identify *E. coli* associated dams of buffalo calves with and without diarrhoea, and 15 samples were found positive for *E .coli* was showed in Table 4.3.

Table 4.3: Detection of *Escherichia coli* positive samples from faeces of buffalo dams using polymerase chain reaction assay

Sr. No.	Animal No. during sample processing	No. of Animal sampled	Parity	Diarrhea Status Yes	No
1	14D	BD182	3rd	-	ND
2	19D	BD 902	1st	D	-
3	17D	BD847	3rd	D	-
4	21D	BD909	2nd	D	-
5	22D	BD935	2nd	D	-
6	34D	BD10	6th	-	ND
7	39D	BD1025	1st	D	-
8	58D	BD16	6th	-	ND
9	10D	BD848	2ndt	D	-
10	47D	BD1038	1st	D	-
11	48D	BD190	2nd	D	-
12	49D	BD967	2nd	D	-
13	55D	BD19	2nd	D	-
14	12D	BD1010	1st	D	-
15	53D	BD24	2nd	D	-

4.4 Detection of *Escherichia coli* from faeces of buffalo calves and dams with occurrence of diarrhoea

Result of positive detection of *E. coli* and occurrence of diarrhoea in faecal samples from buffalo calves and dams was showed in Table 4.4.

Table 4.4: Detection of *Escherichia coli* positive samples from faeces of buffalo calves and dams with occurrence of diarrhoea

Age	Diarrhoegenic Calves			Diarrhoegenic Dams		
	No. with diarrhoea	*E. coli* +ve	Total	No. with diarrhoea	*E. coli* +ve	Total
Day 1-2 Days	05(31.25)	08(50.00)	16(42.12)	06(33.33)	07(38.89)	18(45.00)
Day 3-3 Months	09(64.29)	10(71.42)	14(36.84)	05(35.71)	05(35.71)	14(35.00)
4-6 Months	04(50.00)	05(62.50)	08(21.05)	02(25.00)	03(37.50)	08(20.00)
Total	18(47.37)	23(60.52)	38	13(32.50)	15(37.50)	40

4.5 Detection of *Escherichia coli phoA gene, virulence genes* and *antibacterial resistance genes*

Result of positive detection of *E. coli phoA genes, virulence genes* and *antibacterial resistance genes* of *E. coli* after examining faecal samples from buffalo calves and dams was depicted in Table 4.5.

Table 4.5: Detection of *Escherichia coli phoA gene, virulence genes* and *antibacterial resistance genes*

Sample	Genes								
	E coli	*Virulence*						*Antibacterial Resistance*	
	PhoA	tsh	EAEC	eaeA	ST	Afa	eaeA	tetA	sul1
1	+VE	+VE	-VE	+VE	-VE	-VE	+VE	+VE	+VE
2	+VE	+VE	-VE	+VE	-VE	-VE	+VE	+VE	+VE
3	+VE	+VE	-VE	+VE	-VE	-VE	+VE	+VE	+VE
4	+VE	+VE	-VE	+VE	-VE	-VE	+VE	+VE	+VE
5	+VE	+VE	-VE	+VE	-VE	-VE	+VE	+VE	+VE

4.6 Chi-square analysis of *Escherichia coli* from faeces of buffalo calves and dams

Result of chi-square analysis of *Escherichia coli* positive samples detected from faeces of buffalo calves and dams was depicted in Table 4.6. It indicated that P value was 0.04 which was less than 0.05 and therefore the correlation of *Escherichia coli* positive detection between calves and dams is significant.

Table 4.6: Chi-square analysis of *Escherichia coli* positive samples from faeces of buffalo calves and dams

Group	*Escherichia coli*		Total
	Yes (%)	No (%)	
Calf	23(60.53)	15(39.47)	38(48.72)
Dam	15(37.50)	25(62.50)	40(51.28)
Total	38(48.72)	40(51.28)	78

X^2 = 4.136, If P ≤ 0.05, significant level

P = 0.04

4.7 Chi-square analysis of positive diarrhoea cases in buffalo calves and dams

Result of chi-square analysis of occurrence of diarrhoea cases detected from faeces of buffalo calves and dams was depicted in Table 4.7. It indicated that P value was 0.825 which was greater than 0.05 and therefore the correlation of diarrhoea cases detected from faeces of buffalo calves and dams was not significant.

Table 4.7: Chi-square analysis of positive diarrhoea cases in buffalo calves and dams

Group	Diarrhoea		Total (%)
	Yes	No	
Calf	19(50.00)	19(50.00)	38(48.72)
Dam	21(52.50)	19(47.50)	40(51.28)
Total	40(51.28)	38(48.72)	78

X^2= 0.049, If P ≤ 0.05, significant level
P=0.825

4.8 Chi-square analysis of *Escherichia coli* positive samples from faeces of buffalo calves and their dams with diarrhoea

Result of chi-square analysis of *Escherichia coli* positive samples with diarrhoea detected from faeces of buffalo calves and dams was depicted in Table 4.8. It indicated that P value was 0.514 which was greater than 0.05 and therefore the correlation of *Escherichia coli* positive detection and diarrhoea cases between calves and dams was not significant.

Table 4.8: Chi-square analysis of *Escherichia coli* positive samples from faeces of buffalo calves and their dams with diarrhoea

Diarrhoea	Group		Total (%)
	Calf (%)	Dam (%)	
Yes	18(78.26)	13(86.66)	31(81.58)
No	05(21.74)	02(13.33)	07(18.42)
Total	23(60.53)	15(39.47)	38

X^2 = 0.427, If P ≤ 0.05, significant level
P = 0.514

4.9 Chi-square analysis of *Escherichia coli* positive samples from faeces of buffalo calves with diarrhoea based on sex of calves

Result of chi-square analysis of *Escherichia coli* positive samples with diarrhoea based on sex of buffalo calves was depicted in Table 4.9. It indicated that P value was 0.514 which was greater than 0.05 and therefore the correlation of *Escherichia coli* positive detection with diarrhoea cases between male and female calves was not significant.

Table 4.9: Chi-square analysis of *Escherichia coli* positive samples from faeces of buffalo calves with diarrhoea based on sex of calves

Diarrhoea	Sex		Total (%)
	Female (%)	Male (%)	
Yes	13(41.94)	18(58.06)	31(81.58)
No	02(28.57)	05(71.43)	07(18.42)
Total	15(39.47)	23(60.53)	38

X^2 = 0.427, If P ≤ 0.05, significant level
P = 0.514,

4.10 Chi-square analysis of *Escherichia coli* positive samples from faeces of buffalo dams with diarrhoea according to status of their parity

Result of chi-square analysis of *Escherichia coli* positive samples with diarrhoea detected from faeces of buffalo dams according to status of their parity was depicted in Table 4.10. It indicated that P value was 3.0 and was greater than 0.05. Therefore the correlation of *Escherichia coli* positive detection with diarrhoea cases according to parity status of the dams was not significant.

Table 4.10: Chi-square analysis of *Escherichia coli* positive samples from faeces of buffalo dams with diarrhoea according to status of their parity

Diarrhea	Parity				Total (%)
	1(%)	2 (%)	3 (%)	6 (%)	
Yes	04(30.77)	08(61.54)	01(07.69)	00(00.00)	13(86.67)
No	00(00.00)	00(00.00)	00(00.00)	02(100.0)	02(13.33)
Total	04(26.67)	08(53.33)	01(06.67)	02(13.33)	15

X^2 = 15.000, P = 3

4.11 Chi-square analysis of *Escherichia coli* positive samples from faeces of buffalo calves and their dams with diarrhoea according to age of calves

Result of chi-square analysis of *Escherichia coli* positive samples with diarrhoea based on age of buffalo calves was depicted in Table 4.11. It indicates that P value were 0.007, 0.001and 0.427 for calves of Day1 to 2 days of age, Day 3 to 3 months of age and 4 to 6 months of age respectively. Values for the first 2 groups were less than 0.05 and therefore the correlations of *Escherichia coli* positive detection with diarrhoea cases according to ages in the first 2 groups were significant. But for calves in the 3rd group of 4 to 6 months the values was more than 0.05 and therefore not significant.However P value of chi-square analysis of dams of these calves was 0.001 which was less than 0.05 and is at significant level.

Table 4.11: Chi-square analysis of *Escherichia coli* positive samples from faeces of buffalo calves and their dams with diarrhoea according to age of calves

Age group of calves			*E coli*		Total (%)
			Yes (%)	No (%)	
1-2 Days	Diarrhea	Yes	05(31.25)	00(00.00)	05(31.25)
		No	03(18.75)	08(50.00)	11(68.75)
	Total		08(50.00)	08(50.00)	16
Dam	Diarrhea	Yes	13(32.50)	08(20.00)	21(52.50)
		No	02(05.00)	17(42.50)	19(47.50)
	Total		15(37.50)	25(62.50)	40
Day 3 - 3 Months	Diarrhea	Yes	09(64.29)	00(00.00)	09(64.29)
		No	01(07.14)	04(28.57)	05(35.71)
	Total		10(71.43)	04(28.57)	14
4-6 Months	Diarrhea	Yes	04(57.14)	01(14.29)	05(71.43)
		No	01(14.29)	01(14.29)	02(28.57)
	Total		05(71.43)	02(28.57)	07

Group 1- $X^2 = 7.273$, P = 0.007
Dams - $X^2 = 11.235$, P = 0.001
Group 2 - $X^2 = 10.080$, P = 0.001
Group 3 - $X^2 = 0.630$, P = 0.427

4.12 A summary of result of chi-square analysis of *Escherichia coli* positive samples with diarrhoea based on age of buffalo calves was depicted in Table 4.12. It indicates that P value were 0.007, 0.001and 0.427 for calves of Day1 to 2 days of age, Day 3 to 3 months of age and 4 to 6 months of age respectively. Values for the first 2 groups were less than 0.05 and therefore the correlations of *Escherichia coli* positive detection with diarrhoea cases according to ages in the first 2 groups were significant. But for calves in the 3rd group of 4 to 6 months the values was more than 0.05 and therefore not significant.However P value of chi-square analysis of dams of these calves was 0.001 which was less than 0.05 and is at significant level.

Table 4.12: Chi-square analysis of *Escherichia coli* positive samples from faecse of buffalo calves with diarrhea according to their ages

Age group of calves	Total	*E coli* Positive	Diarrhoea Positive
1-2 days	16	08(50.00)	05(31.25)
Day 3-3 Months	14	10(71.43)	09(64.29)
4-6 Months	08	05(62.50)	04(50.00)
Total	38	23(60.53)	18(47.37)

Group 1- $X^2 = 7.273$, P = 0.007
Group 2 - $X^2 = 10.080$, P = 0.001
Group 3 - $X^2 = 0.630$, P = 0.427

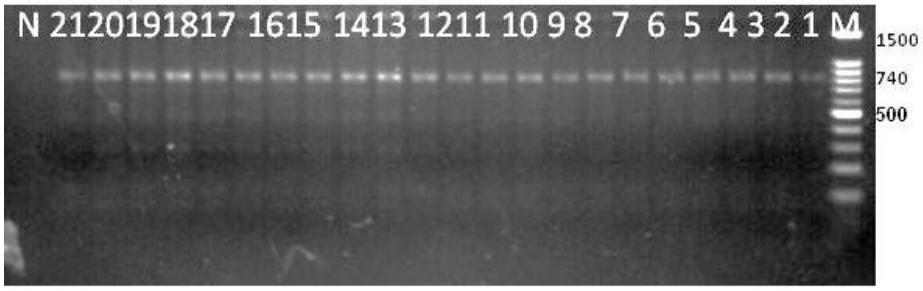


Figure 4.1: ***Escherichia coli phoA*** **positive genes detected by PCR and resolved by agarose gel electrophoresis [Lane 1 (M): 100bp plus DNA ladder (Fermentas, USA); Lane 1-21: 740 bp PCR product; Lane N: negative template control]**

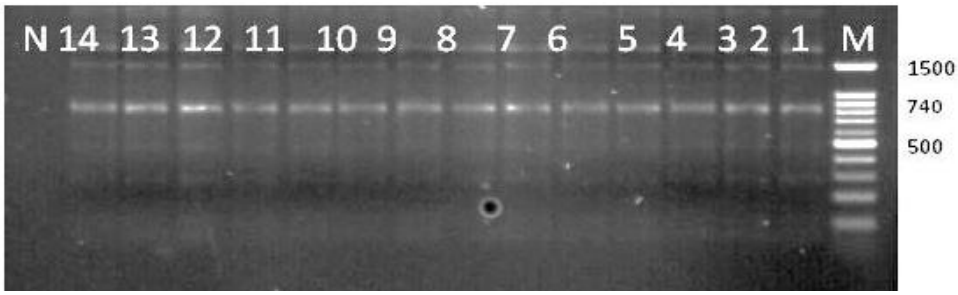


Figure 4.2: ***Escherichia coli phoA*** **positive genes detected by PCR and resolved by agarose gel electrophoresis [Lane 1 (M): 100bp plus DNA ladder (Fermentas, USA); Lane 1-14: 740 bp PCR product; Lane N: negative template control]**

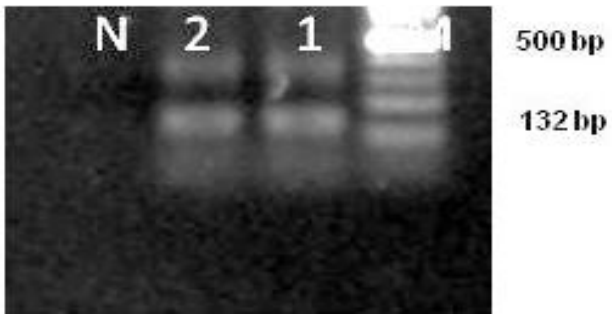


Figure 4.3: ***Escherichia coli LT*** **positive genes detected by PCR and resolved by agarose gel electrophoresis [Lane 1 (M): 100bp plus DNA ladder (Fermentas, USA); Lane 1-2: 132 bp PCR product; Lane N: negative template control]**

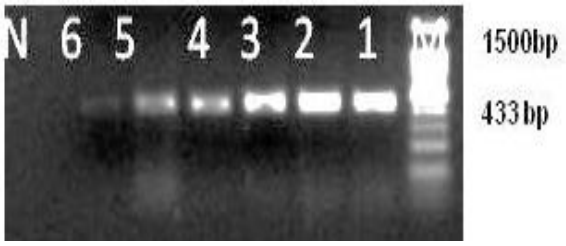


Figure 4.4: ***Escherichia coli SUL1*** **positive genes detected by PCR and resolved by agarose gel electrophoresis [Lane 1 (M): 100bp plus DNA ladder (Fermentas, USA); Lane 1-6: 433 bp PCR product; Lane N: negative template control]**

Figure 4.5: ***Escherichia coli eaeA*** **positive genes detected by PCR and resolved by agarose gel electrophoresis [Lane 1 (M): 100bp plus DNA ladder (Fermentas, USA); Lane 1-8: 248 bp PCR product; Lane N: negative template control]**

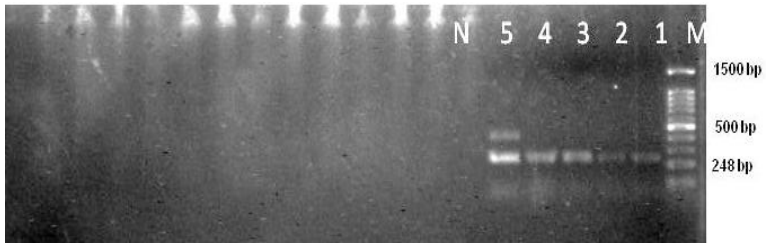


Figure 4.6: ***Escherichia coli eaeA*** **positive genes detected by PCR and resolved by agarose gel electrophoresis [Lane 1 (M): 100bp plus DNA ladder (Fermentas, USA); Lane 1-5: 248 bp PCR product; Lane N: negative template control]**

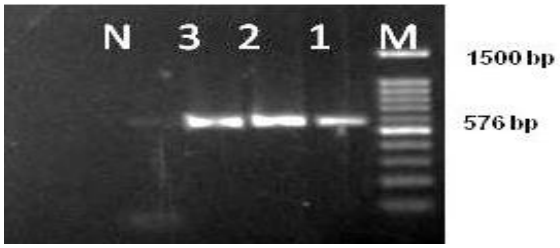


Figure 4.7: ***Escherichia coli Tet A*** **positive genes detected by PCR and resolved by agarose gel electrophoresis [Lane 1 (M): 100bp plus DNA ladder (Fermentas, USA); Lane 1-3: 576 bp PCR product; Lane N: negative template control]**

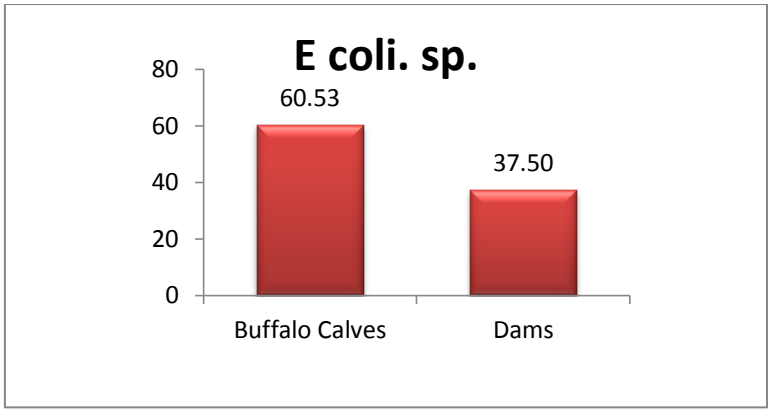


Figure 4.8: Percentages of *Escherichia coli* positive samples from buffalo calves and dams

4.1 Detection of *Salmonella* from faeces of buffalo calves and their dams

Examination of 78 faecal samples by PCR assay revealed a positive detection of 11 samples from buffalo calves and 2 from buffalo dams after examining of 38 and 40 samples respectively as showed in Table 4.13.

Table 4.13: Detection of *Salmonella* positive samples from faeces of buffalo calves and their dams using polymerase chain reaction assay

Sr. No.	Animal No. during processing	No. of Animal sampled	Sex	Date of Birth	Diarrhea Status	
					Yes	No
1	3D	BC81	M	03-08-2017	D	-
2	4D	BC1374	F	03-07-2017	D	-
3	11D	BC1279	M	03-09-2016	D	-
4	30D	BC1369	M	01-07-2018	D	-
5	31D	BD791	4^{TH}	ADULT	D	-
6	35D	BC1371	F	02-07-2017	D	-
7	38D	BC80	M	23-07-2017	D	-
8	41D	BC79	F	22-07-2017	D	-
9	52D	BD49	1^{ST}	ADULT	D	-
10	50D	BC2.5M	F	2MONTHS	D	-
11	36D	BC22D	F	22DAYS	D	-
12	7D	BC1311	M	11-11-2017	D	-
13	43D	BC2M	F	2 MONTHS	D	-

4.14 Detection of *Salmonella* from faeces of buffalo calves

Examination of 38 faecal samples from diarrhoeic and non-diarrrhoeic calves conducted using PCR during the study period was done to identify *Salmonella* associated with calf diarrhoea and, 11 samples were found to be positive for *Salmonella* as showed in Table 4.14.

Table 4.14: Detection of *Salmonella* positive samples from faeces of buffalo calves using polymerase chain reaction assay

Sr. No.	Animal No. during processing	No. of Animal sampled	Sex	Date of Birth	Diarrhea status	
					Yes	No
1	3D	BC81	M	03-08-2017	D	-
2	4D	BC1374	F	31-07-2017	D	-
3	7D	BC1311	M	11-11-2017	D	-
4	11D	BC1279	M	03-09-2016	D	-
5	30D	BC1369	M	01-07-2018	D	-
6	35D	BC1371	F	02-07-2017	D	-
7	36D	BC22D	F	22DAYS	D	-
8	38D	BC80	M	23-07-2017	D	-
9	41D	BC79	F	22-07-2017	D	-
10	43D	BC2M	F	2 MONTHS	D	-
11	50D	BC2.5M	F	2MONTHS	D	-

4.15 Detection of *Salmonella* positive samples from faeces of buffalo dams using polymerase chain reaction assay

Polymerase chain reaction (PCR) examination of 40 faecal samples from diarrhoeic and non-diarrrhoeic buffalo dams conducted during the study was to identify *E. coli* infection in dams of buffalo calves with and without diarrhoea and only 2 samples were found positive for *Salmonella* as depicted in Table 4.15.

Table 4.15: Detection of *Salmonella* positive samples from faeces of buffalo dams using polymerase chain reaction assay

Sr. No.	Animal No. during processing	No. of Animal Sampled	Parity	D.O.B.	Diarrhea Status	
					Yes	No
1	31D	BD 791	4^{th}	ADULT	D	-
2	52D	BD 49	1^{st}	ADULT	D	-

4.16 Detection of *Salmonella* positive samples in buffalo calves and their dams with diarrhoea

Result of positive detection of *Salmonella* and occurrence of diarrhoea in faecal samples from buffalo calves and dams was showed in Table 4.16.

Table 4.16: Detection of *Salmonella* positive samples in buffalo calves and their dams with diarrhoea

Age	Diarrhaegenic calves			Diarrhoegenic dams		
	No. with Diarrhea	*Salmonella* +ve	Total	No. with Diarrhea	*Salmonella* +ve	Total
Day 1-2 Days	05(31.25)	06(37.50)	16(42.12)	02(11.11)	02(11.11)	18(45.00)
Day 3-3 Months	03(21.43)	03(21.43)	14(36.84)	00(00.00)	00(00.00)	14(35.00)
4-6 Months	02(25.00)	02(25.00)	08(21.05)	00(00.00)	00(00.00)	08(20.00)
Total	10(26.32)	11(28.95)	38	02(05.00)	02(50.00)	40

4.17: Positive detection of *Salmonella genus specific genes, salmonella typhi* genes and *Salmonella virulence genes*

An examination of a total of 38 faecal samples from calves with and without diarrhoea for detection of different *Salmonella genes*, 11 were found positive for genus specific and other genes in buffalo calves but only 2 positive samples were detected for the same genes in samples from buffalo dams after examining a total of 40. *stn, invA* and *sef* are virulence genes of *Salmonella* detected numbering 7, 6 and 5 samples respectively. *Salmonella typhi* genes were detected in all *Salmonella* positive samples detected. However, *Salmonella enteritidis* gene was not detected in all screened samples including *Salmonella* positive samples as showed in Table 4.17.

Table 4.17: Positive detection of *Salmonella genus specific genes, salmonella typhi* genes and *Salmonella virulence genes*

Sample	Genes					
	Salmonella genes			*Virulence genes*		
	Genus Specific	*S. typhi*	*S.enteritidis*	*inv*	*Sef*	*Stn*
1	+ VE	+ VE	-VE	+ VE	+VE	+ VE
2	+ VE	+ VE	-VE	+ VE	+VE	+ VE
3	+ VE	+ VE	-VE	+ VE	+VE	+ VE
4	+ VE	+ VE	-VE	+ VE	+VE	+ VE
5	+ VE	+ VE	-VE	+ VE	+VE	+ VE

4.18 Chi-square analysis of *Salmonella* positive samples in buffalo calves and dams

Result of chi-square analysis of *Salmonella* positive samples detected from faeces of buffalo calves and dams was depicted in Table 4.18. It indicated that P value was 0.005 which was less than 0.05 and therefore the correlation of *Salmonella* positive detection between calves and dams was significant.

Table 4.18: Chi-square analysis of *Salmonella* positive samples in buffalo calves and dams

Group	*Salmonella*		Total (%)
	Yes (%)	No (%)	
Calf	11(28.95)	27(71.05)	38(48.72)
Dam	02(05.00)	38(95.00)	40(51.28)
Total	13(16.67)	65(83.33)	78

X^2= 8.046, P = 0.005, Significant since P ≤ 0.05

4.19 Chi-square analysis of positive *Salmonella* samples of buffalo calves and dams with diarrhoea

Result of chi-square analysis of occurrence of diarrhoea cases detected from faeces of buffalo calves and dams was depicted in Table 4.19. It indicated that P value was 0.657 which was greater than 0.05 and therefore the correlation of diarrhoea cases detected from faeces of buffalo calves and dams was not significant.

Table 4.19: Chi-square analysis of positive *Salmonella* samples of buffalo calves and dams with diarrhoea

Diarrhea	Group		Total (%)
	Calf	Dam	
Yes	10(90.91)	02(100.00)	12(92.31)
No	01(09.09)	00(00.00)	01(07.69)
Total	11(84.62)	02(15.38)	13

X^2 = 0.197, P = 0.657
P ≥ 0.05, not significant

4.20 Chi-square analysis of positive *Salmonella* samples of buffalo calves with diarrhoea according to sex of the calves

Result of chi-square analysis of *Salmonella* positive samples with diarrhoea based on sex of buffalo calves was depicted in Table 4.20. It indicated that P value was 0.657 which was greater than 0.05 and therefore the correlation of *Escherichia coli* positive detection with diarrhoea cases between male and female calves was not significant.

Table 4.20: Chi-square analysis of positive *Salmonella* samples of buffalo calves with diarrhoea according to sex of the calves

Diarrhea	Sex		Total (%)
	Female (%)	Male (%)	
Yes	02(16.66)	10(76.92)	12(92.31)
No	00(00.00)	01(100.00)	01(07.69)
Total	02(15.38)	11(84.62)	13

X^2 = 0.197, P = 0.657, P ≥ 0.05, not significant

4.21 Chi-square analysis of positive *Salmonella* samples of buffalo dams with diarrhoea based on parity status of the dams

Result of chi-square analysis of *Salmonella* positive samples with diarrhoea detected from faeces of buffalo dams according to status of their parity was depicted in Table 4.21. It indicated that P value was 2.0 and was greater than 0.05. Therefore the correlation of *Salmonella* positive detection with diarrhoea cases according to parity status of the dams was not significant.

Table 4.21: Chi-square analysis of positive *Salmonella* samples of buffalo dams with diarrhoea based on parity status of the dams

Diarrhoea	Parity		Total (%)
	1	4	
Yes	01(50.00)	01(50.00)	02(100.00)
Total	01(50.00)	01(50.00)	02

X^2 = 0.0, P = 2.0, P ≥ 0.05, not significant

4.22 Chi-square analysis of positive *Salmonella* samples of buffalo calves and their dams with diarrhoea based on age category of calves

Result of chi-square analysis of *Salmoella* positive samples with diarrhoea based on age of buffalo calves was depicted in Table 4.22. It indicated that P value were 0.000, 0.145 and 0.290 for calves of Day1 to 2 days of age, Day 3 to 3 months of age and 4 to 6 months of age respectively. Values for the first group were less than 0.05 and therefore the correlations of *Salmonella* positive detection with diarrhoea cases according to the ages in the first group was significant. But for calves in the 2nd and 3rd groups of Day 3 to 3 months and 4 to 6 months, the values were more than 0.05 and therefore the correlation was not significant. Similarly P value of chi-square analysis of dams of these calves was 0.168 which was more than 0.05 and therefore was not significant.

Table 4.22: Chi-square analysis of positive *Salmonella* samples of buffalo calves and their dams with diarrhea based on age category of calves

Age group of Calves			*Salmonella*		Total (%)
			Yes (%)	No (%)	
1-2 Days	Diarrhea	Yes	05(31.25)	00(00.00)	05(31.25)
		No	01(06.25)	10(62.50)	11(68.75)
	Total		06(37.50)	10(62.50)	16
Dams	Diarrhea	Yes	02(05.00)	19(47.50)	21(52.50)
		No	00(00.00)	19(47.50)	19(47.50)
	Total		02(05.00)	38(95.00)	40
Day3-3 Months	Diarrhea	Yes	03(21.43)	06(42.86)	09(64.29)
		No	00(00.00)	05(35.71)	05(31.71)
	Total		03(21.43)	11(78.57)	14
4-6 Months	Diarrhea	Yes	02(28.57)	03(42.86)	05(71.43)
		No	00(00.00)	02(28.57)	02(28.57)
	Total		02(28.57)	05(71.43)	7

Group 1 - $X^2 = 12.121$, P = 0.00, Significant since $P \leq 0.05$
Dams - $X^2 = 1.905$, P = 0.168, $P \geq 0.05$, not significant
Group 2 - $X^2 = 2.121$, P = 0.145, $P \geq 0.05$, not significant
Group 3 - $X^2 = 1.120$, P = 0.290, $P \geq 0.05$, not significant

4.23 A summary of chi-square analysis of *Salmonella* positive samples with diarrhoea according to age category of buffalo calves

Result of chi-square analysis of *Salmoella* positive samples with diarrhoea based on age of buffalo calves was depicted in Table 4.23. It indicated that P value were 0.000, 0.145 and 0.290 for calves of Day1 to 2 days of age, Day 3 to 3 months of age and 4 to 6 months of age respectively. Value for the first group was less than 0.05 and therefore the correlations between *Salmonella* positive detection with diarrhoea cases according to the ages in the first group was significant. But for calves in the 2nd and 3rd groups of Day 3 to 3 months and 4 to 6 months the values were more than 0.05 and therefore were not significant. Similarly P value of chi-square analysis of dams of these calves was 0.168 which was more than 0.05 and was not at significant level.

Table 4.23: Chi-square analysis of *Salmonella* positive samples with diarrhea according to age category of buffalo calves

Age group of calves	Total	*Salmonella* Positive	Diarrhoea Positive
1-2 days	16	06(37.50)	05(31.25)
Day 3-3 Months	14	03(21.43)	03(21.43)
4-6 Months	08	02(25.00)	02(25.00)
Total	38	11(28.95)	10(26.32)

Group 1 - $X^2 = 12.121$, P = 0.00, Significant since $P \leq 0.05$
Group 2 - $X^2 = 2.121$, P = 0.145
Group 3 - $X^2 = 1.120$, P = 0.290

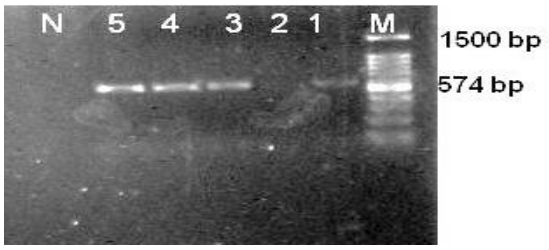


Figure 4.9: ***Salmonella 18SrRNA*** **positive genes detected by PCR and resolved by agarose gel electrophoresis [Lane 1 (M): 100bp plus DNA ladder (Fermentas, USA); Lane 1-5: 574 bp PCR product; Lane N: negative template control]**

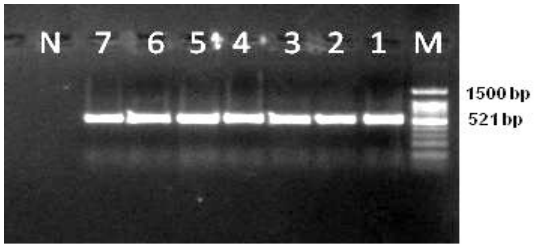


Figure 4.10: ***Salmonella inv*** **positive genes detected by PCR and resolved by agarose gel electrophoresis [Lane 1 (M): 100bp plus DNA ladder (Fermentas, USA); Lane 1-7: 521 bp PCR product; Lane N: negative template control]**

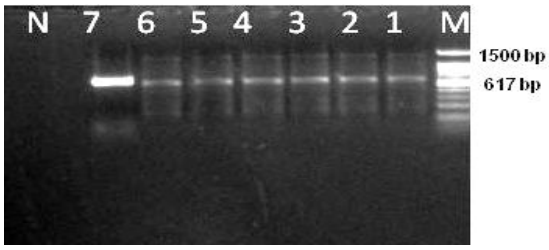


Figure 4.11: ***Salmonella stn*** **positive genes detected by PCR and resolved by agarose gel electrophoresis [Lane 1 (M): 100bp plus DNA ladder (Fermentas, USA); Lane 1-7: 617 bp PCR product; Lane N: negative template control]**

Figure 4.12: ***Salmonella stn*** **positive genes detected by PCR and resolved by agarose gel electrophoresis [Lane 1 (M): 100bp plus DNA ladder (Fermentas, USA); Lane 1-7: 617 bp PCR product; Lane N: negative template control]**

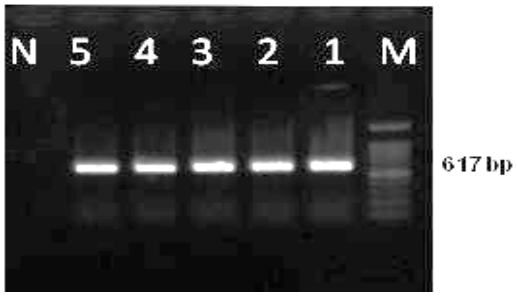


Figure 4.13: *Salmonella stn* positive genes detected by PCR and resolved by agarose gel electrophoresis [Lane 1 (M): 100bp plus DNA ladder (Fermentas, USA); Lane 1-7: 617 bp PCR product; Lane N: negative template control]

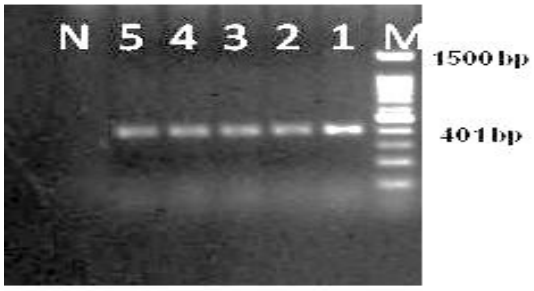


Figure 4.14: *Salmonella typhi* positive genes detected by PCR and resolved by agarose gel electrophoresis [Lane 1 (M): 100bp plus DNA ladder (Fermentas, USA); Lane 1-5: 401 bp PCR product; Lane N: negative template control]

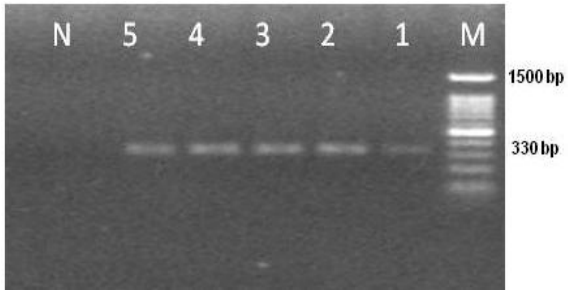


Figure 4.15: *Salmonella sef* positive genes detected by PCR and resolved by agarose gel electrophoresis [Lane 1 (M): 100bp plus DNA ladder (Fermentas, USA); Lane 1-5: 330 bp PCR product; Lane N: negative template control]

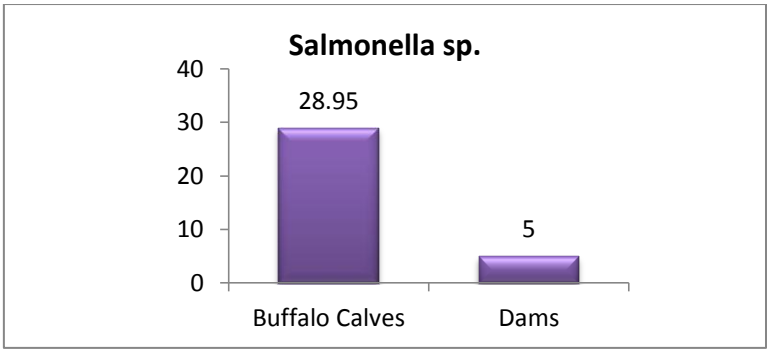


Figure 4.16: Percentages of *Salmonella* positive samples from buffalo calves and dams

4.24 Results of positive detection of samples with *Cryptosporidium* species from faeces of buffalo calves and their dams

An examination of a total of 38 faecal samples from buffalo calves with and without diarrhoea for detection of *Cryptosporidium* positive samples, revealed 10 positive samples in calves while examination of 40 faecal samples of buffalo dams revealed the detection of 17 positive samples.

Table 4.24: Detection of *Cryptosporidium* positive samples from faeces of buffalo calves and their dams using polymerase chain reaction assay

Sr. No.	No. of Animal during processing	No. of Animal sampled	Sex	Date of Birth	Diarrhea status	
					Yes	No
1	2D	BC1359	M	15-05-2017	-	ND
2	3D	BC81	M	03-08-2017	D	-
3	4D	BC1374	F	31-07-2017	D	-
4	15D	BD16	6TH	ADULT	-	ND
5	39D	BD1025	1ST	ADULT	D	-
6	40D	BD185	4TH	ADULT	-	ND
7	41D	BD902	1ST	ADULT	D	-
8	43D	BC2M	F	2 MONTH	D	-
9	44D	BD173	4TH	ADULT	D	-
10	45D	BC1372	M	23-07-2017	-	ND
11	46D	BD182	3RD	ADULT	-	ND
12	47D	BD1038	1ST	ADULT	D	-
13	48D	BD190	2ND	ADULT	D	-
14	49D	BD967	2ND	ADULT	D	-
15	50D	BC1361	M	24-05-2017	-	ND
16	51D	BD998	2ND	ADULT	D	-
17	52D	BD49	1ST	ADULT	D	-
18	53D	BD24	3RD	ADULT	-	ND
19	23D	BD1003	1ST	ADULT	-	ND
20	55D	BD19	2ND	ADULT	D	-
21	56D	BD196	2ND	ADULT	D	-
22	60D	BD185	4TH	ADULT	D	-
23	61D	BC2.5M	F	2.5 MONTHS	D	-
24	62D	BC22D	F	22 DAYS	D	-
25	29D	BC1364	F	12-08-2016	D	-
26	67D	BC1363	M	30-05-2017	D	-
27	68D	BD173	4TH	ADULT	D	-

4.25 Detection of *Cryptosporidium* positive samples from faeces of buffalo calves using polymerase chain reaction assay

Examination of 38 faecal samples from diarrhoeic and non-diarrrhoeic calves conducted using PCR during the study period detected 10 samples positive for *Cryptosporidium* as showed in Table 4.14.

Table 4.25: Detection of *Cryptosporidium* positive samples from faeces of buffalo calves using polymerase chain reaction assay

Sr. No.	Animal No. during processing	No. of Animal sampled	Sex	Date of Birth	Diarrhoea Status	
					Yes	No
1	2D	BC1359	M	15-05-2017	-	ND
2	3D	BC81	M	03-08-2017	D	-
3	4D	BC1374	F	31-07-2017	D	-
4	41D	LPM79	F	22-07-2017	D	-
5	43D	BC2M	F	2 MONTHS	D	-
6	45D	BC1372	M	23-07-2017	-	ND
7	50D	BC1361	M	24-05-2017	-	ND
8	29D	BC1364	F	12-08-2016	D	-
9	61D	BC2.5M	F	2.5 MONTHS	D	-
10	62D	BC22D	F	22 DAYS	D	-

4.26 Detection of *Cryptosporidium* positive samples from faeces of buffalo dams using polymerase chain reaction assay

Examination of 40 faecal samples from diarrhoeic and non-diarrrhoeic buffalo dams conducted during the study detected 17 samples positive for *Cryptosporidium* as depicted in Table 4.15.

Table 4.26: Detection of *Cryptosporidium* positive samples from faeces of buffalo dams using polymerase chain reaction assay

Sr. No.	Animal No.during sample processing	No. of Animal sampled	Parity	Date of Birth	Diarrhea Status	
					Yes	No
1	23D	BD1003	1ST Lactation	ADULT	-	ND
2	39D	BD1025	1ST Lactation	ADULT	D	-
3	40D	BD185	4TH Lactation	ADULT	D	
4	44D	BD178	4TH Lactation	ADULT	D	-
5	47D	BD1038	1ST Lactation	ADULT	D	-
6	48D	BD190	2ND Lactation	ADULT	D	-
7	49D	BD967	2ND Lactation	ADULT	D	-
8	51D	BD998	2ND Lactation	ADULT	D	-
9	52D	BD49	1ST Lactation	ADULT	D	-
10	53D	BD24	3RD Lactation	ADULT	D	ND
11	55D	BD19	2ND Lactation	ADULT	-	-
12	31D	BD791	4TH Lactation	ADULT	D	-
13	60D	BD848	2ND Lactation	ADULT	D	-
14	68D	BD173	4TH Lactation	ADULT	D	-
15	58D	BD16	4TH Lactation	ADULT	-	ND
16	14D	BD182	3RD Lactation	ADULT	-	ND
17	21D	BD902	1ST Lactation	ADULT	D	-

4.27 Detection of *Cryptosporidium* positive samples from faeces of buffalo calves and dams with diarrhoea

Result of positive detection of *Cryptosporidium* and occurrence of diarrhoea in faecal samples of buffalo calves and dams was showed in Table 4.16.

Table 4.27: Detection of *Cryptosporidium* positive samples from faeces of buffalo calves and dams with diarrhoea

Age	Diarhaegenic Calves			Diarhaegenic Dams		
	No. with Diarrhea	*Crypto-sporidium* +ve	Total	No. with Diarrhea	*Crypto -sporidium* +ve	Total
Day 1-2 Days	01(06.25)	02(12.50)	16(42.11)	09(50.00)	09(50.00)	18(45.00)
Day3-3 Months	06(42.86)	08(57.14)	14(36.84)	01(07.12)	05(35.71)	14(35.00)
4-6 Months	00(00.00)	00(00.00)	08(21.05)	02(25.00)	03(37.50)	08(20.00)
Total	07(18.42)	10(26.32)	38	12(30.00)	17(42.50)	40

4.28 Chi-square analysis of *Cryptosporidium* positive samples with diarrhoea in buffalo calves and dams

Result of chi-square analysis for occurrence of diarrhoea cases detected from faeces of buffalo calves and dams was depicted in Table 4.19. It indicated that P value was 0.11 which was greater than 0.05 and therefore the correlation of diarrhoea cases detected from faeces between buffalo calves and damswas not significant.

Table 4.28: Chi-square analysis of *Cryptosporidium* positive samples with diarrhoea in buffalo calves and dams

Diarrhea	Group		Total (%)
	Calf (%)	Dam (%)	
Yes	07(30.43)	16(69.57)	23(85.19)
No	03(75.00)	01(25.00)	04(14.81)
Total	10(37.04)	17(62.96)	27

X^2 = 2.20 P =0.11

4.29 Chi-square analysis of *Cryptosporidium* positive samples from faeces of buffalo calves with diarrhea based on sex of calves

Result of chi-square analysis of *Cryptosporidium* positive samples with diarrhoea based on sex of buffalo calves was depicted in Table 4.20. It indicated that P value was 0.088 which was greater than 0.05 and therefore the correlation of*Cryptosporidium* positive detection with diarrhoea cases between male and female calves was not significant.

Table 4.29: Chi-square analysis of *Cryptosporidium* positive samples from faeces of buffalo calves with diarrhea based on sex of calves

Diarrhea	Sex		Total (%)
	Female (%)	Male (%)	
Yes	16(69.57)	07(30.43)	23(85.19)
No	01(25.00)	03(75.00)	04(14.81)
Total	17(62.96)	10(37.04)	27

X^2 = 2.902, P = 0.088

4.30 Chi-square analysis of cryptosporidium positive samples of buffalo dams with diarrhea according to parity status

Result of chi-square analysis of *Cryptosporidium* positive samples with diarrhoea detected from faeces of buffalo dams according to status of their parity was depicted in Table 4.21. It indicated that P value was 2.0 and was greater than 0.05. Therefore the correlation of*Cryptosporidium* positive detection with diarrhoea cases according to parity status of the dams was not significant.

Table 4.30: Chi-square analysis of cryptosporidium positive samples of buffalo dams with diarrhea according to parity status

Diarrhea	Parity			Total (%)
	1 (%)	2 (%)	4 (%)	
Yes	04(25.00)	09(56.25)	03(18.75)	16 (94.12)
No	01(100.00)	00(00.00)	00(00.00)	01(05.88)
Total	05(29.41)	09(52.94)	03(17.65)	17

X^2 = 2.550, P = 2

4.31 Chi-square analysis of *Cryptosporidium* positive samples from faeces of buffalo calves and their dams with diarrhea according to age of calves

Result of chi-square analysis of *Cryptosporidium* positive samples with diarrhoea based on age of buffalo calves was depicted in Table 4.22. It indicated that P value were 0.541, 0.334 and 0.83 for calves of Day1 to 2 days of age, Day 3 to 3 months of age and 4 to 6 months of age respectively. Values for all the three groups were less than 0.05 and therefore the correlations of *Cryptosporidium* positive detection with diarrhoea cases according to the ages in all the three groups were not significant. But for P value of chi-square analysis of dams of these calves was 0.00 which was less than 0.05 and was therefore significant.

Table 4.31: Chi-square analysis of *Cryptosporidium* positive samples from faeces of buffalo calves and their dams with diarrhea according to age of calves

Age group of Calves			*Cryptosporidium*		Total (%)
			Yes (%)	No (%)	
1-2 Days	Diarrhea	Yes	01 (06.25)	04 (25.00)	05 (31.25)
		No	01 (06.25)	10 (62.50)	11 (68.75)
	Total		02 (12.50)	14 (87.50)	16
Dams	Diarrhea	Yes	16 (40.00)	05 (12.50)	21 (52.50)
		No	01 (02.50)	18 (45.00)	19 (47.50)
	Total		17 (42.50)	23 (57.50)	40
Day 3-3 Months	Diarrhea	Yes	06 (42.86)	03 (21.43)	09 (64.29)
		No	02 (58.71)	03 (21.43)	05 (35.71)
	Total		08 (57.14)	06 (42.86)	14
4-6 Months	Diarrhea	Yes	00 (00.00)	05 (71.43)	05 (71.43)
		No	00 (00.00)	02 (28.57)	02 (28.57)
	Total		00 (00.00)	07(100.00)	07

Group 1 - X^2 = 0.374, P = 0.541
Dams - X^2 = 20.534 P = 0.00
Group 2 - X^2 = 0.933, P = 0.334
Group 3 - X^2 = 4.14, P = 0.83

4.32 Chi-square analysis of *Cryptosporidium* positive samples from faeces of buffalo calves with diarrhea according to age of calves

Result of chi-square analysis of *Cryptosporidium* positive samples with diarrhoea based on age of buffalo calves was depicted in Table 4.23. It indicated that P value were 0.541, 0.334 and 0.83 for calves of Day1 to 2 days of age, Day 3 to 3 months of age and 4 to 6 months of age respectively. Values for all the three groups were less than 0.05 and therefore the correlations of *Cryptosporidium* positive detection with diarrhoea cases according to the ages in all the three groups were not significant. But for P value of chi-square analysis of dams of these calves was 0.00 which was less than 0.05 and was therefore significant.

Table 4.32: Chi-square analysis of *Cryptosporidium* positive samples from faeces of buffalo calves with diarrhea according to age of calves

Age group of calves	Total	*Cryptosporidium* positive	Diarrhea Positive
1-2 Days	16	02(12.50)	01(06.25)
Day 3-3 Months	14	08(57.14)	06(42.86)
4-6 Months	08	00(00.00)	00(00.00)
Total	38	10(26.32)	07(18.42)

Group 1 - X^2 = 0.374, P = 0.541
Dams - X^2 = 20.534 P = 0.00
Group 2 - X^2 = 0.933, P = 0.334
Group 3 - X^2 = 4.14, P = 0.83

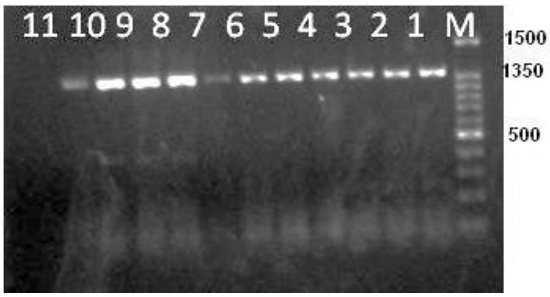


Figure 4.17: *Cryptosporidium* positive genes detected at 1,350 bp resolved by agarose gel electrophoresis [Lane 1 (M): 100bp plus DNA ladder (Fermentas, USA); Lane 1-11: 1,350 bp PCR product; Lane N: negative template control]

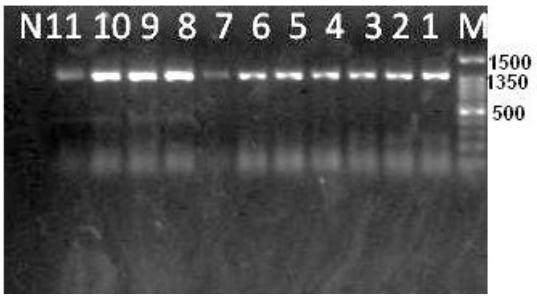


Figure 4.18: *Cryptosporidium* positive genes detected at 1,350 bp resolved by agarose gel electrophoresis [Lane 1 (M):100bp plus DNA ladder (Fermentas, USA); Lane 1-11: 1,350 bp PCR product; Lane N: negative template control]

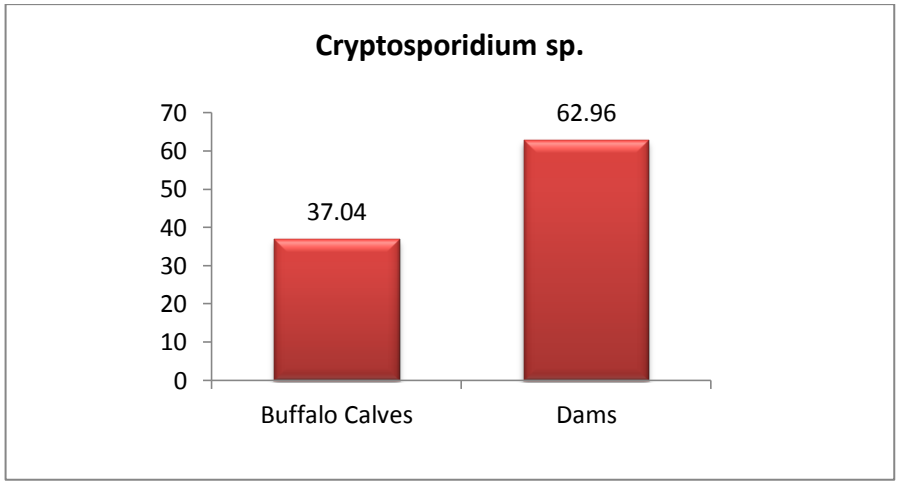


Figure 4.19: Percentages of *Cryptosporidium* positive samples of buffalo calves and dams

Table 4.33: Detection of *Escherichia coli*, *Salmonella* and *Cryptosporidium* positive samples from faeces of buffalo calves

Sr. No.	Infection Type	No. of Animals Examined	No. Positive (%)
1	*E coli* species	38	23 (52.27)
2	*Salmonella* species	38	11 (25.00)
3	*Cryptosporidium* species	38	10 (22.73)
		Total	44

Table 4.34: Detection of *Escherichia coli, Salmonella* and *Cryptosporidium* positive samples from faeces of buffalo dams

Sr. No.	Infection Type	No. of Animals Examined	No. Positive (%)
1	*E coli* species	40	15 (44.12)
2	*Salmonella* Species	40	02 (05.88)
3	*Cryptosporidium* Species	40	17 (50.00)
		Total	34

Table 4.35: Detection of *Escherichia coli, Salmonella* and *Cryptosporidium* positive samples from faeces of buffalo calves and dams

Sr. No.	Infection Type	No. of Animals Examined		No. positive		Total (%)
		Buffalo Calves	Buffalo Dams	Buffalo Calves (%)	Buffalo Dams (%)	
1	*E coli* species	38	40	23(29.49)	15 (19.23)	38 (48.72)
2	*Salmonella* species	38	40	11 (1410)	02 (02.56)	13 (16.67)
3	*Cryptosporidium* species	38	40	10 (12.82)	17 (21.80)	27 (34.62)
	Total			44	34	

Table 4.36: Prevalence of *Escherichia coli*, *Salmonella*, and *Cryptosporidium* positive samples according to parity status of buffalo dams

Parity Status	Infection Status						Total (%)
	Escherichia coli		*Salmonella*		*Cryptosporidium*		
	+VE (%)	-VE (%)	+VE (%)	-VE (%)	+VE (%)	-VE (%)	
1	04(03.33)	05(04.17)	01(00.83)	08(06.67)	05(04.17)	04(10.00)	09(22.50)
2	08(06.67)	05(04.17)	00(00.00)	13(10.83)	09(07.50)	04(03.33)	13(32.50)
3	01(00.83)	03(02.50)	00(00.00)	04(03.33)	00(00.00)	04(03.33)	04(10.00)
4	00(03.33)	12(10.00)	01(00.83)	11(09.17)	03(02.50)	09(07.50)	12(30.00)
6	02(01.67)	00(00.00)	00(00.00)	02(01.67)	00(00.00)	02(01.67)	02(05.00)
Total	15(12.50)	25(20.83)	2(01.67)	38(31.67)	17(14.17)	23(19.17)	40

Table 4.37: Detection of *Escherichia coli*, *Salmonella* and *Cryptosporidium* positive samples from faeces of buffalo calves and dams without diarrhea

Age	Non - Diarrhaegenic Calves					Non -Diarhaegenic Dams				
	No with no Diarrhea (%)	Total	Infection type			No. with no Diarrhea (%)	Total	Infection type		
			E coli (%)	*Salmonella* (%)	*Cryptosporidium* (%)			*E coli* (%)	*Salmonella* (%)	*Cryptosporidium* (%)
Day 1-2 Days	04(50.00)	16	02(25.00)	00(00.00)	02 (25.00)	01(25.00)	18	01(25.00)	00(00.00)	00(00.00)
Day 3-3 Months	03(37.50)	14	01(12.50)	00(00.00)	02 (25.00)	01(25.00)	14	00 (0.00)	00(00.00)	01(25.00)
4-6 Months	01(25.00)	08	01(12.50)	00(00.00)	00 (00.00)	02(50.00)	08	01(25.00)	00(00.00)	01(00.00)
Total	08(100.00)	38	04(50.00)	00(00.00)	04 (50.00)	04(100.00)	40	02.(50.00)	00(00.00)	02(25.00)

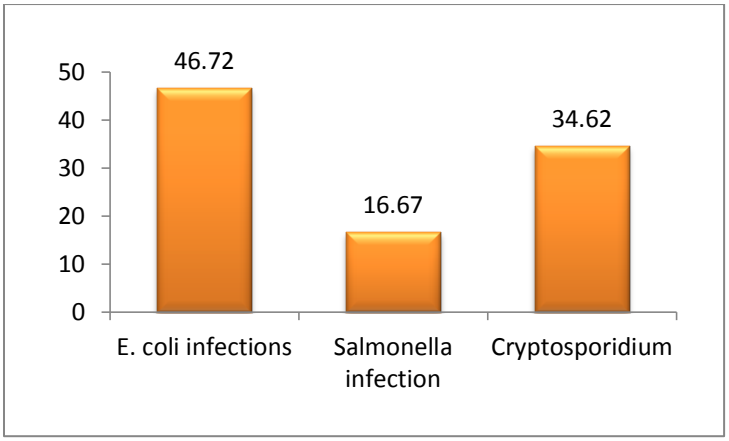


Figure 4.20: A comparative percentages of *Escherichia coli*, *Salmonella* and *Cryptosporidium* species positive samples from buffalo calves and dams combined

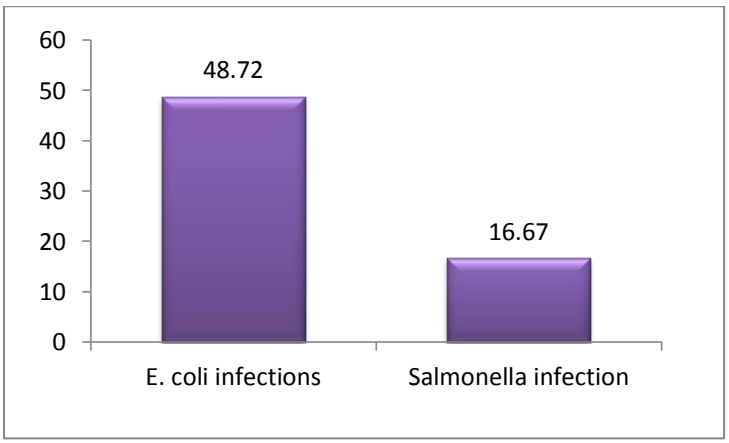


Figure 4.21: A comparative percentages of *Escherichia coli* and *Salmonella* species positive samples from buffalo calves and dams combined

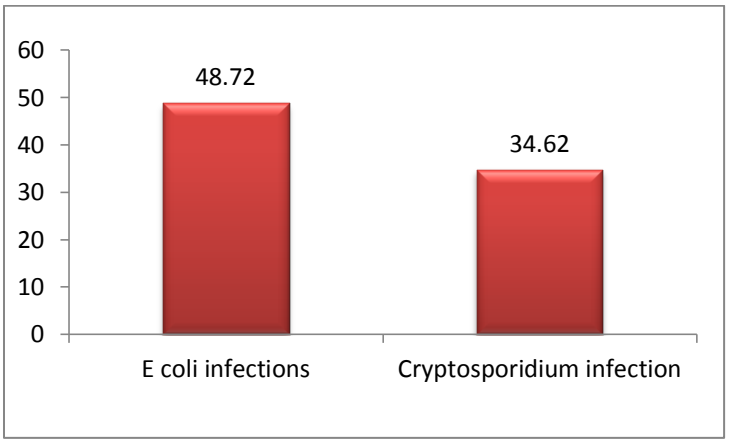


Figure 4.22: A comparative percentages of *Escherichia coli* and *Cryptosporidium* species positive samples from buffalo calves and dam combined

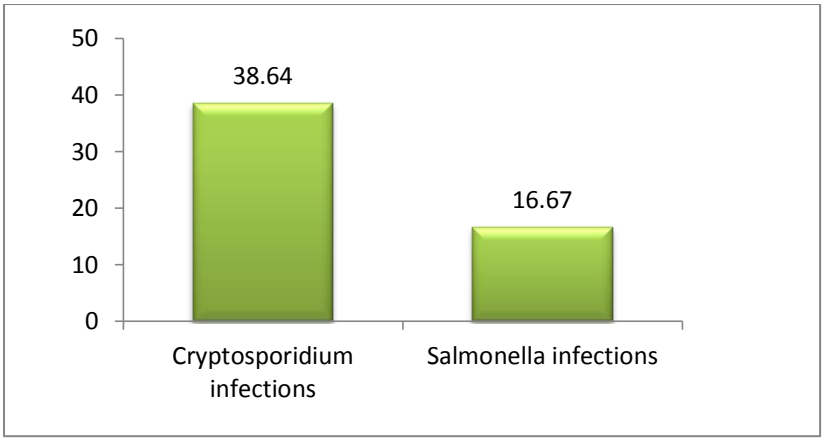
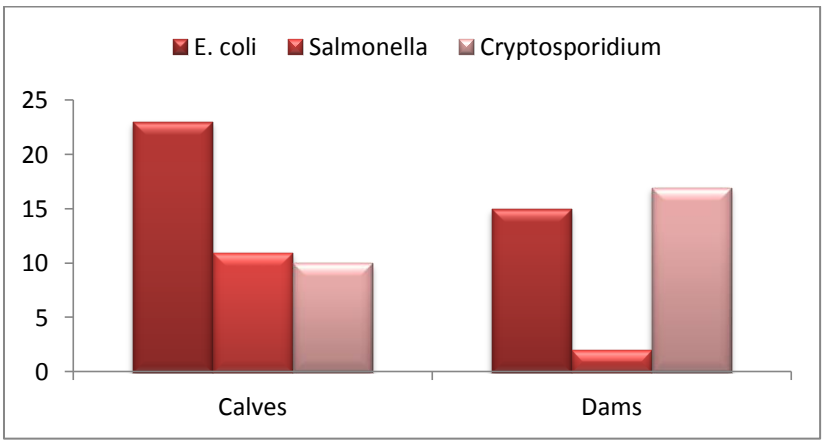
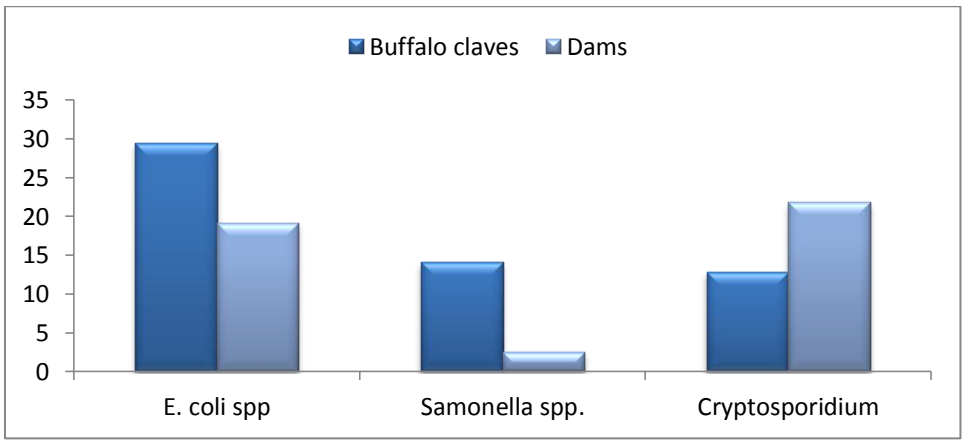


Figure 4.23: A comparative percentages of *Salmonella* and *Cryptosporidium* species positive samples of buffalo calves and dams combined



Animal category

Figure 4.24: Prevalence of *Escherichia coli, Salmonella* and *Cryptosporidium* species from buffalo calves and dams combined



Infection Status

Figure 4.25: Percentages of *Escherichia coli, Salmonella* and *Cryptosporidium* species positive samples from buffalo calves and dams

4.5 Results of Non- detection of Rotavirus and Coronavirus from faecal samples of Buffalo calves and their dams

4.5.1 RNA-PAGE analysis

All 78 faecal samples examined and analysed, diarrheic and non-diarrheic from Buffalo Calves and Dams, were found negative for Rotavirus and Coronavirus by RNA-PAGE analysis shown in figure 1.

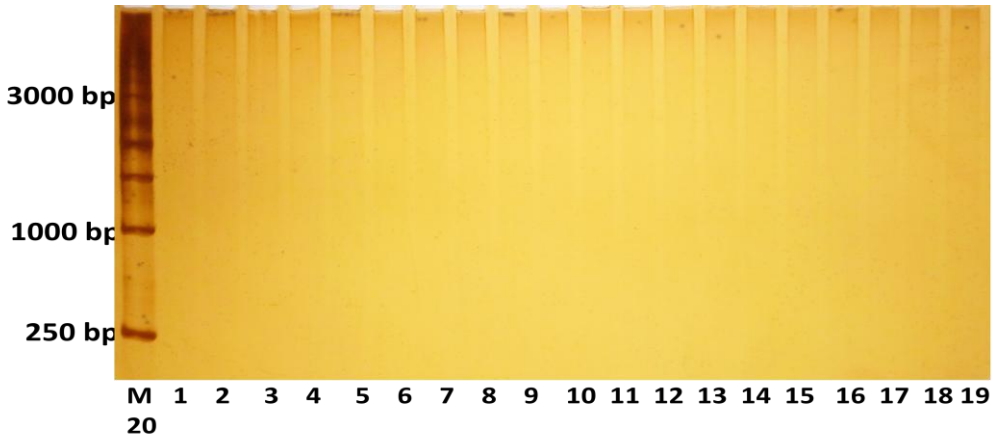


Figure 4.26: RNA-PAGE detection of rotavirus positive samples from faeces of buffalo calves and dams. None of the Samples were found positive for rotavirus. Lane M (20) represents the ladder for rotavirus while Lane 1 to 19 is showing negative results from the samples.

RT-PCR

All samples were negative as revealed by RNA-PAGE and amplification by Bov9com5 and Bov9com3 Primers did not yielded an expected product of 1,013 bp as shown in figure 2.

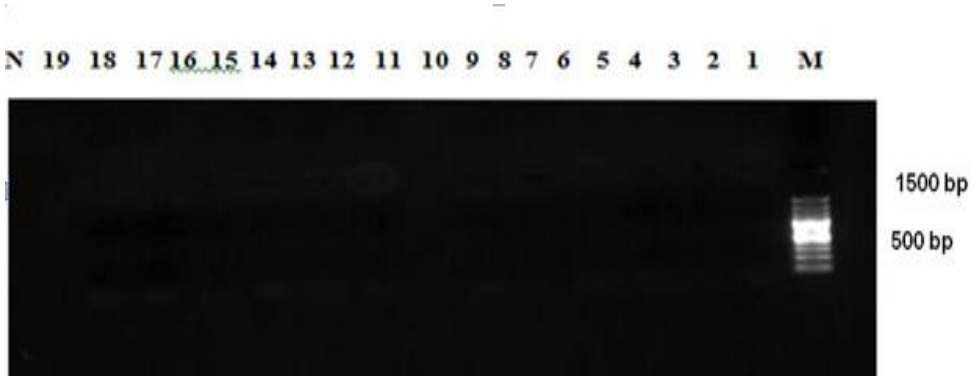


Figure 4.27: RT-PCR revealed negative detection of rotavirus after using Bov9com5 and Bov9com3 Primer as indicated in Lane 1 to 19. Lane M is 100bp plus RNA ladder and Lane N represents negative template control.

4.38 Results of infection in buffalo calves of Day - 1 to 2 - Days of age

LPM82 and LPM83 have no diarrhoea and no evidence of infection by any infectious agent. They are seemingly resisant calves. Where as BC1265 and BC 1268 each had only *E coli* positive detection but all were diarrhoeic. It may be considered as the main agent.

Table 4.38: Multiple infections in buffalo calves of Day - 1 to 2 - Days of age

Sr. No	Calf No.	Sex	D.O.B.	Diarrhea Status		Infection Status				
				Yes	No	Bacteria		Parasite	Virus	
						Ecoli	*Salmonella*	*Cryptosporidium*	Rota	Corona
1	BC81	M	03.08.2017	D	-	+VE	+VE	+VE	-VE	-VE
2	BC1374	F	31-07-2017	-	ND	-VE	-VE	+VE	-VE	-VE
3	BC1369	M	01-07-2018	D	-	+VE	+VE	-VE	-VE	-VE
4	BC1371	F	21-07-2017	D	-	+VE	+VE	-VE	-VE	-VE
5	LPM80	M	23-07-2017	D	-	+VE	+VE	-VE	-VE	-VE
6	LPM79	F	22-07-2017	D	-	+VE	+VE	-VE	-VE	-VE
7	BC1373	F	31-07-2017	-	ND	+VE	-VE	-VE	-VE	-VE
8	BC1372	M	23-07-2017	-	ND	+VE	-VE	+VE	-VE	-VE
9	BC1370	F	19-07-2018	-	ND	-VE	-VE	-VE	-VE	-VE
10	BC1375	F	03-08-2017	-	ND	-VE	-VE	-VE	-VE	-VE
11	LPM82	M	08-08-2017	-	ND	-VE	-VE	-VE	-VE	-VE
12	LPM83	F	09-08-2017	-	ND	-VE	-VE	-VE	-VE	-VE
13	BC1317	M	11:11:2016	D	-	+VE	+VE	-VE	-VE	-VE
14	BC69	M	03:08:2016	D	-	+VE	-VE	-VE	-VE	-VE
15	BC1265	M	13:08:2016	D	-	+VE	-VE	-VE	-VE	-VE
16	BC1268	M	19:08:2016	D	-	+VE	-VE	-VE	-VE	-VE

4.39 Results of infections in Buffalo Dams of Calves of Day - 1 to 2 - Days of age

BD 799 and LPM 38 each were non-diarrhoeic and showed resistance to all infectious agents screened. Genes of resistance may pass to their calves.

Table 4.39: Multiple infections in Buffalo Dams of Calves of Day - 1 to 2 - Days of age

Sr. No.	Dam No.	Parity	Diarrhea Status		Infection Status				
			Yes	No	Bacteria		Parasite	Virus	
					E coli	*Salmonella*	*Cryptosporidium*	Rota	Corona
1	BD799	1st Lactation	-	ND	-VE	-VE	-VE	-VE	-VE
2	BD967	2nd Lactation	D	-	+VE	-VE	+VE	-VE	-VE
3	BD791	4th Lactation	D	-	-VE	+VE	+VE	-VE	-VE
4	LPM38	2nd Lactation	-	ND	-VE	-VE	-VE	-VE	-VE
5	BD1025	1st Lactation	D	-	+VE	-VE	+VE	-VE	-VE
6	LPM16	5th Lactation	-	ND	-VE	-VE	-VE	-VE	-VE
7	BD1046	1st Lactation	-	ND	-VE	-VE	-VE	-VE	-VE
8	BD1038	1st Lactation	D	-	+VE	-VE	+VE	-VE	-VE
9	LPM185	4th Lactation	D	-	-VE	-VE	+VE	-VE	-VE
10	BD909	2nd Lactation	D	-	+VE	-VE	-VE	-VE	-VE
11	BD49	1st Lactation	D	-	-VE	+VE	+VE	-VE	-VE
12	LPM10	6th Lactation	-	ND	+VE	-VE	-VE	-VE	-VE
13	BD1068	3th Lactation	-	ND	-VE	-VE	-VE	-VE	-VE
14	BD182	3rd Lactation	-	ND	-VE	-VE	-VE	-VE	-VE
15	BD190	2nd Lactation	D	-	+VE	-VE	+VE	-VE	-VE
16	BD173	4th Lactation	D	-	-VE	-VE	+VE	-VE	-VE
17	BD19	2nd Lactation	D	-	+VE	-VE	+VE	-VE	-VE
18	BD0015	2nd Lactation	ND	-	-VE	-VE	-VE	-VE	-VE

Table 4.40: Multiple infections in buffalo calves of Day -3 to 3 - Months of age

Sr. No.	Calf No.	Sex	D.O.B.	Diarrhea Status		Infection Status				
						Bacteria		Parasite	Virus	
						E coli	*Salmonella*	*Cryptosporidium*	Rota	Corona
1	BC364	F	05-06-2017	-	ND	-VE	-VE	-VE	-VE	-VE
2	BC1365	M	07-06-2017	D	-	+VE	-VE	+VE	-VE	-VE
3	BC1366	M	11-06-2017	-	ND	+VE	-VE	-VE	-VE	-VE
4	BC1358	F	01-05-2017	D	-	+VE	-VE	-VE	-VE	-VE
5	BC1359	M	15-05-2017	-	ND	-VE	-VE	+VE	-VE	-VE
6	BC1360	F	20-05-2017	D	-	+VE	-VE	-VE	-VE	-VE
7	BC77	M	21-05-2017	-	ND	-VE	-VE	-VE	-VE	-VE
8	BC1361	M	24-05-2017	-	ND	-VE	-VE	+VE	-VE	-VE
9	BC1363	M	30-05-2017	D	-	+VE	-VE	+VE	-VE	-VE
10	BC1367	M	14-06-2017	D	-	+VE	-VE	-VE	-VE	-VE
11	BC1368	F	18-06-2017	-	ND	-VE	-VE	-VE	-VE	-VE
12	BC22D	F	22 DAYS	D	-	+VE	+VE	+VE	-VE	-VE
13	BC2M	F	2 MONTH	D	-	+VE	+VE	+VE	-VE	-VE
14	BC2.5M	F	2.5 MONTH	D	-	+VE	+VE	+VE	-VE	-VE

4.41 Result of infections in buffalo dams for calves of Day - 3 to 3 - months of age

LPM177, BD 912, BD 1068, BD 998, BD959, BD675 and BD170 are seemingly resistant dams for all infectious agents under this study.

Table 4.41: Multiple infections in buffalo dams for calves of Day - 3 to 3 - months of age

Sr. No	Dam No.	Parity	Diarrhea Status		Infection Status				
			Yes	No	Bacteria		Parasite	Virus	
					E coli	*Salmonella*	*Cryptosporidium*	Rota	Corona
1	LPM177	4^{TH} Lactation	-	ND	-VE	-VE	-VE	-VE	-VE
2	BD912	2^{ND} Lactation	-	ND	-VE	-VE	-VE	-VE	-VE
3	BD935	2^{ND} Lactation	D	-	+VE	-VE	+VE	-VE	-VE
4	BD1010	1^{ST} Lactation	D	-	+VE	-VE	-VE	-VE	-VE
5	BD1068	1^{ST} Lactation	-	ND	-VE	-VE	-VE	-VE	-VE
6	BD1003	1^{ST} Lactation	-	ND	-VE	-VE	+VE	-VE	-VE
7	BD847	3^{RD} Lactation	D	-	+VE	-VE	-VE	-VE	-VE
8	BD183	3^{RD} Lactation	-	ND	-VE	-VE	-VE	-VE	-VE
9	BD998	2^{ND} Lactation	D	-	-VE	-VE	+VE	-VE	-VE
10	BD959	2^{ND} Lactation	-	ND	-VE	-VE	-VE	-VE	-VE
11	BD848	2^{ND} Lactation	D	-	+VE	-VE	+VE	-VE	-VE
12	BD902	1^{ST} Lactation	D	-	+VE	-VE	+VE	-VE	-VE
13	BD675	3^{RD} Lactation	-	ND	-VE	-VE	-VE	-VE	-VE
14	BD170	4^{TH} Lactation	-	ND	-VE	-VE	-VE	-VE	-VE

4.42 Result of infections in buffalo calves of Month- 4 to 6 - months of age

BC1324, BC75, BC1344 andBC72 are seemingly resistant calves.

Table 4.42: Multiple infections in buffalo calves of Month- 4 to 6 - months of age

Sr. No	Calf No.	Sex	D.O.B.	Diarrhea Status		Infection Status				
						Bacteria		Parasite	Virus	
						E coli	*Salmonella*	*Cryptosporidium*	Rota	Corona
1	BC1311	M	11-11-2017	D	-	+VE	+VE	-VE	-VE	-VE
2	BC1364	F	05-06-2017	D	-	+VE	-VE	+VE	-VE	-VE
3	BC1324	M	01-12-2016	-	ND	-VE	-VE	-VE	-VE	-VE
4	BC75	M	27-12-2016	-	ND	-VE	-VE	-VE	-VE	-VE
5	BC1344	M	23-01-2017	-	ND	-VE	-VE	-VE	-VE	-VE
6	BC1279	M	03-09-2016	D	-	-VE	+VE	-VE	-VE	-VE
7	BC72	F	20-09-2016	-	ND	-VE	-VE	-VE	-VE	-VE
8	BC1377	F	07-10-2016	-	ND	+VE	-VE	-VE	-VE	-VE

4.43 Infections in buffalo dams of calves of Month - 4 to 6 - months of age

BD-170; BD-196; BD-181 and BD-20 are seemingly resistant

Table 4.43: Multiple infections in buffalo dams of calves of Month - 4 to 6 - months of age

Sr. No.	Dam No.	Parity	Diarrhea Status		Infection Status				
			Yes	No	Bacteria		Parasite	Virus	
					E coli	*Salmonella*	*Cryptosporidium*	Rota	Corona
1	BD182	2^{ND}	D	-	+VE	-VE	+VE	-VE	-VE
2	BD170	4^{TH}	-	ND	-VE	-VE	-VE	-VE	-VE
3	BD24	2^{ND}	D	-	+VE	-VE	+VE	-VE	-VE
4	BD196	4^{TH}	-	ND	-VE	-VE	-VE	-VE	-VE
5	BD16	6^{TH}	-	ND	+VE	-VE	+VE	-VE	-VE
6	BD181	4^{TH}	-	ND	-VE	-VE	-VE	-VE	-VE
7	BD178	4^{TH}	D	-	-VE	-VE	-VE	-VE	-VE
8	BD20	4^{TH}	-	ND	-VE	-VE	-VE	-VE	-VE

OBJECTIVE NUMBER 2

Table 4.44: O.D values of bovine Immunoglobulin-G (IgG) at A_{562}

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.148	0.223	1.519	1.512	0.053	0.052	0.048	0.046	0.047	0.048	0.047	0.044
B	0.235	0.167	0.169	0.184	0.049	0.049	0.05	0.047	0.051	0.046	0.106	0.049
C	0.188	0.271	0.308	0.315	0.05	0.047	0.046	0.047	0.048	0.045	0.047	0.045
D	0.255	0.25	2.186	2.182	0.051	0.047	0.049	0.048	0.048	0.047	0.047	0.047
E	0.307	0.32	0.055	0.046	0.046	0.047	0.047	0.05	0.049	0.049	0.045	0.047
F	0.431	0.421	0.058	0.045	0.045	0.044	0.047	0.047	0.049	0.053	0.048	0.049
G	0.673	0.68	0.045	0.045	0.045	0.047	0.045	0.048	0.046	0.05	0.048	0.046
H	1.119	1.113	0.051	0.045	0.046	0.046	0.046	0.044	0.046	0.046	0.047	0.049

4.45 Estimated protein content of bovine IgG

Different dilutions of bovine serum albumin concentrations (BSA) in microgram per milliliter (µg/l) and the average optical density (OD) values taken at absorbence 562 (A_{562}) after processing of BSA for determining protein concentrations using Bicinchoninic acid (BCA) protocol.

Table 4.45:Estimated protein content of bovine IgG= [120ug/ml] x100= 12 mg/ml

BSA Conc (µg/ml)	Average OD/562
20	0.190333
40	0.2295
80	0.2295
120	0.3135
200	0.426
400	0.6765
800	1.116
1200	1.5155
2000	2.184

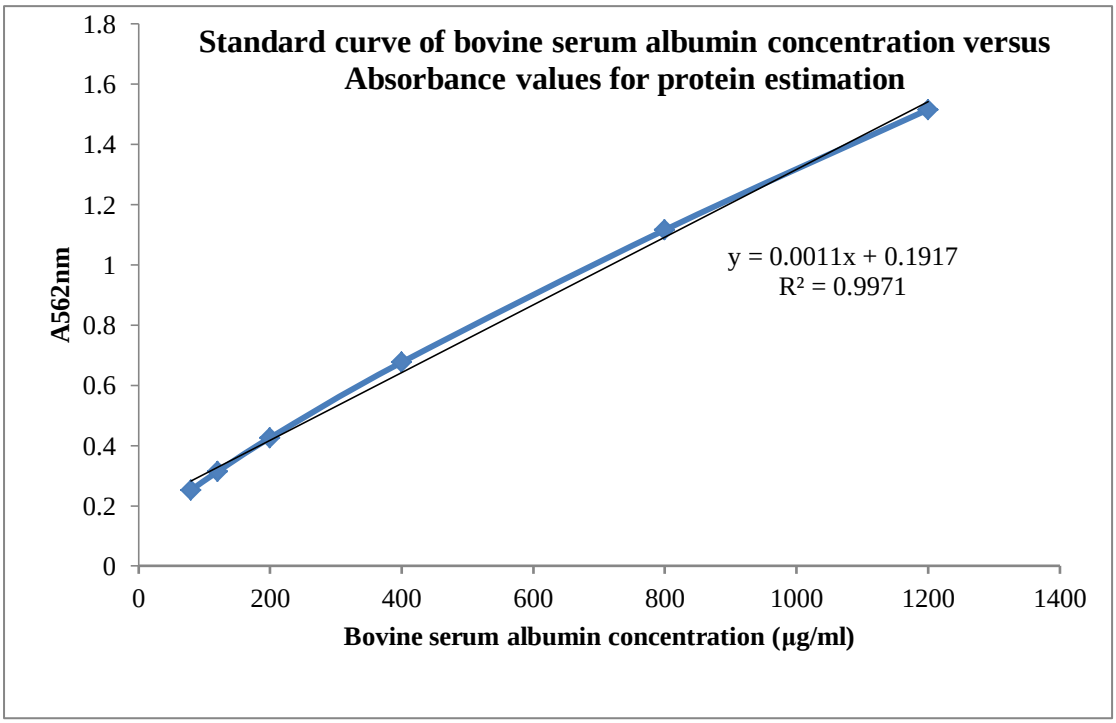


Figure 4.28: Standard curve of bovine serum albumin concentration for protein estimation

Table 4.46: Seriel log dilution of pure bovine IgG

Sr. No.	Conc. of bovine IgG (Positive Control)
1	1.25ug/ml
2	1.0ug/ml
3	0.625ug/ml
4	0.5ug/ml
5	0.3125ug/ml
6	0.15625ug/ml
7	0.078ug/ml
8	0.039ug/ml

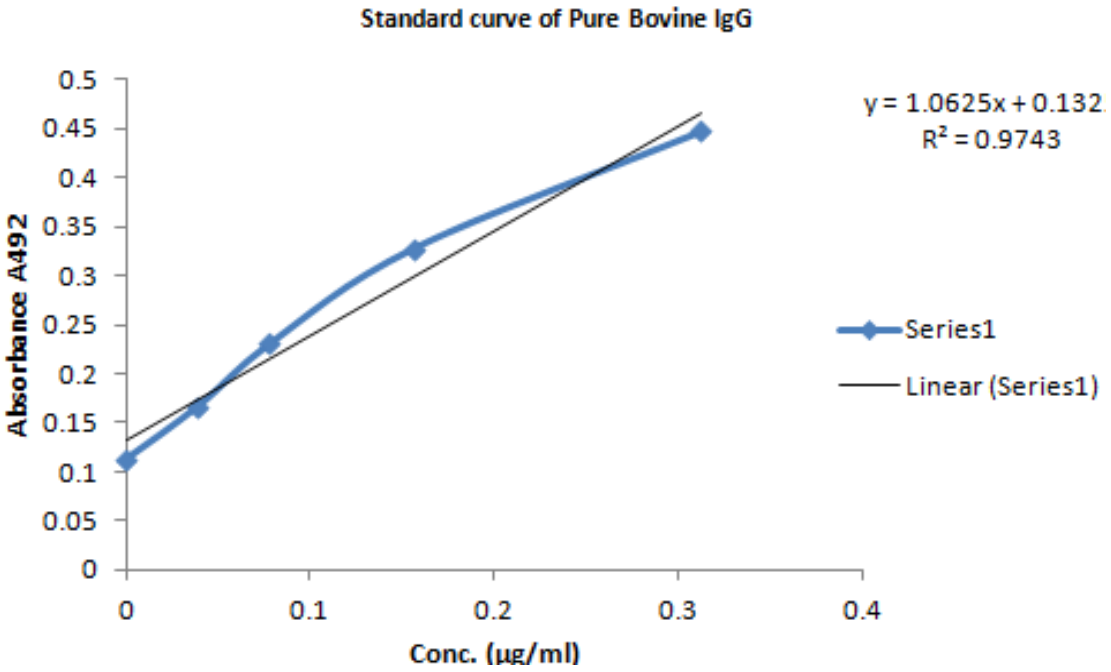


Figure 4.29: Standard curve for quantitation of pure bovine IgG

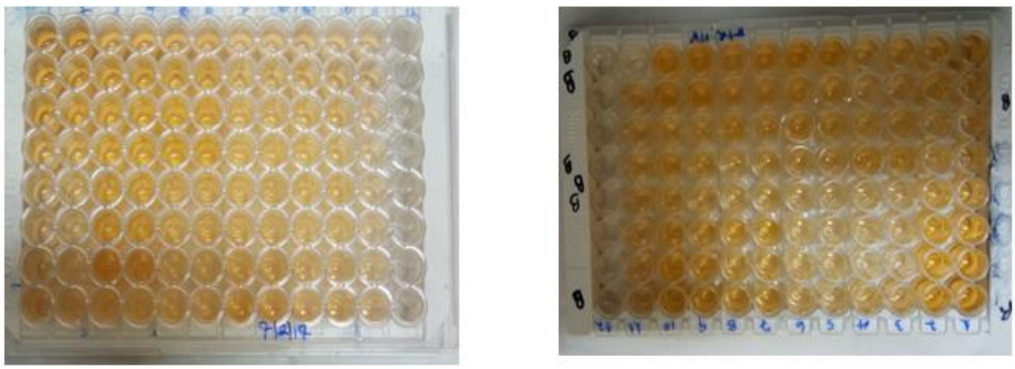


Figure 4.30: Results of sandwich ELISA for detection of bovine IgG from oral fluid, urine, meconium, faeces, colostrums, milk and serum

Table 4.47: Immunoglobulin-G (IgG) concentrations in colostrums samples of buffalo dams

Sample No. (colostrum)	Conc. of IgG	Sample No. (colostrum)	Conc. of IgG
BD185	23826.79739	BD909	3532.679739
BD1056	26934.64052	BD799	15928.10458
BD38	29081.69935	BD49	14444.44444
BD185	17496.73203	BD1038	1990.196078
BD183	27666.66667	BD967	6885.620915
BD1046	24297.38562	BD19	395.4248366
BD16	20705.88235	BD791	26058.82353
BD10	13656.86275	PBST	-

Table 4.48: Immunoglobulin-G (IgG) concentrations in milk samples of buffalo dams

Sample No. (Milk)	Conc. of IgG	Sample No. (Milk)	Conc. of IgG	Sample No. (Milk)	Conc. of IgG
BD181	31.74836601	BD177	36.59150327	BD178	29.87908497
BD176	27.24509804	BD20	29.62418301	BD847	28.94444444
BD170	26.74509804	BD998	18.98366013	BD188	30.24836601
BD1010	23.96405229	BD912	25.28431373	BD1003	18.5751634
BD24	25.6503268	BD1068	23.2254902	BD935	25.61111111
BD182	26.08496732	BD183	24.21568627	BD848	24.70261438
BD173	27.85620915	BD198	30.54901961	PBST	2.843137255
BD959	21.00000000	BD1055	26.41830065	PBST	2.013071895

Table 4.49: Immunoglobulin-G (IgG) concentrations in serum samples of buffalo calves

Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG
BC81	5790.22082	BC1370	5703.470032	BC1364	5507.886435	BC1363	5914.037855	BC1324	6389.589905
BC1374	4883.280757	BC1375	5455.835962	BC1365	5319.400631	BC1367	5762.618297	BC75	6120.662461
BC1369	5223.18612	BC 82	5427.444795	BC1366	5532.334385	BC1368	5614.353312	BC1344	6211.356467
BC1371	5291.009464	BC 83	5711.356467	BC1358	5738.170347	BC22D	5676.656151	BC1279	5947.949527
BC 80	5432.176656	BC1317	5427.444795	BC1359	5904.574132	BC2M	5419.55836	BC72	6251.577287
BC 79	5967.665615	BC69	5876.971609	BC1360	5836.750789	BC2.5M	5893.533123	BC1377	5812.302839
BC1373	5733.438486	BC1265	5769.716088	BC77	5723.18612	BC1311	5533.911672	BC1411	6447.949527
BC1372	5423.501577	BC1268	5533.123028	BC1361	5981.861199	BC1364	6409.305994	BC1412	6085.173502

Table 4.50: Immunoglobulin-G (IgG) concentrations in serum samples of buffalo dams

Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG
BD 799	5917.513683	BD1038	5286.551994	BD 190	5897.576231	BD 1010	5996.481626	BD 902	6033.620016
BD791	5262.283237	BD185	5341.673182	BD 173	2489.835809	BD1068	3350.664582	BD 675	5609.460516
BD 967	5223.612197	BD 49	5414.017341	BD 19	5358.87412	BD1003	5376.075059	BD 170	5782.64269
BD 38	5431.978108	BD 909	4483.580923	BD 15	5729.867084	BD 847	5334.636435	BD 182	5938.623925
BD1025	5395.621579	BD 49	5438.623925	BC1413	5564.894449	BD 183	5918.295543	BD 24	5663.408913
BD 20	2817.044566	BD 10	5551.602815	BD 177	4366.692729	BD 998	5609.460516	BD 196	5957.388585
BD16	5493.745113	BD 1068	5711.884285	BD 912	6016.810008	BD 959	5497.263487	BD181	5965.598124
BD1046	6226.34871	BD 182	5682.173573	BD 935	6085.22283	BD 848	6126.661454	BD 178	6308.053167

Table 4.51: Concentrations of Immunoglobulin-G (IgG) from saliva samples of buffalo calves

Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG
BC1268	0.121294891	BC02	0.285204856	BC1367	0.249340866	BC1377	0.147622661	BC1374	0.212711864
BC 79	0.08592277	BC78	0.256272129	BC80	0.001647834	BC75	0.040301318	BC1373	0.025290845
BC1372	0.09868173	BC1360	0.32090395	BC1369	0.123773394	BC1368	0.106167608	BC81	0.187806026
BC1361	0.07589459	BC1279	0.198128477	BC1369	0.016054614	BC22D	0.251062215	BC1324	0.006550329
BC1365	0.23422782	BC1046	0.24552352	BC1344	0.017890772	BC01	0.251542742	BC1279	0.140566515
BC1371	0.24529192	BC1317	0.017804755	BC182	0.047410546	BC1306	0.027187658	BC1279	0.198128477
BC73	0.001568032	BC75	0.00230698	BC72	0.11646434	BC1268	0.042640364	BC1046	0.24552352
BC69	0.08682347	BC1359	0.106170966	BC1358	0.087900188	BC82	0.186911488	BC1317	0.017804755

Table 4.52: Immunoglobulin-G (IgG) concentrations in oral fluids samples of buffalo calves

Sample No. (Saliva)	Conc. of IgG	Sample No. (Saliva)	Conc. of IgG
BC2.5	0.211632948	BC1265	0.088367052
BC81	0.262933526	BC82	0.202456647
BC1265	0.134031792	-	
BC75	-0.007080925	-	-
BC1375	0.236343931	-	-
BC1374	0.230563584	-	-
BC183	0.269580925	-	-
BC1365	0.080274566	-	-

Table 4.53: Concentrations of Immunoglobulin-G (IgG) from saliva samples of buffalo dams

Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG
BD 24	0.13502829	BD20	0.04317328	BD176	0.020291902	BD176	0.104001883	BD848	0.148634294
BD170	0.04755179	BD176	0.104001883	BD183	0.224858757	BD181	0.021876581	BD183	0.224858757
BD178	0.11247646	BD38	0.193361582	BD177	0.1673258	BD188	0.160596864	BD847	0.208548306
BD912	0.23578154	BD1025	0.24091331	BD16	0.188841808	BD38	0.193361582	BD16	0.188841808
BD1025	0.24091331	BD10	0.21501882	BD791	0.224623352	BD909	0.234934087	BD791	0.224623352
BD10	0.21501882	BD20	0.04317328	BD967	0.186440678	BD1068	0.09373823	BD967	0.186440678
BD1010	0.24190202	BD909	0.234934087	BD935	0.212947269	BD176	0.020291902	BD1317	0.118173258
BD170	0.04755179	BD1068	0.09373823	BD1317	0.118173258	BD1038	0.246029337	BD49	0.233712696

Table 4.54: Immunoglobulin-G (IgG) concentrations in faecal samples of buffalo calves

Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG
BC01	0.435033784	BC1367	0.232398649	BC1375	0.466858108	BC79	0.207407407	BC1311	0.201891892
BC02	0.396013514	BC1365	0.351925676	BC1369	0.520919067	BC1265	0.016255144	BC1324	0.192798354
BC03	0.483074324	BC1306	0.142112483	BC1361	0.325540541	BC1363	0.30027027	BC77	0.137585734
BC05	0.393581081	BC10	0.004938272	BC1373	0.44739369	BC81	0.453378378	BC2M	0.136351166
BC06	0.242364865	BC1279	0.299725652	BC1368	0.369958848	BC22D	0.471587838	BC1359	0.108847737
BC04	0.052194787	BC1364	0.469256757	BC80	0.432871622	BC69	0.115500686	BC1377	0.417533784
BC1372	0.443918919	BC1360	0.024485597	BC1.5	0.361081081	BC1371	0.483141892	BC1268	0.132784636
BC1366	0.437432432	BC82	0.473310811	BC1374	0.596021948	BC82	0.395404664	PBST	0.190603567

Table 4.55: Immunoglobulin-G (IgG) concentrations in faecal samples of buffalo dams

Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG
BD24	0.272567568	BD181	0.115294925	BD791	0.398310811	BD1038	0.021742112	BD799	0.012894376
BD16	0.050405405	BD1068	0.226587838	BD10	0.246364883	BD1038	0.213310811	BD185	0.084636488
BD1046	0.002743484	BD909	0.369290541	BD190	0.203017833	BD183	0.040397805	BD647	0.301114865
BD20	0.087804054	BD1003	0.007887517	BD49	0.372635135	BD16	0.030315501	BD1025	0.327601351
BD967	0.130912162	BD935	0.115439189	BD38	0.307613169	BD967	0.13484225	BD173	0.079290541
BD19	0.129423868	BD1805	0.180439189	BD1010	0.454594595	BD177	0.135665295	BD188	0.291993243
BD912	0.039986283	BD959	0.16902027	BD848	0.005967078	BD176	0.08744856	PBST	0.220507545
BD178	0.047195946	BD182	0.237804054	BD1068	0.021742112	BD998	0.193484225	PBST	0.190603567

Table 4.56: Immunoglobulin-G (IgG) concentrations in urine samples from buffalo calves

Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG
BC 01	0.269966216	BC 04	0.371993243	BC81	0.379121622	BC1358	0.054966216	BC1374	0.406182432
BC 02	0.219189189	BC 05	0.365777027	BC1370	0.423952703	BC1364	0.406114865	BC1265	0.307331081
BC 03	0.236148649	BC07	0.394864865	BC1358	0.054966216	BC79	0.059695946	BC1372	0.286199422
BC 04	0.371993243	BC1375	0.464527027	BC1364	0.406114865	BC1373	0.371452703	BC1374	0.406182432
BC 05	0.365777027	BC1375	0.464527027	BC1373	0.371452703	BC1306	0.101081081	BC1265	0.307331081
BC 01	0.269966216	BC81	0.379121622	BC1317	0.16222973	BC75	0.173885135	BC1306	0.101081081
BC 02	0.219189189	BC1317	0.16222973	BC78	0.345371622	BC1370	0.423952703	BC75	0.173885135
BC 03	0.236148649	BC78	0.345371622	BC1311	0.129121622	BC1311	0.129121622	BC79	0.059695946

Table 4.57: Immunoglobulin-G (IgG) concentrations in urine samples from buffalo dams

Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG	Sample No.	Conc. of IgG
BD 19	0.065101351	BD998	0.086722973	BD20	0.19847973	BD173	0.129594595	BD188	0.050912162
BD122	0.078682432	BD1367	0.39097973	BD24	0.322195946	BD178	0.059358108	BD182	0.097466216
BD935	0.104966216	BD49	0.282736486	BD1036	0.355574324	BD24	0.322195946	BD1046	0.344763514
BD998	0.086722973	BD959	0.198513514	BD791	0.301317568	BD1036	0.355574324	BD188	0.050912162
BD1367	0.39097973	BD49	0.282736486	BD967	0.244222973	BD791	0.301317568	BD182	0.097466216
BD 19	0.065101351	BD181	0.084459459	BD173	0.129594595	BD967	0.244222973	-	-
BD122	0.078682432	BD959	0.198513514	BD178	0.059358108	BD181	0.084459459	-	-
BD935	0.104966216	BD16	0.154797297	BD38	0.344121622	BD1046	0.344763514	-	-

Table 4.58: Immunoglobulin-G (IgG) concentrations in colostrums and sera of buffalo dams and in sera of their respective calves with occurrence of diarrheoa

Sample No. (Dam)	Conc. of IgG (colostrum)	Conc. of IgG (serum)	Sample No (calf)	Conc. of IgG (serum)	Infection Status (Calf)			Diarrhea (Calf)
					E coli	*Salmonella*	*Cryptosporidium*	
BD185	23826.79739	5341.673182	BC 83	5711.356467	-VE	-VE	-VE	ND
BD38	29081.69935	5431.978108	BC 79	5967.665615	+VE	+VE	-VE	D
BD183	27666.66667	5918.295543	BC 77	5723.18612	-VE	-VE	-VE	ND
BD1046	24297.38562	6226.34871	BC1373	5733.438486	+VE	-VE	-VE	ND
BD16	20705.88235	5493.745113	BC 80	5432.176656	+VE	+VE	-VE	D
BD10	13656.86275	5551.602815	BC 82	5427.444795	-VE	-VE	-VE	ND

Table 4.59: Immunoglobulin-G (IgG) concentrations in colostrums and sera of buffalo dams and in sera of their respective calves with occurrence of diarrheoa

Sample No. (Dam)	Conc. of IgG (colostrum)	Conc. of IgG (serum)	Sample No. (calf)	Conc. of IgG (serum)	Infection Status (calf)			Diarrhea (calf)
					E coli	*Salmonella*	*Cryptosporidium*	
BD909	3532.679739	4483.580923	BC1375	5455.835962	-VE	-VE	-VE	ND
BD799	15928.10458	5917.513683	BC1369	5223.18612	+VE	+VE	-VE	D
BD49	14444.44444	5414.017341	BC 81	5790.22082	+VE	+VE	+VE	D
BD1038	1990.196078	5286.551994	BC1374	4883.280757	-VE	-VE	+VE	ND
BD967	6885.620915	5223.612197	BC1370	5291.009464	-VE	-VE	-VE	ND
BD19	395.4248366	5358.87412	BC1317	5427.444795	+VE	+VE	-VE	D
BD791	26058.82353	5262.283237	BC1371	5291.009464	+VE	+VE	-VE	D

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Table 4.60: NOD2/ CARD 15 gene expression status in infected buffalo dams

Sr. No.	Animal sampled	NOD2/CARD15 Scores	Infection Status	
			E coli	*Salmonella*
1	BD 791	+VE	-VE	+VE
2	BD 1046	+VE	-VE	-VE
3	BD 799	+VE	-VE	-VE
4	BD 10	+VE	+VE	-VE
5	BD 49	+VE	-VE	+VE
6	BD 38	+VE	-VE	-VE

Table 4.61: TLR4 gene expression status in infected buffalo calves

Sr. No.	Animal Sampled	TLR4 Scores	Infection Status	
			E coli	*Salmonella*
1	BC 1371	+VE	+VE	+VE
2	BC 79	+VE	+VE	-VE
3	BC 1373	+VE	+VE	-VE
4	BC 1375	+VE	-VE	-VE
5	BC 83	+VE	-VE	-VE
6	BC 81	+VE	+VE	+VE
7	BC 1374	+VE	+VE	+VE

Table 4.62: NOD2/ CARD15 gene expression status in infected buffalo calves with and without diarrhea

Sr. No.	Animal Sampled	NOD2/ CARD15 Scores	Infection status		IgG Conc. (serum) (Calf)	Diarrhea Status
			E coli	*Salmonella*		
1	BC 1371	+VE	+VE	+VE	5291.009464	D
2	BC 79	+VE	+VE	+VE	5967.665615	D
3	BC 81	+VE	+VE	+VE	5790.22082	D
4	BC 82	+VE	-VE	-VE	5427.444795	ND
5	BC 83	+VE	-VE	-VE	5711.356467	ND
6	BC 1373	+VE	+VE	-VE	5733.438486	ND
7	BC 1375	+VE	-VE	-VE	5455.835962	ND
8	BC 1372	+VE	-VE	-VE	5423.501577	ND
9	BC 72	+VE	+VE	+VE	6251.577287	ND
10	BC 1374	+VE	+VE	+VE	4883.280757	ND

Table 4.63: NOD2/ CARD 15 gene expression status in infected buffalo dams with and without diarrhea

Sr. No.	Animal sampled	NOD2/ CARD15 Scores	Infection Status		IgG Conc. (serum) (calf)	Diarrhea Status
			Ecoli	*Salmonella*		
1	BD 791	+VE	-VE	+VE	5262.283237	D
2	BD 1046	+VE	-VE	-VE	6226.34871	ND
3	BD 799	+VE	-VE	-VE	5917.513683	ND
4	BD 10	+VE	+VE	-VE	5551.602815	ND
5	BD 49	+VE	-VE	+VE	5438.623925	D
6	BD 38	+VE	-VE	-VE	5431.978108	ND

Table 4.64: TLR4 gene expression status in infected buffalo calves with and without diarrhea

Sr. No.	Animal Sampled	TLR4 Scores	Infection Status		IgG Conc. (serum) (calf)	Diarrhea Status
			E coli	*Salmonella*		
1	BC 1371	+VE	+VE	+VE	5291.009464	D
2	BC 79	+VE	+VE	-VE	5967.665615	D
3	BC 1373	+VE	+VE	-VE	5733.438486	ND
4	BC 1375	+VE	-VE	-VE	5455.835962	ND
5	BC 83	+VE	-VE	-VE	5711.356467	ND
6	BC 81	+VE	+VE	+VE	5790.22082	D
7	BC 1374	+VE	+VE	+VE	4883.280757	ND

Table 4.65: TLR4 gene expression status in infected buffalo dams with and without diarrhea

Sr. No.	Animal sampled	TLR4 Scores	Infection Status		IgG Conc. (serum) (Dam)	Diarrhea Status
			E coli	*Salmonella*		
1	BD 791	+VE	-VE	+VE	5262.283237	D
2	BD 1046	+VE	-VE	-VE	6226.34871	ND
3	BD 799	+VE	-VE	-VE	5917.513683	ND
4	BD 10	+VE	+VE	-VE	5551.602815	ND
5	BD 49	+VE	-VE	+VE	5438.623925	D
6	BD 38	+VE	-VE	-VE	5431.978108	ND

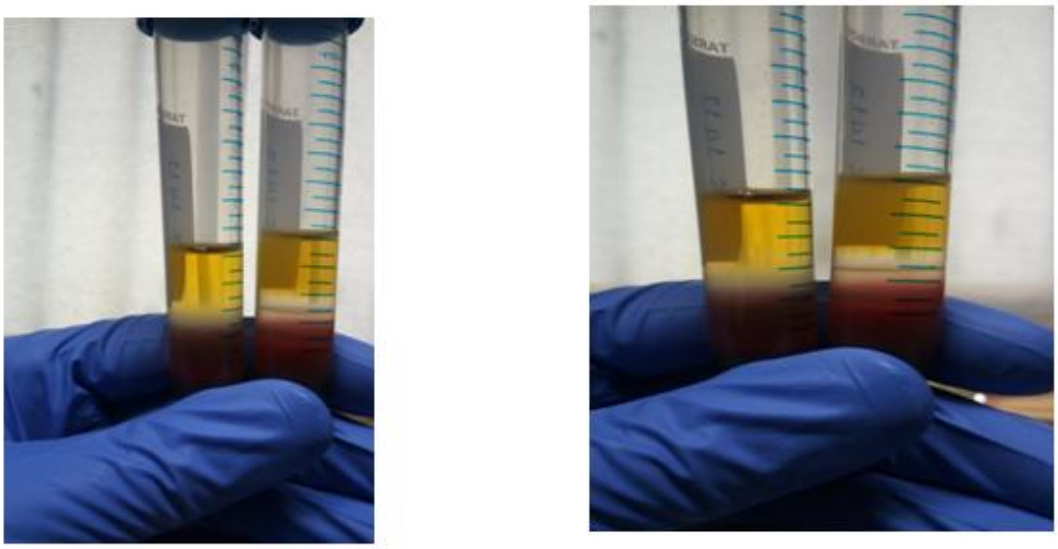

Figure 4.31: Result of pheripheral blood mononuclear cells (PBMC) extraction from blood plasma.White layer is a collection of PBMCs.

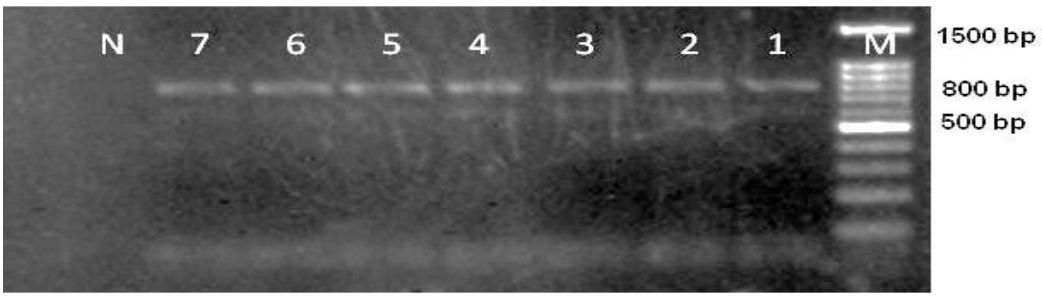

Figure 4.32: Positive *TLR4 gene* transcript detected at 800 bp resolved by agarose gel electrophoresis [Lane 1 (M) :100bp plus DNA ladder (Fermentas, USA); Lane 1-7: 800 bp PCR product; Lane N: negative template control]

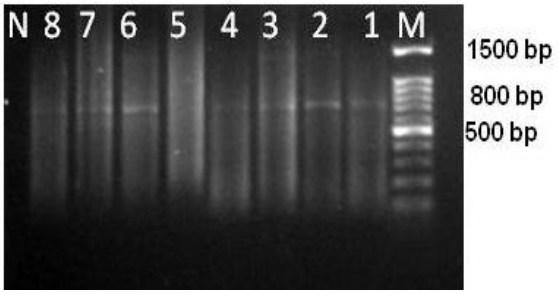

Figure 4.33: Positive*TLR4 gene* transcript detected at 800 bp and resolved by agarose gel electrophoresis [Lane 1 (M): 100bp plus DNA ladder (Fermentas, USA); Lane 1-8: 800 bp PCR product; Lane N: negative template control]

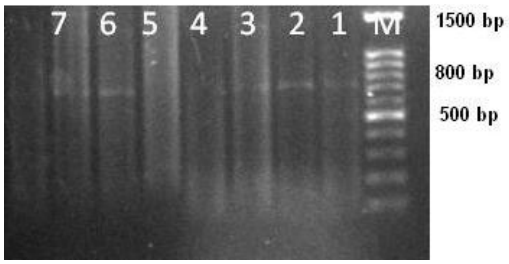

Figure 4.34: Positive *TLR4 gene* transcript detected at 800 bp and resolved by agarose gel electrophoresis [Lane 1 (M):100bp plus DNA ladder (Fermentas, USA); Lane 1-7:800 bp PCR produc].

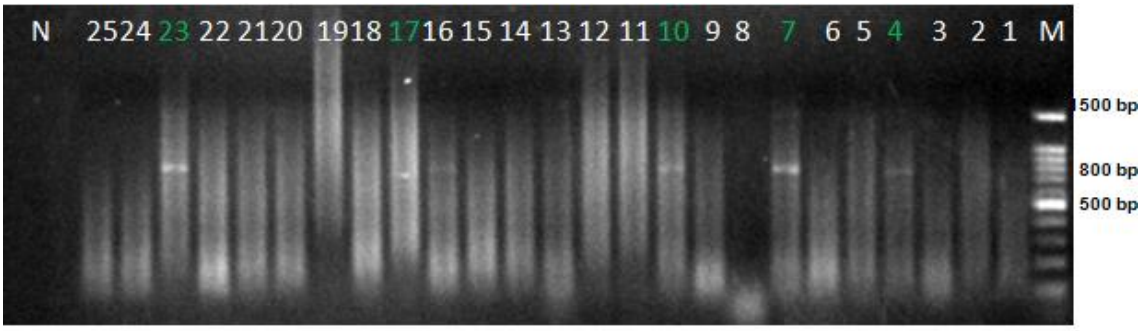

Figure 4.35: Positive *TLR4 gene* transcript detected at 800 bp and resolved by agarose gel electrophoresis [Lane 1 (M) :100bp plus DNA ladder (Fermentas, USA); Lane 4, 7, 10, 16, 22: 800 bp PCR product; Lane 1-3, 5-6, 8-9, 11-15, 17-21, 23-25: negative PCR product; Lane N: negative template control]

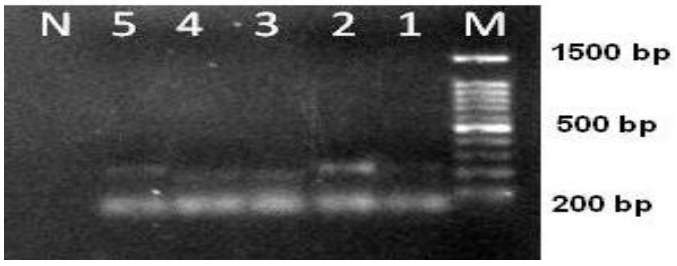

Figure 4.36: Positive *CARD15/NOD2 gene* transcript detected at 200 bp and resolved by agarose gel electrophoresis [Lane 1 (M) :100bp plus DNA ladder (Fermentas, USA); Lane 2, 3, 4 and 5: 200 bp PCR product; Lane 1 is negative PCR product; N: negative template control]

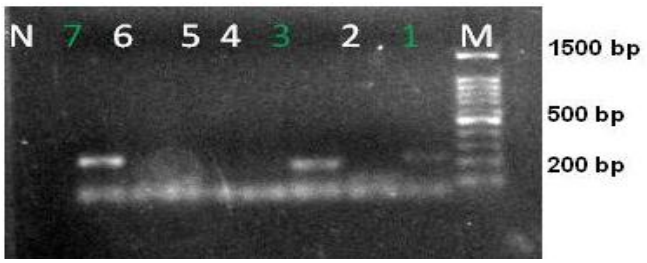

Figure 4.37: Positive *CARD15/NOD2 gene* transcript detected at 200 bp and resolved by agarose gel electrophoresis [Lane 1 (M):100bp plus DNA ladder (Fermentas, USA); Lane 1, 3, and 7: 200 bp PCR product; Lane 2, 4 and 5 are negative PCR product; N: negative template control]

Figure 4.38: Positive *CARD15/NOD2 gene* transcript detected at 200 bp and resolved by agarose gel electrophoresis [Lane 1 (M) :100bp plus DNA ladder (Fermentas, USA); Lane 6, 16, and 17: 200 bp PCR product; Lane 1-5, 7-15, and 18 are negative PCR product; N: negative template control]

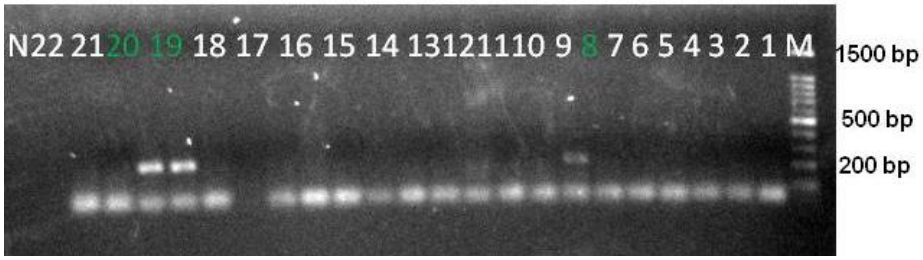

Figure 4.39: Positive *CARD15/NOD2 gene* transcript detected at 200 bp and resolved by agarose gel electrophoresis [Lane 1 (M) :100bp plus DNA ladder (Fermentas, USA); Lane 6, 16, and 17: 200 bp PCR product; Lane 1-5, 7-15, and 18 are negative PCR product; N: negative template control]

CHAPTER-V

DISCUSSION

Diarrhoea is the most commonly reported calf disease and a major cause of calf morbidity and mortality worldwide (Gitau *et al*. 1994; Bendali *et al.* 1999; Bazeley 2003; Svensson *et al*. 2006; Millemann 2009; Marce *et al.* 2010). Diarrhea in young calves is the main cause of economic losses through poor growth, morbidity and mortality (Constable 2004; Gaber 2004), and the role played by *Escherichia coli* (*E. coli*) in producing diarrhea in calves has received a great attention by many researchers (Shahrani *et al*., 2014). In the present study 78 faecal samples were collected from buffalo calves and their dams. Calves were categorized into 3 groups of Day- 1 to 2 -Days, Day -3 to 3- Months and a group of 4 to 6 Months of age during sample collection. A total of 38 positive faecal samples with *Escherichia coli* were recorded. The findings agrees with results of Bendali *et al.* (1999a) who recorded *Escherichia coli* as predominant pathogen when patterns of diarrhea in newborn beef calves was studied in south-west France. Garcia *et al.* (2000) reported similar records of predominance of *Escherichia coli* in studies on Rotavirus and concurrent infections with other enteropathogens in neonatal diarrheic dairy calves in Spain. This agrees with records of Paul *et al*., (2010), who showed that *E.coli* was isolated with an incidence of 50%.

Meanwhile, other researchers isolated *E.coli* from calves with lower incidence as described by Anwarullah *et al*., (2014), who isolated *E.coli* with an incidence of 14.6%. On the other hand, higher number of *E.coli* was recorded by Majueeb *et al*., (2014) who isolated *E.coli* with an incidence of 72.8%.

Most studies revealed higher prevalence of *E.coli* isolation from diarrheagenic and non-diarrheagenic buffalo calves of young ages of one and two weeks. The higher prevalence of *E. coli* in these ages may be due to poor managemental practices and predisposing factors like overcrowding and malnutrition, which are supposed to be a primary cause of immunosuppression. Although the role of these factors may not be prominent in the present case and yet substantial degrees of positive detections were recorded in the present studies probably because, these animals are reared in an organized farm under intensive conditions. Furthermore, *E. coli* is a commensal organism and is responsible for diarrhea in calves, particularly those receiving less or

no maternal antibodies through colostrum (Malik *et al*., 2012) especially in farms where milk is mainly used for commercial purposes, which is exactly the case in animals sampled for the current studies.

Detection of the alkaline phosphastase gene (*phoA*) showed that 100% isolates were positive for the *phoA* gene. This result agree with Chang *et al*., (1986) and Kong *et al*., (1999) who reported that *phoA* gene is a housekeeping gene present in all *E.coli* strains.

The detection of the *eaeA* (intimin) gene by PCR showed that 27% of examined isolates were positive for the *eaeA* gene with 15 and 10 of the genes detected respectively from calves and dams. This result agrees with findings of Hala (2012) who detected the *eaeA* gene by 20% but disagree with that of Nguyen *et al*., (2011) who detected the *eaeA* gene by 9.8% and Mohammadi *et al*., (2013) who reported that all of their isolates were *eaeA*-negative. The intimin gene detection is mainly linked to the EPEC pathotype (Beraldo *et al*., 2014) and *eaeA*-positive strains are considered to be more virulent to human than the *eaeA*-negative ones. This indicates a possible participation of buffalo calves in the zoonotic transmission of pathogenic *E.coli*.

The *tsh* gene encodes a temperature-sensitive hemagglutinin of *E.coli*, first identified by Provence and Curtiss (1994). Also the *tsh* protein was the first identified member of an expanding subclass of the IgA protease family of autotransporters present in *Shigella* spp. and numerous pathotypes of *E. coli* (Stathopoulos *et al*., 1999). In the present study result of PCR for the detection of the *tsh* gene showed that 100% of detected *E. coli* were positive for the genes. This nearly agrees with Janßen *et al*. (2001) and Saidenberg *et al*., (2013) who detected the *tsh* gene in 85.3% and 78.3%, respectively, whereas it is contrary to findings of Mohamed *et al*., (2014), Delicato *et al*., (2003) and Ewers *et al*., (2004), who detected a *tsh* positive frequency of 28%, 39.5% and 53.3%, respectively. However, those authors detected the *tsh* gene from the APEC isolated from poultry, where as the *E. coli* detection in the present study was recorded from faecal samples of Buffalo Calves and their Dams. This could indicate a possibility of either the expression of the *tsh* gene is under-estimated in different animal species or a poultry-to-buffalo transmission of APEC and/or plasmid transfer is possible. Different animal species are generally reared together in the country side and this allows the continuous interaction between the microbial environments of those species with its possible inter-species mixing requiring a more

detailed study of the co-existence of such mixed microbial populations. Further, this may indicate a possible wider role of buffalo calves and their dams as reservoir for extra-intestinal infections to human.

Antibiotics are widely used in the treatment and prevention of disease in the veterinary practice as well as serving as growth enhancer in animals. To date, there are many reports regarding *E. coli* resistance to antibiotics in many countries and regions (Johns *et al*., 2012, Szmolka and Nagy, 2013).

The results of detection for *E.coli* antibiotic resistance genes in the present study revealed level of resistance to Oxytetracycline. This was in agreement with Shahrani, *et al*. (2014) who found 98.09% resistance of *E. coli* against tetracycline in .diarrheaic calves in Iran and Balasubramaniam *et al*., (2014) reported the detection of *E. coli* resistance genes against tetracycline from poultry as 88% from India. But the findings of Nizza *et al*., (2010) recorded *E.coli* tetracycline resistance genes as 34%. Sulfanamide resistant genes among faecal samples with *E.coli* detection was also recorded in the present study. High resistance of *E. coli* to Sulfamethoxazole-trimethoprime (90.31%) was observed by Shahrani *et al*., (2014). This is quite important as Sulfamethoxazole-trimethoprime and tetracycline are commonly used in veterinary and human practices. Furthermore, the detection of the sulfonamide resistant genes from the present studies was positive for the *sul1* gene. Similarly higher record was reported by Nelson *et al*., (2014) who detected the *sul1* gene in 73% of samples examined. On the contrary, Hilbert ., (2011), Momtaz *et al*., (2013), Dehkordi *et al*., (2014) and Shahrani *et al*., (2014), detected variable percentages of lower and higher values of *sul1* gene with 39.50%, 82.78%, 18% and 90.31%, respectively.

Detection of tsh gene of *Escherichia coli* from species other than poultry is one of the rare results recorded from diarrheagenic buffalo calves and their dams as witnessed in the present studies. This may suggest a wider distribution of the *tsh*-carriers than it was estimated. Furthermore, these results along with that for detection of antibiotic resistance genes suggest that buffaloes, like poultry have equal risk and zoonotic potential of pathogenic *E. coli* species transfer to human. Antimicrobial resistance is one of the major problems of some magnitude that is bedeviling the veterinary and medical practices especially, where animals and humans are in close and continuous contact. This paved ways for the transfer of resistance stains within and between different animal species as well as between animals and humans. This

exerts negative consequences in the control of pathogenic *E. coli* and treatment of *E. coli*-induced diseases in different hosts.

The present study was conducted to detect the presence of Salmonella infection from diarrhaeic and non-diarrhaeic buffalo calves and their dams using polymerase chain reaction (PCR) and recorded the prevalence of 12(28.95%) and 2(05.00%) respectively from calves and dams after respectively examining 38 and 40 buffalo calves and dams. Although the investigations and the outcome involved both Calves and Dams, the basis for comparison dwell more on Buffalo Calves as the target of syndrome of calf diarrhea. Similar observations were recorded by Fahmy *et al*, (2017) after examining faeces of Calves from feedlot farms in Egypt but the percentage of *Salmonella* isolated from diarrheic Calves was 07% and a zero record of results from Dams. The findings of El-Shehedi *et al.*, 2015 who isolated Salmonella species with 6.1% from faecal samples of diarrheic Calves in Egypt was lower compared with the findings of the present study. Similarly lower percentages for detection of Salmonella isolates were reported by various workers such as Haggag and Khaliel , 2002 with the record of 4%; Younis *et al*., 2009, 4.09%; Garcia *et al*., 2000, 1.8%; Achá *et al*., 2004, 2% and Osama *et al.*, 2011, 1.56% . Although substantial percentages of Salmonella detection were recorded by El-Seedy *et al*., 2016, with 18%; Youssef and El-Haig , 2012 with 18.66; Seleim *et al.*,2004 with 17% and Riad *et al.*, 1998 with the record of 18.2%, these are lower when compared to the findings recorded in the present study.

The variations in prevalence of *Salmonella* among apparently healthy and diarrheic calves reported in different countries reflect the effect of wide range of different management risk factors (Vanselow e*t al*., 2007; Alam *et al*., 2009; Jones, 2011).

An entirely contrary view of total lack of detection of any Salmonella genes or isolates were presented by the findings of Wani *et al*.,(2013) who after examining faecal samples of Buffalo Calves for detection *Escherichia coli* and Salmonella genes only reported a positive presence of that of *E. coli* from Kashmir, in India.

This negative detection was further supported by reports from other parts of India and Mozambique according to Hussain and Saikia (2000) and Acha *et al.*, (2004) respectively.

Although most investigations relating to calf diarrhea are concern with records of infections from calves only, positive salmonella gene detection from buffalo dams

were also recorded in the present study, although the number was very low represented as only 05.00%. In the overall, 13(16.67%) positive detection of Salmonella after screening 38 buffalo calves and 40 buffalo dams was considered low, especially as it was the least number of positive detections when compared with *Escherichia coli* and *Cryptosporidium* genes from same study. Lower detection may be attributed to different factors. In this study all detected genes for *Salmonella* were recorded from young calves less than 3 months, with only two records from the dams. Calves may be at greater risk of infection than adults due to their naive immune system and in addition to that the presence of concurrent infection with multiple enteric pathogens (*Escherichia coli, Cryptosporidium*) recorded in the present study adds to this problem. This ascertions was supported by the findings of Divers and Peek (2008).

In addition to the detection of genus specific genes of *Salmonella*, *Salmonella typhimurium* was the only species detected in the present study. This contradicts the outcome of positive detection of Salmonella by serotyping made by array of researchers across the globe, where the predominance of *S. enteritidis* serovar among diarrheic Calves were recorded especially by Youssef and El-Haig, (2012); Younis *et al*., (2009); Seleim *et al*., (2004); Moussa *et al*., (2010) all from Egypt. This report was further substantiated by the findings of researchers across Europe as presented by Murray (1994) and Smith-Palmer *et al.,* (2003). *InvA* target gene is located on *Salmonella*, pathogenicity island 1 (SPI1) which is essential for the invasion of epithelial cells by *Salmonella*. This gene is highly conserved in almost all *Salmonella* species (Serotypes) and has been used as a potential target for *Salmonella* detection (Jeong *et al.,* 2011). Conventional PCR can contribute to meeting the need of fast identification and detection methods in disease monitoring and control. However, despite its specificity and sensitivity, thorough investigations should incorporate the use of other conventional methods such as bacterial culture.

Cryptosporidiosis caused by infection with *Cryptosporidium* species as one of the major aetiological agents of neonatal diarrhoea in calves and may be due to several factors like early contamination after birth by contact with their dams, contaminated litters, asymptomatic carriers and contaminated environment (Castro-Hermida *et al*., 2002). This may however be applicable for other aetiological agent associated with calf diarrhea.

Although cryptosporidiosis in animals and birds has been reported from Egypt, Brazil, USA, Czech Republic, Malaysia, Tanzania, Spain, Morocco, France, UK, Canada, Japan, Oman, Poland, Iran and China, only a few published reports of this disease in animals are available from India (Kumar *et al*., 2005).

The present study was conducted to detect the prevalence of Cryptosporidial infection in diarrheic and non-diarrhaeic Buffalo Calves stratified into three groupings according to their ages. Similarly, samples were collected from their respective Buffalo Dams for detection of Cryptosporidium genes using polymerase chain reaction (PCR) in both Calves and Dams. A total prevalence of 10 (26.32) and 17 (42.50) were respectively recorded from Buffalo Calves and Dams with and without diarrhea. An overall prevalence of 27 (34.62) was recorded from a total of 38 Calves and 40 Dams examined. Polymerase chain reaction (PCR) detected positive samples alongside standard positive control and yielded 1,325bp band.

In India Cryptosporidiosis was reported for the first time in Uttar Pradesh (Dubey *et al*., 1992) and later in Calcutta (Chattopadhyay *et al*., 2000; Das *et al*., 2003), Pondicherry (Kumar *et al*., 2004), Andra Pradesh (Shobhamani, 2005), UP (Jayabal and Ray, 2005) ,West Bengal (Roy *et al*., 2006) and Punjab (Singh *et al*., 2006). The prevalence varied depending upon the age of the animal and other geographical and management practices.

It was observed that the majority of the animals between 1-6 months of age were found to have Cryptosporidiosis caused by *Cryptosporidium parvum*, compared to those above six months and one year of age. Similar observations were made by Ongerth and Stibbs (1989), Shobhamani (2005), Jayabal and Ray (2005), Roy *et al*. (2006), and Mehdiazami (2007) who reported higher rates of infection among calves less than 6 months of age. The study indicated that the younger animals were highly susceptible to infection with Cryptosporidiosis compared to adult animals.

On the contrary, cross-sectional study conducted to determine the prevalence of bovine Cryptosporidiosis used 110 fecal samples of crossbred diarrhoeic Calves from two different areas (Muktagacha, Mymensingh and Shajadpur, Sirajgonj) in Bangladesh during April 2012 to September 2014.An overall prevalence of Cryptosporidiosis in crossbred Calves was 28.18% (31/110) by rapid detection kit. Higher prevalence of Cryptosporidiosis was found in the Calves from Shajadpur (29.76%) than in Calves from Muktagacha (23.08%) (Khair *et al*.,2014).The prevalence of Cryptosporidiosis was significantly ($p<0.001$) higher in Calves between

1-2 months (70%) age group than less than one month age group (24.49%). Cryptosporidiosis was however not observed in Calves over two months of age. The prevalence of Cryptosporidiosis was higher in males (34.75%) than females (24.64%) although not statistically significant. It was evident that these findings tallied with the outcome of the current study especially as there was positive PCR detection of Cryptosporidium with the bands at 1,325bp.Similarly high rates of detection was evident in both studies, except that records revealed higher prevalence in the current studies. This makes us to borrow a leaf from the ascersions of Khair *et al*, (2014) who reported that the prevalence of Cryptosporidiosis in bovine in areas examined in Bangladash was under diagnosed and the clinical status of infection was potentially high to apply such claim to current investigation in Hisar, Haryana state, India.

A similar result was obtained by Mallinah *et al.,* (2009) who studied the prevalence of Cryptosporidiosis by screening 455 bovine faecal samples collected from five different organized dairy farms and Veterinary hospitals located in and around Bangalore, South India. Although faecal samples were examined by Sheather's sugar flotation method for detection of oocysts and recorded 5.71 percent positive for Cryptosporidiosis. Sevinc *et al.,* (2009) has a specie wise identification as *Cryptosporidium parvum* and *Cryptosporidium andersoni* based on the morphology and micrometry of the oocysts by Modified Ziehl-Nelsen staining, Kinyoun'ning method and Safranin methylene blue staining methods. The prevalence and intensity of Cryptosporidiosis was found more in calves of less than one month of age compared to adults and more frequently seen in diarrheic than in non-diarrheic sampled animals. On the contrary, Cryptosporidium infection was detected more in dams than calves in the present study and more in non-diarrheic than in diarrheic animals.The sex wise prevalence of Cryptosporidiosis as observed by the former was more in females compared to males and this concurred with the findings in the present study with highest prevalence in buffalo dams as exclusively females. A statistically significant difference was found between sexes and age prevalence of Cryptosporidiosis in the calves. Three hundred (300) faecal samples from diarrhoeic and non - diarrhoeic calves screened for the presence of Cryptosporidium infections revealed a positive detection of *Cryptosporidium parvum* using acid-fast staining method (Ziehl Neelsen) and ELISA kit as reported by Sevinc *et al.,* 2009. Calves were grouped according to their age as follows: 1-10, 10-20, 20-30, 30-45 and >45 days. The prevalence of infection in diarrhoeic and non diarrhoeic Calves was 63.92% and 9.85%,

respectively. Cryptosporidium infection was detected in 50.75%, 35.71%, 25.45%, 14.71% and 13.24% respectively in the age groups of examined calves (Sevinc *et al.*, 2009). This is however a contrary record of prevalence rate detected in the present study which employed PCR as a different method of detection and using different criteria of animal groupings during sampling.

Bovine Rotaviruses and Coronaviruses are the most common identified viruses in diarrhoea of neonatal food animals (Holland 1990; Athanassious *et al*., 1994). Array of investigators reported a positive detection of Rotavirus group A with good percentages in their prevalence (21.1 %) as was indicated by Alfieri *et al*. (2006) and suggested that Rotavirus is one of the more important causative agents in neonatal calf diarrhoea.

Rotavirus represents one of the major causes of neonatal mortality in dairy buffaloes in India as reported by series of researchers. However, despite such importance, rotaviruses have not been demonstrated in any of the diarrhoeic and non-diarrheic faecal samples of buffalo calves of less than 6 months age group and their respective dams examined using conventional and modern molecular tools of RNA-PAGE, RT-PCR in the present study.This contradicts the findings of Sagar (2008) who reported 20% and 2.7% incidence of bovine group A rotavirus in Bovine Calves in India. However, in a study of rotavirus in Kolkata, Nataraju *et al*. (2009) showed 10.52% (10/95) samples with characteristic of group A rotavirus-like and long-type electropherotype (e-type) pattern and 4.21% (4/95) samples with the characteristic of group B rotavirus long-type of electropherotype pattern in Buffalo Calves in Kolkata, Eastern India. Similarly, Niture *et al*. (2011) detected rotavirus in 7.22% Buffalo Calves, 7.40% in poultry and 19.75% in human faecal samples in Western India. Chitambar *et al.* (2011) detected group A RV in 2.8% apparently healthy and 14.3% diarrheic animals in Pune, Western India. Differences in the incidence of rotavirus were probably due to season and climatic factors such as rainfall, temperature and relative humidity. This claim was supported by the findings of Dhama *et al*. (2009) who attributed temperature variations or chilling during winter in farms as enhancer to severity of rotaviral infection.

Although, samples were screened and recorded as RNA-PAGE negative in the present study, they were subjected to further scruitiny by RT-PCR for amplification of VP7 gene of group A rotaviruses. Bov9Com5 and Bov9Com3 primer pairs used in this study was attributed to specificity for bovine rotavirus in revealing the expected

product of 1,013 bp for VP7 gene. Although negative in our study, such an expected size of 1,013 bp of VP7 gene amplicon was obtained by Mondal *et al*. (2011).

Findings of the present study obtained by RNA PAGE are non-comparable to that obtained by Dash *et al*. (2011) who detected rotavirus in 16.83% diarrheic calves from Mathura province of India as was also the case with Ghosh *et al*. (2007) who detected rotavirus in 22% diarrheic bovine samples from West Bengal. The non detection of roatavirus infection obtained in the present study is typical rare of group A mammalian rotavirus especially in diarrheic calves. It was an established fact from earlier studies that group A rotaviruses are the major cause of diarrhea in calves all over the world (Okada and Matsumoto 2002; Saravanan *et al*., 2006).

The results obtained in the present study following RT-PCR for the identification of G genotype is also in complete disagreement with the results of Malik *et al*. (2012), in which they found 52.9% of G3 and 47% of mixed G types in the samples. The occurrence of zero percentages of G8 in samples in the present study depicts lack of concurrence with the reports of Beg *et al*. (2010), who reported the occurrence of 9.67% of G8 from Srinagar. In a similar study conducted by Fukai *et al*. (1999) a prevalence of 4.7% by G8 genotype of the bovine group A rotavirus was reported which also represents a total disagreement with findings in the present study.

It is therefore concluded that despite a non detection of bovine rotavirus from buffalo calves and dams further research efforts should be intensified for a better surveillance that helps in achieving stringent control measures.

Although series of researchers (Schroeder *et al.* 1985; McDougall and Cullum 1999; Vermunt 2002; Svensson *et al.* 2003; Parkinson *et al*. 2010; Izzo *et al*. 2011), have identified Coronavirus as the major causal pathogen for infectious diarrhoea in Calves, and stressed that pathogens and species of pathogens vary in their virulence (Howe *et al*. 2008), both within and between-countries as relates to Calf diarrhoea. However, examinations of faecal samples using PCR in the present study did not detect the occurrence of Coronavirus in both Buffalo Calves and their Dams. This concurs with the ascertions of Vermunt (2002) that Coronaviral infection, although present in New Zealand, appears to be of little consequence in Calf diarrhea because of low detection of the virus. The same, minimally pathogenic relationship between coronavirus and Calves has also been noted in the Netherlands (Bartels *et al*. 2010). Similarly a Dutch study related to Calf diarrhea showed that the prevalence of

coronavirus infection was low (only affecting one or two calves) when compared with *Clostridium perfringens* bacteria (Bartels *et al.* 2010).

An independently similar study on the incidence of Rotavirus and Coronavirus in fecal samples collected from different localities covering Menofiya governorate, Egypt during the period from November 2014 to March 2015 using direct sandwich ELISA for antigen detection of the virus revealed an occurrence of highest rates of diarrhea in 1st group, followed by 2nd group, then 3rd group of animals sampled.Similar observation was reported by others, [Lorino *et al*., (2005) ; El-Naker *et al*., (2007) and Lorenz *et al*., (2011)], who recorded the incidence rate of diarrhea during neonatal period as high in the first days of Calves' age. Coronavirus are ubiquitous and as a result, most of the animals, including pregnant cows coming from intensive livestock farms, have specific antibodies against these pathogens. The antibodies produced by cows in response to natural immunization or vaccination are transmitted to the calf at birth via the colostrum (Radostits *et al*., 2007 and Morshedi *et al*., 2010), but the diagnosis of Coronavirus infection was based primarily on the detection of virus or viral antigen in the faeces. However, there are a variety of diagnostic methods available for the detection of Coronavirus including PCR, ELISA, Electron microscope and Immune electron microscope (Cho *et al*., 2010 and Jakobsson 2013).

ELISA is one of the essential methods in the determination of viral antigens and has the good qualities of being fast and having the capability to handle a big number of samples at the same time (Duman and Aycan 2010 and Jakobsson 2013). It is widely used for viral antigen detection from the faeces of diarrheic calves (Ali *et al.*, 2008; Dhama *et al*., 2009, Badiei *et al*., 2010 and El-Bagoury *et al*., 2014). Coronavirus infection is most often transmited through a faecal-oral route and Calves are most often infected by contact with other calves, primarily or secondarily through objects, feeds and water. Calves can also be infected by virus shed by the Dam at birth. The infected calves shed virus through the faeces from the second day of infection and the shedding may last for 7-8 days. (Malik *et al*., 2005; Dhama *et al*., 2009; Suresh *et al*., 2013 and Collins *et al*., 2014).Examinationof 200 faecal samples revealved the detection of 51(25.5%) positive faecal samples using direct sandwich ELISA kit for detection of Coronavirus antigen. This result may be related to virus shedding in outbreaks in non vaccinated populations of Calves (Brandão *et al*., 2007; Oliveira Filho *et al*., 2007 and Gay *et al*., 2012).

Depending on the age of the calf, some pathogens are more likely to be the cause of diarrhea; Coronavirus mostly affect calves aged 5-20 days old, although can affect calves up to several months of age (Reidy *et al*., 2006; Dash *et al*., 2011 and Gay *et al.*, 2012).

It is well known that the timely feeding of adequate volumes of colostrum is a key factor in ensuring early passive transfer in the neonatal calf. However, farms continue to struggle with colostrum management and it has been reported that many Calves may not receive the first colostrum meal until 6 h of life or later (Vasseur *et al*., 2010). The majority of studies conducted in regards to delaying colostrum feeding and its effects on the passive transfer of IgG were conducted more than 30 years ago, and whether these results hold true using current day colostrum recommendations (3-4 L of colostrum containing ≥ 50 g of IgG per L), standardized colostrum quality and volume among treatments warrants further research. However there was a report that feeding colostrum in a delayed manner affects the prevalence of intestinal bacteria, essential for development of the mucosal immune system and providing energy for intestinal cells during early life (Vasseur *et al*., 2010).

Similarly, Oikonomou *et al*., (2013) reported that although the successful passive transfer of IgG is essential in ensuring a healthy dairy calf, the composition and establishment of GIT microbiota has also been associated with health and disease outcomes. The delivery of colostrum is essential in establishing the early life gut microbiota as it has been shown that withholding colostrum feeding can decrease the abundance of total bacteria within the small intestine during the first 12 h of life (Malmuthuge *et al*., 2015).

In ruminants, colostrum is a vital source of immunoglobulins that provide passive immunity for their offspring during the neonatal period. It is suggested that colostral immunoglobulin G (IgG) concentration varies between and within breeds and could also be affected by maternal factors (Vasseur *et al*., 2010). Effects of litter type and parturition number on colostral IgG concentration were determined for estimation of IgG concentrations in colostrum. They found that the concentration of colostral IgG could be influenced by breed but not by litter type and parturition number. The present study involved a detection of IgG concentrations from buffalo cows of same breed but of different parity status and thus basis for comparative assessments parity wise with findings of early workers.

Sandwiched- ELISA was the method used in the present study for the assessement of IgG concentration in colostrums of buffalo dams, the basis for detection was the same with that of early workers even though their assessments also involved possible differences in IgG concentrations between types of breeds involved in the study. However, simple presence or absence of IgG in the colostrum is the only requirement to ascertain the success of passive transfer to neonatal calves in the current study but further to that IgG concentrations in the colostrums sample were also established.

The amount of immunoglobulins in milk varies and factors influencing their concentration also vary (Korhonen *et al*., 2000; Krol *et al.*, 2012). Different authors have indicated that concentration of immunoglobulins G in the cow milk varies depending not only on the degree of udder infection but also is considerably affected by the cow age, lactation period, keeping conditions, and feeding (McFadden *et al*., 1997; Korcina *et al*., 2012). Although the basis for detection of IgG in the present study was a simple presence or absence for assessing passive transfer to calves, the investigations further established the concentrations of IgG from milk samples from cows at different lactation stages. These are comparable to values obtained by a number of researchers: The mean levels obtained for IgG in the whole population studied are 0.29±0.14 mg/ml, a value close to the range referred to as normal for mature milk, which is 0.3-0.5 mg/ml (Collin *et al*., 2002). Similar IgG values were obtained by other researchers as well: 0.30–0.60 (Pakkanen, 1997; Krol *et al*., 2010). In the analysis of bovine IgG in milk, Grapper *et al*. (2007) reported a higher IgG value 0.72 mg/ml. The highest concentration of the immunoglobulin G in milk 2.05±0.83 mg/ml was indicated by Latvian researchers (Korcina *et al*., 2012).

The age of the cow and the number of lactations are considered as two relevant factors, which determine IgG concentration in milk. The poorest source of IgG proved to be the milk obtained from the 1st and 2nd lactations cows (0.26 and 0.15 mg/ml). Older cows, in the 5th and 6th lactations, produced milk with higher concentration of IgG compared to younger ones (0.41• and 0.11 mg/ml). These findings are similar with that of Krol *et al*. (2010; 2012) who reports of research where primiparous cows were showned to produce significantly less IgG as compared to cows at 2 to 4 lactations ($P \leq 0.05$) and older ($P \leq 0.01$). The lowest level of IgG was found in the 1st lactation (0.454• and 0.16 mg/ml) and in subsequent lactations IgG compounds increased gradually (Krol *et al*., 2012).

James *et al*. (2001) reported the suitability in human patients of using oral-fluid samples for determining the prevalence of immunity to vaccine-preventable infections such as measles, rubella and Hepatitis-B (HBc) by screening paired blood and oral-fluid samples from 853 individuals of all ages from a rural Ethiopian community. Enhanced IgG antibody capture (GAC) enzyme-linked immunosorbent assays (ELISAs), and anti-HBc antibodies with a prototype GACELISA for measles- and rubella-specific antibodies and anti-HBc antibodies were respectively used.The results of 98% and 87% respectively for sensitivity and specificity suggested that oral fluid has potentials to replace serum in IgG antibody prevalence surveys. The present study screened oral fluid for IgG presence in the samples for simple detection for assessment of successful transfer of IgG from Buffalo Dams to their respective neonates, but the quantification to determine the concentrations of IgG in oral fluid samples further revealed the level of protection provided by the amount present. Similarly, the success of IgG detection from oral fluid samples recorded in the present study corroborates with the assertions made by earlier workers that the non-invasiveness in sample collection will serve as a remedy to the problems of Iconophobia in human patients with its unparallelled relevance in animal welfare as a remedy to the problems of cruelty in Veterinary practice.

Selective immunoglobulin A (IgA) deficiency is the most common primary immunodeficiency in humans and may be associated with chronic gastrointestinal disease. This observation has led to the suggestion that the high susceptibility of German shepherd dogs (GSD) to chronic enteropathies is related to a deficiency in mucosal IgA production. Relative deficiencies of IgA has been reported in the serum, saliva, tears, and faeces of GSD both with and without alimentary disease, however, the findings of different studies are not consistent. The aim of this study was to confirm whether a relative deficiency of IgA exists in the faeces of GSD (Peters *et al*., 2004). Faecal IgA, IgM, and IgG were measured by capture enzyme-linked immunosorbent assays. Faecal IgG concentrations in the four breed groups were not significantly different. These findings do not support the hypothesis that GSD have a relative deficiency in faecal IgA. Differences in immunoglobulin concentrations measured from a single defecation, between individuals of the same breed and between breeds, as well as the lack of an internal control molecule, make the determination of a normal reference range for all dogs impossible. Therefore, the usefulness of faecal immunoglobulin quantification for the assessment of intestinal

immunoglobulin secretion in dogs is limited. Because of the lack of data to make comparison on the relative concentrations of IgG from faecal samples in both Bufflalo Calves and Dams, inferences were deduced with results obtained from a different species. It is in liu of this therefore that the above findings on measurement of Ig concentrations in the faeces of healthy dogs were considered for discussions and in this respect, the outcome of the present study yielded a better reference point as reasonable concentrations of IgG were detected in the faeces of both Buffalo Calves and Dams, although some of these samples were from infected animals as opposed to findings from earlier workers, who recorded values from healthy dogs only. Measurement of IgG levels in stool requires an extensive preparation of the stool to obtain a clear globulin-containing fraction and the preparation losses may be considerable so that quantitative levels quoted can only be considered to be estimates.

Toll-like receptor 4 (TLR4) plays an important role in recognizing the conserved patterns unique to microbial surfaces called pathogen associated molecular patterns (PAMPs), and lipopolysaccharides (LPS) was reported to stimulate the expression of the bovine antibacterial peptide-encoding gene via the activation of NF-kB (Diamond *et al*., 2000; Ghosh and Karin, 2002), and induces inflammation that contributes to an immune response. It was proven that the TLR4 gene affects several diseases, including sepsis, immune-deficiencies, atherosclerosis and asthma (Donald *et al*., 2004). A research work permitting the alignments of the bovine TLR4 coding sequence (CDS) and human TLR4 CDS reveal a high similarity of 85%, and their corresponding proteins are 76% similar (Diamond *et al*., 2000; Ghosh and Karin, 2002). The results of cDNA sequence analysis and protein domain of TLR4 inferred that bovine TLR4 might induce immune response for disease resistance of pathogen infection by the signal transduction pathway. This has supported the findings in the present study where positive gene expression for TLR4 was recorded in both buffalo calves and dams with and without infections.

Expressions of TLR4 mRNA were also assessed by Fujisawa *et al*, (2006) using semi-quantitative RT-PCR trinitrobenzene sulfuric acid (TNBS)-treated rat colon where different regions of the affected colonic tissue were separated into mucosal and muscular regions to establish any difference in mRNA expressions between them. TLR4 as the representative PAMP receptors involved in mediating inflammatory responses, show a higher expression of TLRs in the mucosal layer

compared to that in the muscle layer. Similarly, variable expression patterns based on band intensity were recorded in the present study where TLR4 expressions were identified in both PBMC's and colostral leucocytes of Buffalo Calves and Dams respectively.

The detection of gene expressions of both TLR4 and CARD15/NOD2 recorded in the present study in both buffalo calves and dams with and without infection, tally with the traditional understanding reported by Rakoff-Nahoum (2004) that TLR recognition triggers the innate immune system leading to an inflammatory response which serves as a protective role of TLR activation by intestinal commensal bacteria. Under steady state conditions activation of TLRs by commensal microflora was critical for tissue repair and protection against intestinal injury and associated mortality. Furthermore, in vivo it is likely that innate immune pattern recognition receptors are not triggered by single ligands but rather that complex activation of multiple receptors takes place concurrently due to the many TLR and NOD ligand motifs present on a microbial pathogen. (Sansonetti 2004; Philpott 2004).The later further postulated that dual signalling via both TLR and NOD pathways may be necessary for efficient innate immune responses and that in the presence of abnormal NOD2 this process is compromised resulting in an abnormal initial defense against commensal and pathogenic bacteria or an abnormal tolerance mechanism which is critical in maintaining controlled activation of the immune system in the intestine.

TLR4 have been associated with host recognition of bacterial pathogen (Koedel *et al*., 2003; Malley *et al*., 2003; Schroder *et al*., 2003; Yoshimura *et al.*, 1999). Whereas these TLRs are likely to serve as the first line receptors for pathogens, the NOD proteins might play a major role in a subsequent phase of infection. Since TLRs mediate NF-kB activation and NF-kB binding sites have been identified in the NOD2 promoter (Gutierrez *et al*., 2002; Rosenstiel *et al*., 2003), recognition of bacterial pathogens by the TLRs might cause the up-regulation of NOD2 and thereby facilitate the immune response of the host against this pathogen. In line with this hypothesis, the penetration of epithelial and endothelial cells by bacteria is initiated during the first hours after infection, and it is most pronounced after 4–6 h (5). mRNA levels of NOD2 increased within a similar time frame, suggesting that NOD-mediated NF-kB activation might play an important role in this subsequent phase of host responses against these pathogens. These assertions validates the findings in the gene

expression aspect of these studies were both TLR4 and CARD15/NOD2 genes expressions were recorded.

The NOD-dependent NF-kB activation by intact or inactive bacterial pathogen is most likely due to cell wall peptidoglycan. NOD2 has been found to mediate cell activation by a muramyldipeptide conserved in basically all kinds of peptidoglycans (Girardin *et al.*, 2003; Inohara *et al*., 2003),

Thus, NOD2-deficient mice as well as the recently generated NOD1 knockout mice will be of invaluable help to further elucidate the precise role of these proteins in host defense (Chamaillard *et al.*, 2003; Girardin ., 2003; Pauleau and Murray *et al* ., 2003).

Overall, besides bacterial cell recognition by TLRs, NOD2 activation seems to play an important role in host cell activation by internalized bacterial pathogens. Downstream of NOD2 and RIP2, signal-transducing molecules like IRAK, IRAK2, TRAF6, NIK, TAB2, and TAK1 might mediate NF-kB-dependent cell activation. Knowledge about the molecular interaction of bacterial pathogens with target cells may pave the way to innovative therapeutic strategies.

CHAPTER-VI

SUMMARY AND CONCLUSION

SUMMARY

1 There was positive detection of *Escherichia coli* including some of their virulence genes and antibiotic resistance genes.

2 *Salmonella* genus specific genes, *Salmonella typhi*, and some *Salmonella virulence genes* were detected but the specie *Salmonella enteritidis* was not detected.

3 *Cryptosporidium* species were detected in the present study in diarrheoic and non-diarrheoic buffalo calves and dams.

4 Rotavirus and Coronavirus were not detected.

5 Bovine IgG concentration was detected from colostrum, milk, urine, oral fluid, meconium, faeces and serum samples.

6 High concentrations of IgG were detected from colostrum, milk and serum while low concentration of same was detected from meconium, faeces, oral fluid and urine samples.

7 Success of passive transfer of immunity from buffalo dams to their calves was confirmed by detection of variable concentrations of Bovine IgG in screened calves of all age groups.

8 TLR4 and CARD15/NOD2 gene transcripts were detected from PBMC's of buffalo calves.

9 TLR4 and CARD15/NOD2 gene transcripts were detected from colostral leucocytes of buffalo dams.

CONCLUSIONS

1 Despite evidences of interplay of innate and adaptive immune responses witnessed in the present study, a syndrome of diaorrhea was still recorded and this shows the levels of resistance, tolerance and susceptibility exhibited by the calves and dams.

2 The outcome affirms that diarrhea syndrome is still considered to be a threat to the survival of neonatal calves and by extension a major contributor to loss/reduction of replacement heifers in the subsequent production circles.

3. It is therefore suggested that further research on relevance of immunotherapy as an alternative to antibiotic therapy inaddition to an indepth understanding of roles of genetic immunity which is essential for selection of breeds and individuals with optimal genetic potentials especially for maximum yield used in production circle.
4. It is also adviced that sequencing of detected pathogens and other genes should be conducted to further validate and augment the findings recorded in the present studies. This will enable tracing of the evolutionary relationship with other pathogens and related genes from different regions across the globe.

BIBLIOGRAPHY

Achá, S.J., Kuhn, I., Jonsson, P., Mbazima, G., Katouli M.and Mollby R.S (2004). Studies on calf diarrhoea in Mozambique: prevalence of bacterial pathogens. *Acta Vet* Scand., 45: 27-36.

Akira, S (2003). Toll-like Receptor Signaling. *J. Biol. Chem.* 278, 38105-38108.

Alam, M. J., Renter, D.G., Ives, S.E., Thomson, D.U., Sanderson, M.W., Hollis L.C. and Nagaraja, T.G (2009). Potential associations between fecal shedding ofSalmonella in feedlot cattle treated for apparent respiratory disease and subsequent adverse health outcomes. *Veterinary Research*, 40: 02.

Alfieri, A. A., Parazzi, M. E., Takiuchi, E., Medici, K. C and Alfieri, A. F (2006). Frequency of group A rotavirus in diarrhoeic calves in Brazilian cattle herds, 1998-2002. *Trop. Anim. Health Prod.* 38, 521-526.

Ali, Y.H., Khalafalla, A.I., Gaffar, M.E., Peenz, I. and Steel, A.D (2008). Detection and isolation of group A rotavirus from camel calves in Sudan. *Vet. Arhiv*, 78:477-485.

Ammar, S., Mokhtaria, K.., Tahar, B., Amar, A., Redha, B., Yuva, B., Mohamed, H., Abdellatif, N. and Laid, B (2014). Prevalence of rotavirus (GARV) and coronavirus (BCoV) associated with neonatal diarrhea in calves in westernnAlgeria Asian Pac J Trop Biomed, 4(1):S318-S322.

Anwarullah, M., Khan, J. A., Khan, M. S., Ashraf, K.., and Avais, M (2014). Prevalence of salmonella and *Escherichia coli* associated with diarrhea in buffalo and cow calves. *Buffalo Bull.* 33: 3. *Appl. Microbiol.* 51, 595-599.

Athanassious, R., Marsolais, G., Assaf, R., Dba, S., Descoteaux, J. P., Dulude, S., and Montpetit, C (1994). Detection of bovine coronavirus and type A rotavirus in neonatal calf diarrhea and winter dysentery of cattle in Quebec: Evaluation of threediagnostic methods. Can. Vet. J. 35, 163-169.

Bagg, I. (1991). The influence of dental status on the detection of IgG class anti-viral antibodies in human saliva. *Archives of Oral Biology*, 36: 221–226.

Balasubramaniam, A., Arthanari, E. M., Suresh, P., and Sukumar, K (2014). Detection of tetracycline resistance determinant *tetA* gene and antimicrobial resistance pattern in *Escherichia coli* isolates recovered from healthy layer chickens, Vet. World 7(9), 635-638.

Bannerman, D.D., Tupper, J.C., Erwert, R.D., Winn, R.K. and Harlan, J.M (2002). Divergence of bacterial lipopolysaccharide pro-apoptotic signaling downstream of IRAK-1. *J. Biol. Chem.* 277, 8048-8053.

Bartels, C.J.M., Holzhauer, M., Jorritsma, R., Swart, W.A.J.M., and Lam T.J.G.M (2010). Prevalence, prediction and risk factors of enteropathogens in normal and non-normal faeces of young Dutch dairy calves. *Preventive Veterinary Medicine*, 93(2-3), 162-169.

Bazeley, K (2003). Investigation of diarrhoea in the neonatal calf. *In Practice* 25(3), 152-159.

Beg, S. A., Wani, S. A., Hussain, I. and Bhat, M. A. (2010). Determination of G and P type diversity of group A rotaviruses in faecal samples of diarrhoeic calves in Kashmir, India. *Lett. Appl. Microbiol*. 51, 595-599.

Bendali, F., Sanaa, M., Bichet, H., and Schelcher, F (1999). Risk factors associated with diarrhoea in newborn calves. *Veterinary Research* 30(5), 509-522.

Beraldo, L.G., Borges, C., Maluta, R.P., Cardozo, M.V., Rigobelo, E.C., and Avila, F. A (2014).Detection of Shiga toxigenic (STEC) and enteropathogenic (EPEC) *Escherichia coli* in dairy buffalo. *Vet. Microbiol.* http://dx.doi.org/10.1016/ j.vetmic, .01.023.

Bisi-Johnson, M.A., Obi, C.L., Vasaikar, S.D., Baba, K.A., and Hattori, T (2011). Molecular basis of virulence in clinical isolates of *Escherichia coli* and Salmonella species from a tertiary hospital in the Eastern Cape, South Africa. Gut Pathogens 2011, 3:9.

Brandão, P.E., Villareal, L.Y., De Souza, S.L., Richtzenhain, L.J., and Jerez, J.A (2007). Mixed infections by bovine coronavirus, rotavirus and Cryptosporidium parvum in an outbreak of neonatal diarrhea in beef cattle. *Arq Inst Biol* (Sao Paulo), 74:33-34.

Castro-Hermida, J. A., Gonzäles-Losada, Y. A., Meso-Menendez, M., and Ares-Mazäs, E (2002). A study of *Cryptosporidiosis* in a cohort of neonatal calves. *Vet. Parasitol*. 106**,** 11-17.

Cavanagh, D (2005). Coronaviridae: a review of coronaviruses and toroviruses. *In:* Schmidt, A., Wolff, M. H. and Weber, O. (eds.) Coronaviruses with Special Emphasis on First Insights Concerning SARS. Basel/Switzerland: Birkhäuser Verlag.

Chalmers, R.M. and Katzer, F (2013). Looking for *Cryptosporidium*: the application of advances in detection and diagnosis, *Trends in Parasitology* 29, 237- 251.

Chamaillard, M., Philpott, D., Girardin, S. E., Zouali, H., Lesage, S., Chareyre, F. Bui, T. H., Giovannini, M., Zaehringer, U., Penard-Lacronique, V., Sansonetti, P. J., Hugot, J.P and Thomas, G (2003). Gene–environment interaction modulated by allelic heterogeneity in inflammatory diseases, *Proceedings of National Academy of Science*, USA, 100 (6): 3455–3460

Chang, C. N, Kuang, W. J and Chen E. Y (1986). Nucleotide sequence of alkaline phosphatase gene of *Escherichia coli*, *Gene* 44 (1): 121 - 125.

Chattopadhyay, U. K., Chowdhury, D., Dasgupta, C. K. A. and Pramanik K (2000). Prevalence of cryptosporidiosis in man and animals in and around Calcutta, *J. Vet. Parasitol*, 14, 167-168.

Chigerwe, M., Tyler, J.W., Schultz, L.G., Middleton, J.R., Steevens, B.J. and Spain, J..N (2008). Effect of commensal microflora by toll-like receptors is required for intestinal homeostasis' *Cell*; 118(2):229-41

Chitambar, S. D., Arora, R., Kolpe, A..B., Yadav, M.M. and Raut, C.G (2011). Molecular characterization of unusual bovine group A rotavirus G8P14 strains identified in Western India: Emergence of P [14] genotype. *Veterinary Microbiology.* 148: 384–388.

Cho, Y. I., Kim, W. I., Liu, S. Kinyon, J. M., and Yoon, K.. J (2010). Development of a panel of multiplex real-time polymerase chain reaction assays for simultaneous detection of major agents causing calf diarrhea in faeces, *J Vet Diagn Invest*, 22:509-517.

Chuang, T.H. and Ulevitch, R. J (2000). Cloning and characterization of a sub-family of human Toll-like receptors: hTLR7, hTLR8 and hTLR9, *Eur. Cytokine. Netw.* 11, 372-378.

Clark G, and Gill J (2001)**.** Rotavirus in calves. *Vetscript* 14(11), 12-13.

Collin R., McLaren R., Thomson M., and Malcolm, D (2002). Development and validation of a nephelometric immunoassay for IgG1 in milk, *International Journal of Dairy Science*. 69. P. 27–35.

Collins, P., Mulherin, E., Cashman, O., Lennon, G., Gunn, L., O'Shea, H. and Fanning, S (2014). Detection and characterization of bovine rotavirus in Ireland from 2006–2008, *Irish Veterinary Journal*, 67:13.

Constable, P. D (2004). Antimicrobial use in the treatment of calf diarrhoea, *J. Vet. Int. Med.* 18: 8-17.

Das, G., Sarkar, S., Das, P. and Panja, P (2003). *Cryptosporidium* infection of cattle in and around Kolkata, West Bengal, *Indian J. Anim. Hlth.* 42, 142-144.

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

Davis, C. L. and Drackley, J. K (1998). The development, nutrition and management of the young calf., First edition. ed. *Iowa state university press*, Ames, Iowa 50014, Iowa, USA.

De Groot, R. J., Baker, S. C., Baric, R. S., Brown, C. S., Drosten, C., Enjuanes, L., Fouchier, R. A., Galiano, M., Gorbalenya, A. E., Memish, Z. A., Perlman, S., Poon, L. L., Snijder, E. J., Stephens, G. M., Woo, P. C., Zaki, A. M., Zambon, M. and Ziebuhr, J (2013). Middle East respiratory syndrome coronavirus (MERS-CoV): announcement of the Coronavirus Study Group. *Journal of Virology*, 87, 7790-7792.

Dehkordi, F. S., Yazdani, F., Mozafari J. and Valizadeh, Y (2014). Virulence factors, serogroups and antimicrobial resistance properties of *Escherichia coli* strains in fermented dairy products. BMC, *Res.*, Notes. 7. 217.

Delicato, E.R., de Brito, B.G., Gaziri, L.C.J. and Vidotto, M.C (2003).Virulence-associated genes in *Escherichia coli* isolates from poultry with colibacillosis. *Vet. Microbiol.* 94: 97–103.

DeNise, S. K., Robinson, J. D., Stott, G.H. and Armstrong, D.V (1989). Effects of passive immunity on subsequent production in dairy heifers, *Journal of Dairy Science*, 72, 552–554.

Derbigny, W. A., Kerr, M. S. and Johnson, R. M (2005). Pattern recognition molecules activated by *Chlamydia muridarum* infection of cloned murine oviduct epithelial cell lines, *The Journal of Immunology*, **175**: 6065–6075

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

Diamond, G., Kaiser, V., Rhodes, J., Russell, J.P and Bevins C. L (2000). Transcriptional regulation of beta-defencing gene expression in tracheal epithelial cells, *Infect. Immun.* 68, 113-119.

Divers, T.J. and Peek S.F (2008). Rebhun's diseases of dairy cattle. St. Louis: *Saunders Elsevier, Lett. diversity* of group A rotaviruses in faecal samples of diarrhoeic calves in Kashmir, India.

Donald, N.C., David, S.P. and David, A.S (2004). Toll-like receptors in the pathogenesis of human disease. *Nat. Immunol.* 10, 975-979.

Dubey, J. P., Fayer, R. and Rao J. R (1992). Cryptosporidial oocysts in faeces of water buffalo and Zebu calves in India, *J. Vet. Parasitol.* 6, 55-56.

Duman, R. and Aycan, A. E (2010). Prevalence of rotavirus infection in calves with diarrhoea in Konya region, *J. Anim. Vet. Adv.*, 9(1):136-138.

Eckstein, E (1996). Congenital rubella in south India-diagnosis using saliva from infants with cataract. *British Medical Journal*, 312: 161.

Ekperigin, H. E. and Nagaraja, K. V (1998). *Salmonella. Vet. Clin. North Am. Food Anim. Pract.* 14, 17-29.

El-Bagoury, G.F., El-Nahas, E. M., Sharaf El-Deen, S.S. and Salem, S.A.H (2014). Frequency of Rotavirus detection by a sandwitch ELISA in faeces of diarrhoeic bovine calves from Qalubia province, Egypt. *BVMJ*, 27(2):341-347.

El-Naker, Y., El- Sawalhy, A., Youssef, M. and Zeidan, S (2007). Some studies on neonatal calf diarrhea in Egypt, Part 1: Causative agents and some epidemiological aspects *Bull. Anim. Hlth. Prod.* Afr. 56:161-190.

El-Seedy, F.R., Abed, A.H., Yanni H.A. and Abd El-Rahman S.A.A (2016). *Journal of Basic and Applied Sciences*, 5: 45-51.

El-Shehedi, M.A., Mostafa M. E., and Hanan, E.N (2015). Some bacteriological studies on *Salmonella* infection in buffalo calves using PCR technology, *European Scientific Journal*, 11(36): 181-192.

Enjuanes, L., Dediego, M. L., Alvarez, E., Deming, D., Sheahan, T. and Baric, R. S (2008). Vaccines to prevent severe acute respiratory syndrome coronavirus-induced disease. *Virus Research* 133, 45–62.

Eschrig M, Heckert, HP, Goossens, L (2004). Field trial to compare the effectiveness of two different dam vaccines (Rotavec (TM) Corona and Lactovac (R) C) against neonatal diarrhoea in cattle, *Praktische Tierarzt* 85(8), 580.

Ewers, C., Janßen, T., Kießling, S., Philipp H., Wieler, L. H (2004). Molecular epidemiology of avian pathogenic *Escherichia coli* (APEC) isolated from colisepticemia in poultry, *Vet. Microbiol*.104 (1-2):91–101.

Fahmy, M., Yousef, H. M., and Soliman, Y.A (2017). Detection of Salmonellae in the Feces of Feedlot Calves Farms in Egypt, *International Journal of Microbiological Research* 8 (1): 01-08.

Ferwerda, G., Girardin, S.E. , Kullberg, B-J., Le Bourhis, L., de Jong, D. J., Langenberg, D. M. L., van Crevel, R., Adema, G.J.., Ottenhoff, T. H. M., Van der Meer, J. W. M., Netea, M. G (2005). NOD2 and Toll-Like Receptors are nonredundant recognitionsystems of *Mycobacterium tuberculosis*. *PLoS Pathogens*, **1**:0279-0285

Ferwerda, G., Kullberg, B.J., de Jong, D. J.,Girardin, S. E., Langenberg, D.M. L. , van Crevel, R., Ottenhoff, T. H. M. , Van der Meer, J.W. M. and Netea, M.G (2007). *Mycobacterium paratuberculosis* is recognized by Toll-like receptors and NOD2, *Journal of Leukocyte Biology*, 82: 1011–1018

Fleenor, W.A. and Stott G. H (1980). Hydrometer test for estimation of immunoglobulin concentration in bovine colostrums *J. Dairy Sci.* 63:3838-3844.

Fukai, K., Sakai, T. Hirou, M. and Itou T (1999). Prevalence of calf diarrhoea caused by bovine group A rotavirus carrying G serotype specificity, *Vet. Microbiol*, 66, 301-311.

Gaber, S.A (2004). Polymerase chain reaction and DNA electrophoretic pattern of certain types of *Escherichia coli*, M.V.Sc Thesis, Fac. Vet. Med. Alexandria Univ. Egypt.

Gapper L. W., Copestake D. E. J., Otter D. E. and Indyk H. E (2007). Analysis of bovine immunoglobulin G in milk, colostrum and dietary supplements: A review. *Anal. Bioanal. Chem.* 389. P. 93–109.

Garcia L. S., Bruckner, D. A., Brewer, T. C. and Shimizu, R. Y (1983). Techniques for the recovery and identification of *Cryptosporidium* oocysts from stool specimens, *J. Clin. Micro biol.*, 18:185-190.

Garcia, A., Ruiz-Santa-Quinteria, J. A., Orden, J. A., Cid, D., Sanz, R., Gomez-Bautista, M. and De La Fuente, R (2000). Rotavirus and concurrent infections with other enteropathogens in neonatal diarrhoeic in dairy calves in Spain. *Comp. Immun, Microbiol. Infect. Dis.* 23, 175-183.

Garland, A. J. M (1974). The inhibitory activity of secretions in cattle against foot and mouth disease virus, PhD thesis, London School of Hygiene and Tropical Medicine. DOI: 10.17037/PUBS.00878722.

Gay, C.C., Hodgson, J. C., Lofstedt, J. and Bolin, S. R (2012). Diarrhoea in neonatal ruminants, intestinal diseases in ruminants: Merck Veterinary Manual. (Online), available from Http://www.merckmanuals.com/vet/print//digestive_system/intestinadiseasesin_ruminants/diarrhea_in_neonatal_ ruminants.html.

Ghosh, S. and Karin, M (2002). Missing pieces in the NF-kB puzzle, *Cell*, 109 (Suppl.), 81-96.

Ghosh, S., Varghese, V and Samajdar S (2007). Evidence for bovine origin of VP4 and VP7 genes of human group A rotavirus G6P [14] and G10P [14] strains, *J. Clin. Microbiol*. 45, 2751- 2753.

Girardin, S.E., Boneca, I.G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G.,Philpott, D.J. and Sansonetti, P.J (2003). NOD2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection, *The Journal of Biological Chemistry*, 278:8869–8872

Gitau, G., McDermott J. J, Waltnertoews D., Lissemore K. D, Osumo J. M and Muriuki D (1994). Factors influencing calf morbidity and mortality in smallholder dairy farms in Kiambu district of Kenya, *Preventive Veterinary Medicine* 21(2), 167-177, 1994.

Godden, S (2008). Colostrum management for dairy calves, *Vet. Clin. North Am. Food Anim. Pract*. 24:19-39.

Griot-Wenk, M. E., Busato, A., Welle, M., Racine, B. P., Weilenmann, R., Tschudi, P. and Tipold A (1999). Total serum IgE and IgA antibody levels in healthy dogs of different breeds and exposed to different environments, *Res. Vet. Sci.* 67:239–243.

Gulliksen, S.M., Jor, E., Lie, K.I., Hamnes, I.S., Loken, T. and Akerstedt, J (2009). Enteropathogens and risk factors for diarrhoea in Norwegian dairy calves, *J Dairy Sci*., 92:5057-5066.

Gutierrez, O., Pipaon, C., Inohara, N., Fontalba, A. and Ogura, Y (2002). Induction of NOD2 in myelomonocytic and intestinal epithelial cells via Nuclear Factor-kappa B activation, *The Journal of Biological Chemistry*, 277: 41701-41705.

Haeger, J. D., Hambruch, N. and Pfarrer, C (2016). The bovine placenta *in vivo* and *in vitro*, *Theriogenology,* 86(1):306-312.

Haggag, Y.N. and Khaliel, S. A (2002). Public health importance of certain bacteria isolated from calves and small ruminants, In *The Preceding's of 2nd Vet. Cong. Fac. Vet. Med.*, Minufiya Univ., Egypt, 2(1): 173-184.

Hala, A. A (2012). Characterization of *E.coli* isolated from cattle and buffalo calves suffering from diarrhea, *M.V.Sc Thesis*, Fac. Vet. Med. Alexandria Univ.Egypt.

Heinrichs, A. J. and Radostits, O (2001). Health and Production Management of Dairy Calves and Replacement Heifers, In: Radostits, O.M. (Ed.): *Herd Health. Food Animal Production Medicine*. 3rd Ed., Saunders Company, USA. pp. 333-395.

Hilbert, D.W (2011). Uropathogenic *Escherichia coli*: The Pre-Eminent Urinary Tract Infection Pathogen. *Nova Science Publishers*, Hauppauge (pp. 1-67).

Holland, R. E (1990). Some infectious causes of diarrhea in young farm animals, *Clin. Microbiol. Rev*. 3, 345-375.

Howe L, Sugiarto H and Squires R. A (2008). Use of polymerase chain reaction for differentiation of Group A bovine rotavirus G6, G8 and G10 genotypes in the North Island of New Zealand. *New Zealand Veterinary Journal* 56(5), 218-221.

Hu, Q., Tu J., Han, X., Zhu Y., Ding C. and Yu S (2011). Development of multiplex PCR assay for rapid detection of Riemerella anatipestifer, *Escherichia coli*, and *Salmonella enterica* simultaneously from ducks, *J. Microbiol. Methods* 87: 64–69.

Hussain I. and Saikia G. K (2000). Isolation and characterization of bacteria from diarrhoeic calves, *Indian J. comp. Microbiol. Immunol. infect. Dis*., 21 (1), 125–127.

Ibekwe, A. M, Murinda, S. E and Graves, A. K (2011). Genetic Diversity and Antimicrobial Resistance of *Escherichia coli* from Human and Animal Sources Uncovers Multiple Resistances from Human Sources, *PLoS ONE*, Vol. 6, Issue 6, e20819.

Inohara, N., Chamaillard, M., McDonald,C., and Nunez,G (2005). NOD-LRR proteins: Role in host-microbial interactions and inflammatory disease, *Annual Reviews Biochemistry*, 74: 355–383.

Inohara, N., Ogura, Y., Fontalba, A., Gutierrez, O., Pons, F., Crespo, J., Fukase, K., Inamura, S., Kusumoto, S., Hashimoto, M.,Foster, S. J., Moran, A. P., Fernandez-Luna, J. L. and Núñez, G (2003). Host recognition of bacterial muramyl dipeptide mediated through NOD2: Implications for Crohn's disease, *The Journal of Biological Chemistry*, 278: 5509-5512.

Izzo, M.M, Kirkland, P.D., Mohler, V.L., Perkins, N.R., Gunn A. A., and Housea, J. K (2011). Prevalence of major enteric pathogens in Australian dairy calves with diarrhea, *Aust Vet J*, 89:167- 173.

Jakobsson, S (2013). Diagnosis and Molecular Epidemiology of Bovine Rotavirus and Coronavirus in Brazil, Degree project within the Veterinary Medicine Program ISSN 1652-8697 Examensarbete, 22.

James, N., Fikre E.D., Wondatir, N., Andrew, J. V., Bernard, J. C., David, W. Brown, G and Felicity, T. C (2001). Has oral fluid the potential to replace serum for the evaluation of population immunity levels? A study of measles, rubella and hepatitis B in rural Ethiopia, *Bulletin of the World Health Organization*, 79.

JanBen, T., Schwarz, C., Preikschat P., Voss M., Philipp H.C., Wieler L.H (2001). Virulence-associated genes in avian pathogenic *Escherichia coli* (APEC) isolated from internal organs of poultry having died from colibacillosis", *Inter. J. Med. Microbiol*., 291(5): 371-378.

Jayabal, L. and Ray, D.D (2005). Cryptosporidial infection in cattle and buffaloes, *Journal* of *Veterinary Parasitology*. 19: 165-166.

Jeong, E.S., Lee, K..S., Heo, S.H., Seo, J.H. and Choi, Y.K (2011). Prevalence of *Salmonella* and *E. coli* in neonatal diarrhoeic calves, *Exp. Anim.*, pp: 60-65.

Johns, I., Verheyen, K., Good, L. and Rycroft, A (2012). Antimicrobial resistance in faecal *Escherichia coli* isolates from horses treated with anti-microbials: A longitudinal study in hospitalised and non-hospitalised horses, *Vet. Microbiol.*159: 381-389.

Jones, F.T (2011). A review of practical *Salmonella* control measures in animal feed, The Journal of *Applied Poultry Research*, 20: 102-113.

Kaper, J. B (2005). Pathogenic *Escherichia coli*, *Int. J. Med. Microbiol.* 295**,** 355-356.

Kapetanovic, R., Nahori, M.A., Balloy, V., Fitting, C., Philpott, D. J., Cavaillon, J.M. and Adib Conquy, M (2007).Contribution of phagocytosis and intracellular sensing for cytokine production by *Staphylococcus aureus* activated macrophages, *Infection and Immunity* ,75(2): 830–837.

Khair, A., Alam, M. M., Rahman, A. K. M. A., Shahiduzzaman, M., Parvez, M. S .and Chowdhury, E. H (2014). Prevalence of cryptosporidiosis in crossbred calves in two selected areas of Bangladesh, *Bangl. J. Vet. Med.*, 12 (2): 185-190.

Kobayashi, K, Inohara N, Hernandez, L.D, Galán, J.E, Núñez, G and Janeway, C.A (2002). RICK/RICK/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature*, 416:194–199. PMID: 11894098.

Kobayashi, K. S., Chamaillard, M., Ogura, Y., Henegariu, O., Inohara, N., Nunez, G., and Flavell, R.A (2005). NOD2-dependent regulation of innate and adaptive immunity in the intestinal tract., *Science*, 307: 731–734

Koedel, U., Angele, B., Rupprecht, T., Wagner, H., Roggenkamp, A., Pfister, H. W., and Kirschning, C. J (2003). *J. Immunol.* 170, 438–444

Kong, R. Y. C., So, C. L., Law, W.F. and Wu, R.S.S (1999). A sensitive and versatile multiplex PCR system for the rapid detection of enterotoxigenic *E. coli* (ETEC), *Abdulgayeid et al. /Alexandria Journal of Veterinary Sciences* 2015, *47: 90-96*

Korcina, I., Antane, V. and Lusis I (2012). The Concentration of Immunoglobulins A, G, and M in cow milk and blood in relation with cow seasonal keeping and pathogens presence in the udder. *Proc. Latv. Univ. Agr.* 27 (322), P. 44–53.

Korhonen, H., Marnila, P. and Gill, H. S (2000). Milk immunoglobulins and complement factors, *British Journal of Nutrition*, 84 (1). P. 75–80.

Krol, J., Brodziak, A., Litvinczuk, Z. and Barlowska, J (2012). Selected factors determining the content of lactoferrin, lysozyme and immunoglobulins G in bovine milk, A search for antibacterial agents edited by Varaprasad Bobbarala Copyright c 2012 In Tech., .7. P. 107–124.

Krol, J., Litvinczuk Z., Brodziak, A. and Barlowska, J (2010). Lactoferrin, lysozyme and immunoglobulin G content in milk of four breeds of cows managed under intensive production system, *Polish Journal of Veterinary Science*, 13 (2). P. 357–361.

Kumar, D., Sreekrishnan, R and Das S.S (2005). *Cryprosporidiosis*: an emerging disease of zoonotic importance, *Procedings of the National Academy of Sciences, India*, 75:160-172.

Kumar, D., Sreekrishnan, R., Das S. S (2004). *Cryptosporidiosis* in man and animals in Pondicherry. *Indian J. Dairy Sci.* 74, 261-263.

Laberge, I., Ibrahim, A., Barta, J. R.and Griffiths M. W (1999). Detection of *Cryptosporidium parvum* in raw milk by PCR and Oligonucleotide Probe Hybridization, *applied and environmental microbiology*, Sept. 1996, p. 3259–3264.

Laemmli, U.K (1970). Cleavage of structural proteins during the assembly of head of bacteriophage

Littler, R. M., Garden, O. A. and Batt R. M (1999). Immunoglobulin concentrations in faecal extracts as a measure of canine mucosal immune function, p. 278.,*In Proceedings of the 1999 British Small Animal Veterinary Association Congress*, British Small Animal Veterinary Association, Gloucester, United Kingdom.

Lofstedt, J., Dohoo, L R. and Duizer, G (1999). Model to predict septicaemia in diarrhoeic calves, *J. Vet. Intern. Med.* 13, 81-88.

Lorenz I, Mee JF, Earley B, More SJ (2011). Calf health from birth to weaning. I, General aspects of disease prevention, *Irish Veterinary Journal,* 64, Article 10.

Lorenz, I (2009). An update on calf diarrhoea part 2: Prevention., *Irish Veterinary Journal* 62(2), 130-133.

Lorino, T., Daudin, J.J., Robin, S. and Sanaa, M (2005). Factors associated with time of neonatal diarrhoea in French beef calves. *Prev Vet Med*; 68:91-102.

Majueeb, U., Rehman, M. R., Javeed, A. S. and Mohd, A. B (2014). Molecular epidemiology and antibiotic resistance pattern of Enteropathogenic *Escherichia coli* isolated from bovines and their handlers in Jammu, *India. J. Adv. Vet. Anim. Res*. 1(4): 177-181.

Malik, S., Verma, A.K., Kumar, A., Gupta, M. K. and Sharma, S. D (2012). Incidence of calf diarrhoea in cattle and buffalo calves in Uttar Pradesh, India, *Asian J. Anim. Vet. Adv.* 7: 1049-1054.

Malik, S.V.S., Barbuddhe, S.B., Rawool, D.B., Vaidya, V.M. and Sahare, A. M (2005). Data sheet on Rotaviruses (Global status of Rotavirus infections in man and animals), *In Animal Health and Reproduction Compentium,* CAB International, Wallingford, UK.

Malik, Y. S., Sharma, K., Vaid, N., Chakravarti, S. Chandrashekar, K. M., Basera, S. S., Singh, R., Minakshi, P. G., Gulati, B. R., Bhilegaonkar, K. N. and Pandey A. B (2012). Frequency of group A rotavirus with mixed G and P genotypes in bovines: predominance of G3 genotype and its emergence in combination with G8/G10 types, *J. Vet. Sci***.** 13, 271-278.

Malley, R., Henneke, P., Morse, S.C., Cieslewicz, M.J., Lipsitch, M., Thompson, C.M., Kurt-Jones, E., Paton, J.C., Wessels, M.R. and Golenbock, D.T (2003). Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection, *Proc. Natl. Acad. Sci*. U.S.A. 100, 1966–1971.

Mallinath, R. H. K., Chikkachowdappa, P. G., Gowda, A. K. J., D'souza P. E (2009). Studies on the prevalence of cryptosporidiosis in bovines in organized dairy farms in and around Bangalore, South India, *Vet. arhiv* 79, 461-470,.

Malmuthuge, N., Griebel, P.J.and Guan. L.L (2015). The gut microbiome and its potential role in the development and function of newborn calf gastrointestinal tract, Front., *Vet. Sci.* 2(36):1-10. 32

Marce, C., Guatteo R., Bareille, N. and Fourichon, C (2010). Calf housing systems across Europe and risk factors for calf infectious diseases, *Animal* 4(9), 1588-1596.

Masters, P. S (2006). The molecular biology of coronaviruses, *Advances in Virus Research***,** 66, 193-292.

McDougall, S. and Cullum A (1999). Effect of Feeding Colostrum of High Immunoglobulin Content on Calf Growth Rates and Disease, *Paper presented at the Proc 16th Annual Seminar, Society of Dairy Cattle Veterinarians NZVA*.

McFadden, T. B., Besser, T. E. and Barrington G. M (1997). Regulation of immunoglobulin transfer into mammary secretions of ruminants in: R.A.S. Welch, D. J. W. Burns, S. R. Davis, C. G. Prosser (Eds.), Milk Composition, *Production and Biotechnology, CAB International,* Wallingford, UK; 9, 133–151.

McGuire, K., Jones, M., Werling D., Williams, J.L., Glass, E.J. and Jann, O (2005). Radiation hybrid mapping of all 10 characterized bovine Toll-like receptors, *Anim. Genet*. 37, 47-50.

McGuirk, S.M. and Collins, M (2004). Managing the production, storage, and delivery of colostrum. *Veterinary Clinics of North America: Food Animal Practice*, 20, 593–603.

Mehdiazami, M (2007). Prevalence of *Cryptosporidium* infection in cattle in Isfahan, Iran. J. Euk. ***Microbiol.*** 54, 100-102.

Millemann, Y (2009). Diagnosis of neonatal calf diarrhea, *Revue de Medecine Veterinaire* 160(8-9), 404-409.

Mohamed, M. A., Shehata, M. A., Rafeek, E (2014). Virulence Genes, Content and Antimicrobial Resistance in *Escherichia coli* from Broiler Chickens, *Vet. Med. Int.*

Mohammadi, P., Abiri, R., Rezaei., M, Salmanzadeh-Ahrabi, S (2013). Isolation of Shiga toxin-producing *Escherichia coli* from raw milk in Kermanshah, Iran. *Iran. J. Microbiol.* 5 (3), 233–238.

Momtaz, H., Dehkordi, F. S., Hosseini, M. J., Sarshar, M., Heidari M (2013). Serogroups, virulence genes and antibiotic resistance in Shiga toxin-producing *Escherichia coli* isolated from diarrhoaeic and non-diarrhoaeic pediatric patients in Iran, *Gut Pathogens*, 5: 39.

Mondal, A., Majee, S. B. and Bannalikar, A. S (2011). Molecular Characterization of VP7 Gene of Bovine Group A Rotavirus by Reverse Transcription-PCR, *Animal Science Reporter*, 5 (1): 27– 33.

Morshedi, A., Rabbani, M., Zahraei, Salehi, T., Rezazadeh, F. and Taghipoor- Bazargani, T (2010). Evaluation of antibody levels against rotavirus and coronavirus in the colostrum of nonvaccinated cows in southern Tehran, *Iran. Int. J. Vet. Res., 4(4):217-219.*

Moussa, I.M., Ashgan, M.H.M.H., Mahmoud, M.H., Mohamed, K.F.H. and Doss, A (2010). Rapid detection of *Salmonella* species in newborne calves by polymerase chain reaction. *International Journal of Genentics and Molecular Biology*, 2: 62-66.

Moyes , K. M. , Drackley , J. K. , Salak-Johnson , J. L. , Morin , D. E. , Hope , J. C. and Loor, J. J (2009). Dietary-induced negative energy balance has minimal effects on innate immunity during a *Streptococcus uberis* mastitis challenge in dairy cows during mid-lactation, *Journal of Dairy Science*, 92 (9):4301-4316

Murray, C.J (1994). *Salmonella* serovars and phage n types in humans and animals in Australia 1987-1992, *Aust. Vet. J*., 71: 78-81.

Nataraju, S. M, Chattopadhyay, U.K and Krishnan, T (2009). A study on the possibility of zoonotic infection in rotaviral diarrhoea among calves and buffalo calves in and around Kolkata, India, *Eur Rev Med Pharmacol Sci.*, 13(1):7-11.

National Animal Health Monitoring System (2007). Dairy 2007, Part 1: Reference of dairy health and management in the United States, US Dept. of Agric- Anim, And Plant Health Insp. Serv.- Vet. Serv., Ft. Collins, CO.

Naylor, J. M (2002). Neonatal ruminal diarrhoae. In: Smith, B.P. Large Animal Internal Medicine. 3. Edition., St. Louis, Missouri, 352-366.

Nelson, J., Abdlelaziz, A., Elbanna, T., Nagiub, H., Abdel Ghani, S (2014). Mechanisms of Resistance to Antibiotics in *Escherichia coli* from Patients with Urinary Tract Infections in Egypt. *J. Am. Sci.* 10 (10): 235-239.

Nguyen, T. D., Vo, T.T., Vu-Khac, H (2011). Virulence factors in *Escherichia coli* isolated from calves with diarrhoaea in Vietnam, *J. Vet. Sci*, 12(2):159–164.

Nigatu, W (1999). Detection of measles specific IgG in oral fluid using an FITC/anti-FITC IgG captureenzyme linked immunosorbent assay (GACELISA). Journal of Virological Methods, 83: 135–144.

Niture, G. S., Karpe, A. G., Prasad, M (2011). Characterization of buffalo, poultry and human rotaviruses in Western India, *Vet. Arhiv*. 81: 307-319.

Nizza, S., Mallardo, K., Marullo, A., Iovane, V., De Martino, L., Pagnini, U (2010). Antibiotic susceptibility of haemolytic *E. coli* strains isolated from diarrhoaeic faeces of buffalo calves, *Italian J. Animal Sci*. 9 (1), e26.

Nokes, D (1998). A comparison of oral fluid and serum for the detection of rubella-specific antibodies in a community study in Addis Ababa, Ethiopia, *Tropical Medicine and International Health*, 3: 258–267.

Ogura, Y., Inohara, N., Benito, A., Chen, F. F., Yamaoka, S. and Nunez, G (2001). NOD2, a NOD1/Apaf-1 family member that is restricted to monocytes and activates NF- kappaβ, *The Journal of Biological Chemistry*, 276: 4812–18

Oikonomou, G., Tiexeria, A.G.V., Foditsch, C., Bicalho, M.L., Machadon, V.S.and Bicalho, R.C (2013). Faecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of *Faecalibacterium* species with health and growth, *PLOS One*. 8(4): e63157. doi:10.1371/journal.pone.0063157.

Okada, N., Matsumoto, Y (2002). Bovine rotavirus G and P types and sequence analysis of the VP7 gene of two G8 bovine rotaviruses from Japan, *Vet. Microbiol.* 84, 297-305.

Oliveira, Filho, J.P., Silva, D.P.G., Pacheco, M.D., Mascarini, L.M., Ribeiro, M. G. (2007). Diarrhoae in Nelore calves: Clinical and etiologic study, *Braz J Vet Res*, 27:419-424.

Ongerth, E and Sibbs, H (1989). Prevalence of *Cryptosporidium* infection in dairy calves in Western Washington, *American Journal* of *Veterinary Research*, 50:1069-1070.

Opitz, B., Puschel, A., Schmeck, B., Hocke, A. C., Rosseau, S., Hammerschmidt, S., Schumann, R. R., Suttorp, N. and Hippenstiel, S (2004). Nucleotide-binding oligomerization domain proteins are innate immune receptors for internalized *Streptococcus pneumonia*, *The Journal of Biological Chemistry*, 279(35): 36426–36432.

Osama, N.M., Adel, F.F. Amani F.A. and Rania F.F (2011). Faecal Shedding of Non-typhoidal *Salmonella* species in Dairy Cattle and their Attendants in Alexandria Suburbs, *Journal of American Science*, 7(9).

Özkan, C., Altuğ, N., Yüksek, N., Kaya, A and Akgül, Y (2011). Assessment of electrocardiographic findings, serum nitric oxide, cardiac troponins and some enzymes in calves with hyperkaliemia related to neonatal diarrhea, *Revue Méd Vét*, 162:171- 176.

Pakkanen, R., Aalto, J (1997). Growth factors and antimicrobial factors of bovine colostrums, *International Dairy Journal*, 7. P. 285–297.

Palta, P. and Madan, M.L (1996). Seasonal variations in the hypophysial responsiveness toGnRH in cycling buffalo *(Bubalus bubalis*)., *Asian-Australas, J. Anim. Sci*. 9: 711-14.

Pant, S. D., Schenkel, F. S., Leyva-Baca, I., Sharma, B. S. and Karrow, N. A (2007). Identification of single nucleotide polymorphisms in bovine CARD15 and their associations with health and production traits in Canadian Holsteins, *BMC Genomics*, 8:421.

Parkinson, T., Vermunt, J., Malmo, J (2010). Chapter 4: Diseases causing diarrhea, *Diseases of Cattle in Australasia, A comprehensive textbook,* 127-181. Wellington: Vetlearn: The New Zealand Veterinary Association Foundation for Continuing Education.

Paul, S.K., Khan, M.S.R., Rashid, M.A, Hassan, J., Mahmud, S.M.S (2010). Isolation and characterization of *Escherichia coli* isolated from buffalo calves in some selected areas of Bangladesh, *Bangl. J. Vet. Med.* 8 (1): 23 – 26.

Pauleau, A.L. and Murray, P.J (2003). Role of NOD2 in the response of macrophages to toll-like receptor agonists, *Molecular and Cellular Biology,* 23(21):7531–7539

Pereira, F.T.V., Oliveira, L.J. da Silva Nunes Barreto, R., Mess, A.., Perecin, F., Bressan, F.F., Mesquita, L.G. Miglino, M.A. Pimentel, J.R. Neto, P.F. and Merielles, F.V (2013). Faetal-maternal interations in the synepitheliochorial placenta using the eGFP cloned cattle model, *PLoS One*, 8(5):e64399.

Perlman, S. and Netland, J (2009). Coronaviruses post-SARS: update on replication and pathogenesis, *Nat Reviews Microbiology,* 7, 439-50.

Perlman, S (1998). Pathogenesis of coronavirus-induced infections, Review of pathological and immunological aspects, *Advances in Experimental Medicine and Biology,* 440, 503-513.

Peters I. R., Calvert, E.L. Hall, E.J. and Day M.J (2004). Measurement of Immunoglobulin Concentrations in the Faeces of Healthy Dogs ,*Clinical and diagnostic laboratory immunology*, p. 841–848 Vol. 11, No. 5 1071-412X/04/$08.00_0 DOI: 10.1128/ CDLI.11.5.841–848.

Petty, R. E., Palmer, N.R., Cassidy, J. T., Tubergen, D. G., and. Sullivan. D. B (1979). The association of autoimmune diseases and anti-IgA antibodies in patients with selective IgA deficiency, *Clin. Exp. Immunol.* 37:83–88.

Philpott D.J, Sorbara M.T, Robertson S.J, Croitoru K and Girardin S.E (1994). NOD proteins: regulators of inflammation produced by lipopolysaccharide (LPS) – stimulated human blood mononuclear leukocytes: partial Provence, D. L., Curtiss R III, Isolation and characterization of a gene involved in hemagglutination by an avian pathogenic *Escherichia coli* strain, *Infect. Immun.* 62:1369–1380.

Philpott, D.J and Girardin, S.E (2004). The role of Toll-like receptors and NOD proteins in bacterial infection, *Mol Immunol*. 41(11):1099-108

Provence, D. L. and Curtiss R (1994). Isolation and characterization of a gene involved in hemagglutination by an avian pathogenic *Escherichia coli* strain, *Infect. Immun*, 62:1369–1380.

Quigley, J. D. and Wolfe T. M (2003). Effects of spray-dried animal plasma in calf milk replacer on health and growth of dairy calves, *J. Dairy Sci.* 86: 586–592.

Quigley, J.D., A. Lago, C. Chapman, P. Erickson, and J. Polo (2013). Evaluation of the Brix refractometer to estimate immunoglobulin G concentration in bovine colostrums, *J. Dairy Sci*. 96:1148-1155.

Radostits, O.M.; Gay, C.C.; Hichcliff, K.W. and Constable, P.D (2007). Veterinary medicine, 10th edition. WB Saunders Co., London, UK. pp: 851-875 and 1286-1305.

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh F., Edberg, S., Medzhitov, R (2004). Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis, Cell; 118 (2):229-41

Randall, L.P., Cooles, S.W., Osborn, M.K., Piddock L.J.V., Woodward M.J (2004). Antibiotic resistance genes, integrons and multiple antibiotic resistances in thirty-five serotypes of *Salmonella enterica* isolated from humans and animals in the UK., *J. Antimicrobial Chemotherap*. 53: 208–216.

Reidy, N., Lennon, G., Fanning, S., Power, E., O'Shea, H (2006). Molecular characterization and analysis of bovine rotavirus strains circulating in Ireland 2002–2004, *Vet Microbiol* 117:242–247.

Riad, E.M., Tanios A. I. and El-Moghny A.F.A (1998). Antigen capture ELISA technique for rapid detection of *Salmonella typhimurium* in faecal samples of diarrhoaeic cow calves, *Assiut Vet. Med. J.*, 39(78): 324-333.

Rings, D. M (1985). Salmonellosis in calves, *Vet. Cum. North Am. Food Anim. Pract*. 1, 529-539.

Rock, F.L., Hardiman, G., Timans, J.C., Kastelein, R.A. and Bazan, J.F (1998). A family of human receptors structurally related to Drosophila Toll-like receptors, *Proc. Natl. Acad. Sci.* 95, 588-593.

Rosenstiel, P., Fantini, M., Brautigam, K., Kuhbacher, T., Waetzig, G. H., Seegert, D., and Schreiber, S (2003). *Gastroenterology* 124, 1001–1009

Roy, S. S., Pramanik A.K., Batabyal, S., Sarkar, S., Das, P (2006). Cryptosporidiosis as an important zoonotic disease: a review article, *Intas polivet*, 7:432-436.

Sagar, P (2008). RNA-PAGE analysis of rotavirus from calves and children. M.V.Sc thesis submitted to Bombay Veterinary College, MAFSU, Nagpur.

Saidenberg, A. A. B., Allegretti, L., Astolfi-Ferreira, C. C. S., Ferreira, A. J .P., Almeida, M. A. and Raso T.F (2013). Some virulence genes of *Escherichia coli* isolated from cloacal swabs of healthy Alagoas Curassows (Pauxi mitu) in Brazil, *Pesquisa Vet. Brasilleira* 33(4):523-527.

Sambrook, J. and Russel, D.W (2001). Molecular cloning, a laboratory manual, third edition, Cold spring harbor laboratory press, Vol **1**, 7.31-7.34.

Sansonetti, P. J (2004). 'War and peace at mucosal surfaces' *Nat Rev Immunol*. 4(12):953-64.

Saravanan, M., Parthiban, M. and Ramadass P (2006). Genotyping of rotavirus of neonatal calves by nested-multiplex PCR in India, *Vet. Arhiv* 76, 497-505.

Schleifer, K.H. and Kandler, O (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications, *Bacteriological Reviews*, 36(4): 407-477

Schro¨der, N. W., Morath, S., Alexander, C., Hamann, L., Hartung, T., Zahringer, U., Gobel, U. B., Weber, J. R. and Schumann. R. R (2003). Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved, *J. Biol. Chem.* 278:15587–15594.

Schroeder, B. , Sproule, R. and Saywell, D (1985). Prevalence of rotavirus in dairy calves as diagnosed by ELISA, *Surveillance New Zealand* 12(2), 2-3.

Seleim, R.S., Sahar, R.M., Novert, M.H. and Gobran, R. A (2004). *Salmonella* infection in calves: virulence proteins and its immunogenic properties, *J. Vet.* online; <http://www.priory.com/vet/salmonella.htm>.

Sevinc, F., Irmak, K. and Sevinc, M (2009). The prevalence of *Cryptosporidium parvum* infection in the diarrhoiec and non- diarrhoeic calves, *VETERINARSKI ARHIV, 79* (5), 461-470.

Shahrani, M., Dehkordi, F. S. and Momtaz, H (2014). Characterization of *Escherichia coli* virulence genes, pathotypes and antibiotic resistance properties in diarrhoeic calves in *Iran, Biol. Res.* 47: (1) 28.

Shobhamani, B (2005). Epidemiological studies on diarrhoea in calves with particular reference to diagnosis and treatment of cryptosporidiosis, *J. Vet. Parasitol.* 19, 77.

Singh, B. B., Sharma, R., Kumar, H., Banga, H. S., Aulakh, R. S., Gill, J. P. S. and Sharma J. K (2006). Prevalence of *Cryptosporidium parvum* infection in Punjab (India) and its association with diarrhea in neonatal dairy calves. *Vet. Parasitol.* 140, 162-165.

Smith-Palmer, A., Stewart, W.C., Mather, H., Greig, A., Cowden J.M. and Reilly, W.J (2003). Epidemiology of *Salmonella enterica serovars enteritidis* and *typhimurium* in animals and people in Scotland between1990 and 2001, *Vet. Rec.*, 153: 517-520.

Sordillo, M. and Streicher, K. L (2002). Mammary Gland Immunity and Mastitis Susceptibility. *Journal of Mammary Gland Biology and Neoplasia*, **7(2)**:135-146

Stathopoulos, C., Provence, D. L., Curtiss, R.III (1999). Characterization of the avian pathogenic *Escherichia coli* hemagglutinin *Tsh*, a member of the immunoglobulin A protease-type family of autotransporters, *Infect Immun.* 67:772–781.

Suresh, T., Rai, R.B., Wani, M.Y., Damodaran, T. and Dhama, K (2013). Detection of bovine rotavirus in neonatal calf diarrhoea by ELISA, FAT and transmission electron microscopy, *International Journal of Current Research* 5(7):1935-1939.

Svensson, C., Hultgren J., Oltenacu, P.A (2006). Morbidity in 3-7-month-old dairy calves in south-western Sweden, and risk factors for diarrhoea and respiratory disease, *Preventive Veterinary Medicine* 74(2-3).

Svensson, C., Lundborg, K., Emanuelson, U. and Olsson, S.O (2003). Morbidity in Swedish dairy calves from birth to 90 days of age and individual calf-level risk factors for infectious diseases. *Preventive Veterinary Medicine* 58(3-4), 179-197.

Szmolka, A. andNagy, B. (2013). Multidrug resistant commensal *Escherichia coli* in animals and its impact for public health. Front Microbiol. 4: 258-270.

Tajik, J., Nazifi, S., Naghib, S.M. and Ghasrodasht, A.R (2012). Comparison of electrocardiographic parameters and serum electrolytes and microelements between single infection of rotavirus and coronavirus and concurrent infection of *Cryptosporidium parvum* with rotavirus and coronavirus in diarrhoeic dairy calves, *Comp Clin Pathol*, 21 241-244.

Takeda, K., Kaisho, T. and Akira, S (2003). Toll-like receptors. *Ann. Rev. Immunol.* 21, 335-376.

Taylor, K. H (2004). Genetic analyses of bovine CARD15, a putative disease resistance gene. Ph.D. Thesis submitted to the Office of Graduate Studies of Texas A&M University.

Thwe, M (1999). Stability of saliva for measuring HIV in the tropics, *Journal of Tropical Pediatrics*, 45: 296–299.

Tyzzer, E.E (1913). *Cryptosporidium parvum* (sp. nov), A coccidian found in the small intestine of the common mouse, *Arch Protistenkunde* 26, 394-412.

Tyzzer, E.E., (1910). An extracellular Coccidium, *Cryptosporidium Muris* (Gen. Et Sp. Nov.), of the gastric Glands of the Common Mouse, *J Med Res* 23, 487-510.

Van Der Hoek, L., Pyrc, K., Jebbink, M. F., Vermeulen-oost, W., Berkhout, R. J., Wolthers, K. C., Wertheim-van Dillen, P. M., Kaandorp, J., Spaargaren, J. and Berkhout, B (2004). Identification of a new human coronavirus, *Nature Medicine*, 10, 368-373.

Vanselow, B.A., Hornitzky, M.A., Walker, K.H., Eamens, G.J., Bailey, G.D., Gill, P.A., Coates, K.., Corney, B., Cronin J. P. and Renilson, S (2007). *Salmonella* and on-farm risk factors in healthy slaughter age cattle and sheep in eastern Australia, *Australian Veterinary Journal*, 85: 498-50.

Vasseur, E., Borderas, F., Cue, R.I., Lefebvre, D., Pellerin, D., Rushen, J., Wade, K.M. and de Passillé, A. M (2010). A survey of dairy calf management practices in Canada that affect animal welfare, *Journal of Dairy Science*, 93, 1307–1316. doi:10.3168/jds.2009-2429.

Vermunt, J (2002). Calf rearing Part 2: The management of neonatal calf diarrhoea, *Vetscript* 15(5), 6-8.

Vyse, A.J (1999). Detection of rubella virus-specific immunoglobulin G in saliva by an amplification-based enzyme-linked immunosorbent assay using monoclonal antibody to fluorescein isothiocyanate, *Journal of Clinical Microbiology*, 37: 391–395.

Wani, S.A., Hussain, I., Beg, S. A., Rather, M.A., Kabli, Z.A., Mir, M.A. and Nishikawa, Y (2013). *Rev. sci. tech. Off. int. Epiz.*, 32 (3), 833-840.

Weaver, D.M., Tyler, J.W., VanMetre, D.C., Hostetler, D.E., Barrington, G.M (2000). Passive transfer of colostral immunoglobulins in calves, *Journal of Veterinary internal medicine*, 14, 569–577.

Weiss, S. and Navas-Martin, S (2005). Coronavirus Pathogenesis and the Emerging Pathogen Severe Acute Respiratory Syndrome Coronavirus, *Microbiology and Molecular Biology Reviews*, 69, 635–664.

Wells, S.J., Dargatz, D.A., Ott, S.L (1996). Factors associated with mortality to 21 days of life in dairy heifers in the United States, *Preventive Veterinary Medicine*, 29, 9–19.

Whitbread, T. J., Batt, R. M. and Garthwaite. G (1984). Relative deficiency of serum IgA in the German shepherd dog: a breed abnormality, *Res. Vet. Sci.* 37:350–352.

Willard, M. D. Simpson, R. B., Fossum, T. W., Cohen, N. D., Delles, E. K.., Kolp, D. L., Carey, D. P. and Reinhart G. A (1994). Characterization of naturally developing small intestinal bacterial overgrowth in 16 German shepherd dogs, *J. Am. Vet. Med. Assoc.* 204:1201–1206.

Woo, P. C., Lau, S. K., Chu, C. M., Chan, K. H., Tsoi, H. W., Huang, Y., Wong, B. H., Poon, R. W., Cai, J. J., Luk, W. K., Poon, L. L., Wong, S. S., Guan, Y., Peiris, J. S. and Yuen, K..Y (2005).Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia., *Journal of Virology*, 79, 884-895.

Wooding, F.B.P (1992). Current topic: the synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production, *Placenta*, 13:101-113.

Yoshimura, A., Lien, E., Ingalls, R. R., Tuomanen, E., Dziarski, R., and Golenbock, D (1999). *J. Immunol.* 163, 1–5

Younis, E.E., Ahmed, A.M.. El-Khodery, S.A., Osman S.A. and El-Naker Y.F (2009). Molecular screening and risk factors of enterotoxigenic *Escherichia coli* and *Salmonella* spp. in diarrhoeic neonatal calves in Egypt, *Res. Vet. Sci.*, 87: 373-379.

Youssef, A.I. and El-Haig M. M (2012). Herd problems and occupational zoonoses of *Salmonella enteric serovars Typhimurium* and *Enteritidis* infection in diarrhoeic cattle and buffalo calves in Egypt, *Int. J. Bioflux Soc.*, 4(3): 118-123.

Zhang, H-J., Qu, J.M., Shao, C.Z., Zhang, J., He, L.X., Yuan, Z.H (2008). *Aspergillus fumigatus* conidia upregulates NOD2 protein expression both *in vitro* and *in vivo, Acta Pharmacol.* Sin., 29 (10). 1202–1208.

APPENDIX I

A1. Wares and biohazard waste-disposal

All glasswares were cleaned by overnight treatment with chromic acid solution (potassium dichromate- concentrated sulphuric acid soln.) or labware detergent followed by thorough rinsing in tap water, deionized water and finally distilled water. Empty glasswares were dried and sterilized at 160°C for more than one hr. in hot air oven. Liquid media in bottles and flasks were sterilized by autoclaving. All plasticwares, such as 15 ml and 50 ml centrifuge tubes, microcentrifuge tubes, storage vials, cryovials, micropipette tips, etc. were pre-sterilized or disposable.

Sterilized materials were handled in Biosafety type III cabinet. All biological materials, contaminated needles, syringes, pipettes, etc. were placed in biohazard bags and disposed after their chemical/heat inactivation.

A2. General reagents and salt solutions

1. 5 M NaCl

NaCl		29.2 g
MilliQ H_2O/DW	*ad*	100 ml

Sterilized by membrane filtration/autoclaving at 15 psi for 20 min.and stored at RT.

2. 1 M $MgCl_2.6H_2O$

$MgCl_2.6H_2O$		20.330 g
MilliQ H_2O/DW	*ad*	100 ml

Sterilized by membrane filtration/autoclaving at 15 psi for 20 min. and stored at 4ºC.

3. 4 N H_2SO_4

Concentrated H_2SO_4	27.240 ml
MilliQ H_2O/DW	62.760 ml

Stored at RT.

4. 1 M Tris.HCl, pH 7.8

Tris base	12.110 g
MilliQ H_2O/DW	80 ml
Conc. HCl	4.5 ml

Adjusted pH to 8.0, final volume made to 100 ml with MilliQ H_2O/DW, sterilized by autoclaving at 15 psi for 20 min. and stored at 4ºC.

5. 0.5 M EDTA

$EDTA.Na_2$	18.610 g
MilliQ H_2O/DW	80 ml

Approximately 2.0 g NaOH pellets added while stirring the solution to adjust pH to 8.0. Final volume made to 100 ml, sterilized by autoclaving at 15 psi for 20 min. Stored at 4ºC.

6. 10X TBE

Tris base		10.8 g
Boric acid		5.5 g
0.5M $EDTA.Na_2$, pH 8.0		4.0 ml
MilliQ H_2O/DW	*ad*	100 ml

Filter-sterilized and stored at RT.

7. Lysozyme stock solution (10 mg/ml)

Lysozyme		10 mg
10 mM tris-HCl, pH 8.0		1.0 ml

8. 1M IPTG (isopropyl thio-β-D-galactoside)

IPTG		1.25 g
MilliQ H_2O/DW	*ad*	5 ml

Filter-Sterilized and stored as 1.0 ml aliquots at −70ºC.

9. 10 N NaOH

NaOH		40 g
MilliQ H_2O/DW	*ad*	100 ml

Stored at RT.

10. Ethidium bromide stock solution (10 mg/ml)

Ethidium bromide		10 mg
MilliQ H_2O/DW		1.0 ml

Stored at RT.

11. 20% Polyethylene glycol (PEG)-8000/2.5M NaCl

PEG-8000		20 g
5 M NaCl		50 ml
MilliQ H_2O/DW	*ad*	100 ml

Sterilized by autoclaving at 15 psi for 20 min. and stored at 4ºC.

12. Trichloroacetic acid (TCA), 100%

TCA		100 g
MilliQ H_2O/DW	*ad*	45.4 ml

Stored at RT.

13. Saturated ammonium sulphate solution

Ammonium sulphate		800 g
MilliQ H_2O/DW		1000 ml

Mixed by stirring O/N, some crystals remained undissolved to indicate 100% saturation and then stored at RT.

14. Sodium dodecyl/lauryl sulphate (SDS), 10% aq. w/v

SDS		10 g
MilliQ H_2O/DW	*ad*	100 ml

Stored at RT.

15. TES (0.2 M tris- 0.5 mM EDTA- 0.5 M sucrose) buffer, pH 8.0

1 M tris.HCl, pH 8.0 .		20.0 ml
0.5 M EDTA.Na_2		0.100 ml
Sucrose		17.110 g
MilliQ H_2O/DW	*ad*	100 ml

16. TES/4 buffer

TES		25 ml
MilliQ H_2O/DW		75 ml

17. Alsever's anticoagulant solution

Glucose		2.050 g
Tri-sodium citrate		0.800 g
Sodium chloride		0.420 g
Citric acid		0.055 g
MilliQ H_2O/DW	*ad*	100 ml

Autocalved at 10 psi for 15 min. and stored at 4ºC.

18. 10X Phosphate buffered saline (1M PB, pH 7.0- 1.5M NaCl)

Na_2HPO_4		83.4 g
KH_2PO_4		56.2 g
NaCl		87.7 g
MilliQ H_2O/DW	*ad*	1000 ml

19. Ampicillin stock solution (100 mg/ml)

Ampicillin		100 mg
Distilled water		1 ml

Sterile filter, store in aliquots at -20ºC

A3. Buffers & reagents for SDS-Polyacrylamide gel elecphoresis

I. Buffers & reagents for SDS-polyacrylamide gel synthesis & sample loading

1. Acrylamide/bis 30% stock solution

Acrylamide		29.2 g
N,N-metylene bis-acrylamide		0.8 g
MilliQ H_2O	*ad*	100 ml

Membrane-filtered and stored at 4ºC.

2. 1.5M Tris-HCl, pH 8.8 (4x resolving gel buffer)

Tris base		18.171 g
MilliQ H_2O	*ad*	50 ml

Adjusted pH to 8.8 with HCl at RT and added MilliQ H_2O to make 100 ml. Membrane-filtered and stored at 4ºC.

3. 1M Tris-HCl, pH 6.8 (8x stacking gel buffer)

Tris base		12.114 g
MilliQ H_2O	*ad*	50 ml

Adjusted pH to 6.8 with HCl at RT and added MilliQ H_2O to make 100 ml. Membrane-filtered and stored at 4ºC.

4. Sodium dodecyl/lauryl sulphate (SDS) (10% aq., w/v)

SDS		10 g
MilliQ H_2O	*ad*	100 ml

5. Ammonium persulphate/peroxodisulphate (APS) (10% aq., w/v)

APS		100 mg
MilliQ H_2O	*ad*	1 ml

Used fresh or within a few days of storing at 4ºC.

6. Electrode buffer (0.025 M Tris- 0.192 M Glycine, pH 8.3- 0.1 % SDS)

Tris base		3.02 g
Glycine		14.4 g
SDS		1 g
MilliQ H_2O	*ad*	1000 ml

7. 2xLaemmli's sample buffer

1M Tris-HCl, pH 6.8		1.25 ml
Glycerol		2 ml
SDS (10%)		4 ml
2- Mercaptoethanol		1 ml
Bromophenol blue		2 mg
MilliQ H_2O	*ad*	10 ml

II. Staining/destaining of proteins in polyacrylamide gel

1. Coomassie brilliant blue R250 (CBBR-250) staining solution

CBBR-250		1 g
Methanol		225 ml
Glacial acetic acid		50 ml
DW/deionized water		225 ml

Filtered through Whatman no. 1 paper/cotton wool pad and stored at RT.

2. CBBR-250 destaining solution

Methanol		225 ml
Glacial acetic acid		50 ml
DW/deionized water		225 ml

A4. Buffers & reagents for ELISA

1. Coating buffer (50mM Phosphate buffer, pH 7.0)

Na_2HPO_4		4.170 g
KH_2PO_4		2.810 g
MilliQ H_2O	*ad*	1000 ml

Filter-sterilized and stored at 4°C.

2. Phosphate buffered saline (PBS) (100mM PB, pH 7.0- 150 mM NaCl)

Na_2HPO_4		8.340 g
KH_2PO_4		5.620 g
NaCl		8.770 g
MilliQ H_2O	*ad*	1000 ml

Filter-sterilized and stored at 4°C.

3. **Washing buffer** (PBST)

PBS, pH 7.0	1000 ml
Tween 20	0.500 ml

Made fresh for use on the same day.

4. **Blocking buffer** (PBST-1%BSA)

BSA, fraction V	1.0 g
PBS, pH 7.0	100 ml
Tween 20	50µl

Made fresh just before use.

5. **Substrate buffer** [Citrate phosphate buffer (CPB), pH 5.0]

Citric acid		470 mg
Na_2HPO_4		730 mg
MilliQ H_2O	*ad*	100 ml

Membrane-filtered and stored at RT.

6. **Substrate/colour development solution**

O-phenylene diamine	40 mg
CPB	100 ml

Made fresh just before use, filter-sterilized, added 20 µl of H_2O_2 (30%) and used immediately.

A 5 Buffers & reagents for Sandwich ELISA for Bovine IgG measurement

1. **Coating buffer** (50mM Phosphate buffer, pH 7.0)

Na_2HPO_4		4.170 g
KH_2PO_4		2.810 g
MilliQ H_2O	*ad*	1000 ml

Filter-sterilized and stored at 4°C.

2. **Phosphate buffered saline** (PBS) (100mM PB, pH 7.0- 150 mM NaCl)

Na_2HPO_4		8.340 g
KH_2PO_4		5.620 g
NaCl		8.770 g
MilliQ H_2O	*ad*	1000 ml

Filter-sterilized and stored at 4°C.

3. **Washing buffer** (PBST)

PBS, pH 7.0	1000 ml
Tween 20	0.500 ml

Made fresh for use on the same day.

4. **Blocking buffer** (PBST-2% normal rabbit serum)

Rabbit serum	2 ml
PBS, pH 7.0	100 ml
Tween 20	50µl

Made fresh just before use.

5. Substrate/colour development solution

Ready to use TMB; kept stored at 4^0 C

6. Stop solution (0.18 M H_2SO_4

Conc. H_2SO_4 (98 % pure)		1 ml
MiliQ H_2O/DW	*ad*	100

Slowly add acid dropwise into 70 ml of water with the help of a glassrod. And then make final volume by adding more water to diluted solution. Store at RT.

Reagents for silver staining

Fixative solution :	0.5 % glacial acetic acid, 10 % ethanol
Staining solution :	0.185 % silver nitrate in GDW
Developer :	3 g o f NaOH pellets in 100ml GDW and 0.75 ml of formaldehyde was added
Stopper :	5 % glacial acetic acid
Storing solution :	10 % ethanol

APPENDIX-II

Reagents used for AGAROSE GEL ELECTROPHORESIS (AGE)

Ethidium bromide (10 mg/ml)

Ethidium bromide	50 mg
DW	5 ml

Stored the solution in amber colored vial at 4ºC.

0.5 M EDTA, pH 8.0

EDTA. 2 H2O	18.61 g
DW to make	100 ml

Adjusted the pH to 8.0 with solid Sodium hydroxide pellets. The solution was filtered through Whatman filter paper no.1 and stored at room temperature.

Tris- acetate-EDTA (TAE) stock solution (50X)

Tris base	121.0 g
Glacial acetic acid	28.5 ml
0.5 M EDTA, pH 8.0	50.0 ml
DW to make	500 ml

For working solution (1X), diluted the stock TAE in DW.

6X Loading dye (Type IV)

Sucrose 40 % w/v in DW

Bromophenol blue 0.25 % w/v in DW

The solution was stored at 4ºC.

TE- Buffer

1.0 M Tris-Hcl 1.0 ml (10.0 mM)

0.5 M EDTA 0.2 ml (1mM)

Mixed with DW to make 100 ml, sterilized by autoclaving at 15 lb pressure (121ºC) for 15 min. and stored at 4ºC.

APPENDIX III

Sources of chemicals, reagents, etc.

I. Biochemical reagents

i. Sigma Chemical Co., USA

ii. Sisco Research Laboratories (SRL), India

iii. Qualigens, Bombay, India

II. Molecular biology reagents

i. Fermentas, USA

ii. New England Biolabs, USA

iii. Promega, USA

iv. Qiagen, Germany

III. Kits & others materials

i. Maxisorp® ELISA microtitre plates - Nunc, Denmark

ii. DNA purification kit- Qiagen, Germany & Promega, USA

iii. Protein estimation kit - Genei, Bangalore, India

iv. QIAquick PCR Purification Kit- Qiagen, Germany

v. RNA extraction using RNeasy Plus Mini Kit- Qiagen, Germany

☑ **All chemicals and reagents used were of AnalR/LR grade.**

☑ **Commonly used chemicals andreagents are not mentioned in this appendix.**

APPENDIX IV

Special instruments/equipments used in the present study

1. **For PCR: Thermal Cycler** ((XP CyclerR gradient thermal cycler, Bioer Technology Ltd. Co., PR China).
2. **For gel phototgraphy/documentation:** Spectroline®, Spectroline Corp., USA
3. **For ELISA/microplate absorbance measurements:** Multiskan®, Thermo Scientific, USA
4. **For MilliQ ultrapure water:** Millipore® ELIX-III, USA
5. **For RNA/DNA quantification**: NanoDrop 2000 spectrophotometer (Thermo Scientific)

ABSTRACT

Title of Research Project	:	**Investigations on common Pathogens of Neonatal Diaorrhea and assessement of Passive transfer of Immunity in Buffalo Calves**
Full Name of the Degree Holder	:	**Ismaila Alhaji Mairiga**
Admission No.	:	**2015V18D**
Title of the Degree	:	**PhD. in Veterinary Science**
Name and Address of Major Advisor	:	**Dr. Yudvir Singh Rana:** **Prof., Dept. of Veterinary MedicineLUVAS,** **Hisar-125 004 (India)**
Degree Awarding University	:	**Lala Lajpat Rai University of Veterinary and Animal Sciences Hisar-125 004 (Haryana), India**
Year of Award of Degree	:	**2018**
Major Subject	:	**Veterinary Medicine**
Total Number of Pages in Thesis	:	**127 + xiv + IX**
Number of Words in Abstract	:	**529**

Keywords: Diaorrhea, Neonates, Buffalo Calves, Dams, Pathogens, Meconial-IgG, Faecal-IgG, Oral fluid-IgG, , Urine-IgG, Colostral-IgG, Milk-IgG, Serum-IgG, TLR4 and CARD15/NOD2 gene transcripts.

Diarrhoea is the most commonly reported calf disease and a major cause of calf morbidity and mortality worldwide Diarrhea in young calves is the main cause of economic losses through poor growth, morbidity and mortality, and the role played by common pathogens of Calf diaorrheaa in producing the syndrome in calves has received a great attention by many researchers and yet the syndrome persist.In the present study 78 faecal samples were collected from Buffalo Calves and their Dams and Calves were categorized into 3 groups of Day- 1 to 2 - Days, Day -3 to 3- Months and a group of 4 to 6 Months of age. A total of 38 positive faecal samples were recorded as *Escherichia coli* positive, including some of their virulence and antibiotic resistance genes. This comprised of 23(60.53) from Buffalo Calves and 15(37.50) from Buffalo Dams. Similarly an examination of same number of samples from same animals with and without diarrhea revealed 11 (28.95) positive faecal samples with genus specific and other genes of Salmonellain from buffalo calves and 2 (05.00) as positive faecal samples from Buffalo Dams after examining a total of 40 faecal samples. stn, invA and sef are virulence genes of Salmonella detected respectively numbering 7, 6 and 5 out of a total of 13 positive faecal samples recorded. *Salmonella typhi* genes were detected in all Salmonella positive faecal samples. However, Salmonella enteritidis gene was not detected in all screened samples including Salmonella positive ones. A similar examination of a total of 38 and 40 faecal samples from Buffalo Calves and Dams with and without diarrhea revealed Cryptosporidium positive samples as10 (37.04) and 17 (62.96) respectively from buffalo calves and Dams. An examination and analysis of 78 diarrheic and non-diarrheic faecal samples from Buffalo Calves and Dams, revealed a negative detection of Rotavirus and Coronavirus by RNA-PAGE and PCR analysis. Bovine IgG concentration was detected from Colostrum, Milk, Urine, Oral fluid, Meconium, Faeces and Serum samples.High concentrations of IgG were detected from Colstrums, Milk and Sera while low concentration of same was detected from Meconium, Faeces, Oral fluid and Urine samples.Success of passive transfer of immunity from Buffalo Dams to their Calves was confirmed by simple detection of variable concentrations of Bovine IgG in screened Calves. TLR4 and CARD15/NOD2 gene transcripts were detected from PBMC's of Buffalo Calves. Similarly, TLR4 and CARD15/NOD2 gene transcripts were detected from Colostral leucocytes of Buffalo Dams. Despite evidences of interplay of Innate and Adaptive immune responses as witnessed in the present study, a syndrome of diarrhea was recorded and this explains some levels of Resistance, Tolerance and Susceptibility exhibited by different Calves and Dams examined during the course of the study. This outcome affirms that diarrhea syndrome is still considered to be a threat to the survival of neonatal Calves and by extension a major contributer to loss/reduction of replacement heifers in subsequent production circles. It is therefore suggested that further research should be intensified on importance of immunotherapy as an alternative to antibiotic therapy in addition to an indepth understanding of roles of genetic immunity essential for selection of breeds and individuals with optimal genetic potentials for maximum yield in the next production circle.

MAJOR ADVISOR **(SIGNATURE OF DEGREE HOLDER)**

HEAD OF DEPARTMENT

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UNDERTAKING OF COPYRIGHT

I, Ismaila Alhaji Mairiga, Admission No. 2015V18D undertake that I give copyright to Lala Lajpat Rai University of veterinary Sciences, Hisar of my thesis entitled "**Investigations on common Pathogens of Neonatal Diaorrhea and the assessement of Passive transfer of Immunity in Buffalo Calves"** I also undertake that patent, if any, arising out of the research work conducted during the programme shall be filled by me only with due permission of the competent authority of LUVAS, Hisar.

Signature of the Student