Isolation and molecular detection of enteric bacteria from neonatal calf in Chittagong

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Roll No. 0115/03

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Dedication

To my parents

who always valued education

above everything else

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This is to certify that we have examined this thesis and have found that it is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made

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The Author
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List of abbreviations and symbols used

Abbreviation and symbol	Elaboration
^{o}C	Degree Celsius
BA	Blood Agar
DLS	Department of Livestock Services
EMB	Eosin Methylene Blue
mg	Milligram
Min	Minute (s)
ml	Milliliter
NCD	Neonatal Calf Diarrhea
Sec	Second (s)
TSB	Tryptone Soya Broth
TSI	Triple Sugar Iron
XLD	Xylose-Lysine-Desoxycholate
µg	Microgram

Summary

Calf mortality due to neonatal calf diarrhea is a major problem in cattle farming in Bangladesh where certain groups of bacteria play crucial role. The aim of the present study was to carry out pathological and molecular investigation on enterotoxigenic *Escherichia coli* (ETEC) and *Salmonella typhimurium* in neonatal calves. During the study, fecal samples were collected from 200 calves (less than 40 days of age) from a total of 64 dairy and beef farms located in Chittagong Metropolitan Area and Patiya Upazila of Chittagong. The study was conducted during June, 2015 to March, 2016 when 5 dead calves with a history of diarrheal illness were examined through necropsy for gross and histopathological screening. Alongside classical culture technique molecular diagnostic tools like Polymerase chain reaction (PCR) was employed to identify the pathogens including *E. coli* and *Salmonella* spp. Two separate primer pairs namely ECO and K99 were used during this study where *ECO* is indicative of *E. coli*, and *K99* is indicative for Enterotoxigenic *E. coli* (ETEC) K99. Besides, *Salmonella* spp. was confirmed by *InvA* gene in PCR. *Typh* and *104* primers were also used to identify *S. typhimurium* and multidrug resistant *S. typhimurium* DT104, respectively. Results indicated 19 (9.5%) cases of ETEC K99 out of 200 isolates, whereas *S. typhimurium* was recovered from only 8 calves (4%) and *S. typhimurium* DT104 was recorded from 2 calves. None of the dead animals was found positive for *S. typhimurium* through PCR. Gross pathological changes observed during necropsy include watery intestinal contents with bad odor, mucus and blood (in two cases) with dilatation of intestinal lumen. Histopathological investigation of intestine showed villous atrophy in all five cases of dead calves. Thickening of intestinal epithelium, sloughing off epithelium, congestion in lamina propria, infiltration of reactive cells were the findings of diarrhea caused by ETEC K99. Two out of five dead calves were found positive for ETEC K99 and one was isolated for mixed infection with *E. coli* and rotavirus in confirmatory molecular identification. Statistically significant difference ($p<0.05$) were observed in the prevalence of *E. coli* and *S. typhimurium* collected from different locations and herd size. Highest prevalence of ETEC K99 and *S. typhimurium* were found in Bakalia (30.43%) and Chawkbazar (27.27%), respectively. Compared to female animals, male calves were mostly affected by both ETEC K99 (13.27%) and *S. typhimurium* (27.27%). Small farms

having <10heads had highest prevalence of ETEC K99 (42.86%). But *S. typhimurium* occurred highest in medium sized farms (5.81%) having 21-50 heads. Multilevel univariable and multivariable logistic regression was used to evaluate the risk factors associated with both infections. *E. coli* was significantly affected by farm size (OR = 3.54, 95% CI: 1.02, 12.78), population density (OR = 1.02, 95% CI: 1.00, 1.05), age (OR = 5.51, 95% CI: 1.59, 19.04), coexistence of other animal species in same pen (OR = 3.24, 95% CI: 1.12, 9.34), suckling as feeding regime (OR = 5.07, 95% CI: 1.43, 17.16). Interestingly, level of dehydration showed inverse association with prevalence of ETEC K99. Infection with *Salmonella* spp. was found to be significantly affected by the source of drinking water supply (OR = 32.28, 95% CI: 5.76-180.94), status of antibiotic therapy (OR = 7.03, 95% CI: 1.30, 37.81) and recent introduction of new calf in the farm (OR = 0.09, 95% CI: 0.01, 0.90). The results of the present study indicate the importance of PCR as rapid, effective and reliable tool alongside conventional culture techniques for screening of ETEC and *Salmonella* spp. when confronted with cases of undifferentiated calf diarrhea. Moreover, identification of the risk factors associated with the spreading of bacteria causing diarrheal illness may be helpful towards developing suitable prevention and control strategies.

Keywords: Calf diarrhea, ETEC, *Salmonella*, Prevalence, Pathology, Risk factors

Chapter-1: Introduction

Livestock is an integral component of the complex farming system in Bangladesh as it not only a source of protein but also a major source of farm power services alongside employment. The livestock sub-sector provides full time employment for 20% of the total population and part-time employment for another 50%. The cattle population in 2015-2016 was 237.85 lakh in the country with milk production record as high as 72.75 lakh metric tons. The GDP contribution of this sub-sector has been a modest 1.66% and GDP growth rate has been 3.21% in the year 2015-2016 (DLS, 2016). However, the sector's actual contribution has been consistently underestimated as the value added in draught power used in farm operation, threshing, sugarcane and oilseed crushing, local transport, dung for cooking fuel and manure for fertilization of crop fields were not taken into account. An estimate of the uncounted sectorial contribution of livestock indicates a foregone value of three times the amount of official GDP attributed to this sector (FAO, 1990). To fight with malnutrition problem we need to address growth in edible livestock products. A bunch of commingled factors such as technical, institutional, and social, are limiting the development of the livestock sector in Bangladesh. Previous studies have identified many areas of concern that restrict the potential development of the livestock sector; outbreak of diseases being the most important one (Abdul-Rahman and Hailes, 2000).

National Animal Health Monitoring System (NAHMS) for U.S. dairy reported that 57% of weaning calf mortality in 2007 was due to diarrhea while calves less than 1 month old were most susceptible (USDA, 2007) and a similar mortality rate (53.4%) for dairy calves due to calf diarrhea was reported in Korea (Hur *et al.*, 2013). The economic loss associated with calf death in Norway where calf production is 280,000 heads per year was estimated to be approximately 10 million US dollars in 2006 (Østerås *et al.*, 2007). Among different factors causing economic loses to Bangladeshi dairy industry, calf mortality is considered as the preeminent element. Infectious diseases are often considered as the principal cause of calf mortality, especially gastro-intestinal disorders (Daros *et al.*, 2014) and respiratory problems (Gulliksen *et al.*, 2009). Moreover, farms with high calf mortality are regarded as of having poor welfare at the farm level. A high

death rate renders an economic burden to the farm that needs to be addressed with utmost importance. It is well established that management practices influence morbidity and mortality in dairy calves. As an example management and feeding of high-quality colostrums can lessen calf mortality and fortify immunity (Abdul-Rahman and Hailes, 2000).

Neonatal calf diarrhea (NCD) relics as an important cause of morbidity and mortality in neonatal calves (Constable, 2004). Diarrhea which is a multifactorial disease, remains the most ususal cause of deaths in newborn calves, even though foremost risk factors that has long been identified, numbers of calves losses due to diarrhea are not declining (Snodgrass *et al.,* 1986). Several enteropathogens were recovered from neonatal calf with diarrhea, their relative prevalence varies geographically but the most conjoint prevalent infections in most areas are *Escherichia coli,* rotavirus, and coronavirus, *C. Perfringens*, *Salmonella* spp. and *Cryptosporidium* spp. (Snodgrass *et al.,* 1986; Garcia *et al.,* 2000).

Earlier reports indicated that Enterotoxigenic *E. coli* (ETEC) infection is the most common type of colibacillosis of newborn animals, travelers and children in the developing world (Nagy and Fekete, 2005). Diarrhea producing *E. coli* possess colonization antigens or adhesions that enable the bacteria to colonize the small intestines (Chakraborty *et al.*, 2001). Notable that expression of K99 fimbriae (or F5 ETEC) accounts for nearly all cases of ETEC infection found in newborn calves (Jay *et al.*, 2004). The common features of ETEC infections in different species are that bacteria adhere to the small intestinal epithelial cells and thereby colonize the gut. They also secrete proteins or peptides (enterotoxins), usually stable toxin (ST) which stimulates the small intestine for increased water and electrolyte secretion and/or decreased fluid absorption. The ability of adhesion of ETEC to intestinal epithelial cells is mainly due to the production of thin (3-7 nm) proteinaceous surface appendages (fimbriae or pili) which can be morphologically, biologically and antigenically different on various strains. The virulence characteristics of ETEC are strongly dependent on the production of adhesins (fimbriae) and enterotoxins. ETEC causing neonatal calf diarrhea possess F5 (K99) as adhesion factor or fimbrae (Bendali *et al.*, 1999).

Salmonella infection in calves is considered as one of the major worldwide problem. Substantial economic losses due to salmonellosis usually results from mortality and poor growth of infected animals as well as transmission potentials to humans (Smith *et al.*, 2004). While it may be convenient to focus on the principal infectious causes of calf diarrhea, notable that this is due to the interaction between a number of related risk factors, (Crouch *et al.*, 2001; Langoni *et al.*, 2004). A number of risk factors are thought to be linked with the etiology of neonatal calf diarrhea including management and environmental factors (Lundborg *et al.*, 2005). All other different risk factors associated with *Salmonella* (Fossler *et al.*, 2005; Davison *et al.*, 2006), and *E. coli* (Schouten *et al.*, 2004; Kuhnert *et al.*, 2005) have been described earlier by several authors . Recently a new multidrug resistant variant *S. typhimurium* DT104 had evolved. It expresses chromosomal resistance to ampicillin, chloramphenicol, tetracycline, sulphonamide, and streptomycin. It was reported to cause epidemics of salmonellosis in humans and animals in the UK, France, and other European countries (Bartels *et al.*, 2010).

The prevalence of ETEC K99 is not reported in Bangladesh. Prevalence of ETEC were reported by different investigators such as 8% (Wani *et al.*, 2013), 9.73% (Wani *et al.*, 2003), 9% (Bandyopadhyay *et al.*, 2011) in India; 10.36% in Egypt (Younis *et al.*, 2009); 5.4% in Korea (Choi and Chae, 1999); 8.33% in China (Cheng *et al.*, 2005); 1.5% in Japan (Yamamoto and Nakazawa, 1997); 5% in Spain (Blanco *et al.*, 1993).

Table 1: Worldwide prevalence of ETEC K99 in diarrhea

Sl. No.	Country	Species	Sample size	Prevalence	Reference
01	Egypt	Diarrheic calf, up to 3 months, feces	127	ETEC- 96(75.6%) 70% *E. coli*serogroups were positive to K99.	El-Seedy *et al.* (2016)
02	Kashmir valley, India	Calf, up to 4 months, feces	70	ETEC-06 (8.6%)	Manzoor *et al.* (2015)
03	Tehran, Iran	Diarrheic calf, Rectal swab	127	ETEC by mPCR-14 (11%). All ETEC were positive to K99 (F5)	Pourtaghi *et al.* (2015)
04	Mymensingh, Bangladesh	Healthy calf, Rectal swab	128	Only *E. coli* – 96(75%)	Ansari *et al.* (2014)
05	Iran	Diarrheic calf, Rectal swab	298	*E. coli* 268 (89.93%) K99 16 (5.3%)	Shams *et al.* (2012)
06	Egypt	Diarrheic calf, Rectal swab	220	*E. coli* -193 (87.73%) K99 - 20 (10.36%)	Younis *et al.* (2009)
07	India	Diarrheic buffalo calf	50	ETEC 10 (20%)	Singh *et al.* (2007)
08	China	Diarrheic calf, Rectal swab	227	K99 -13 (5.7%)	Wang *et al.* (2006)
09	Mozambique, Southeast Africa	Both healthy and Diarrheic calf, up to 6 months, feces	Healthy - 330 Diarrheic- 63	***E. coli*** 55(87.3%) in diarrheic calf 88(26.7%) in healthy calf **K99** 22(40%) in diarrheic calf 14(16%) in healthy calf	Acha *et al.* (2004)
10	France	Diarrheic calf, Rectal swab	3080	ETEC (20.3%)	Bendali *et al.* (1999)

Prevalence of *S. typhimurium* were reported 14.4% in India (Murugkar *et al.*, 2005); 24% in England (Ward et al., 1990). Prevalence of multidrug resistant strain *S. typhimurium* DT104 was reported 15.3% in rat (Yokoyama *et al.*, 2007) and 14.3% of *S. typhimurium* borne diarrhea in calves in Japan (Sameshima *et al.*, 2000).

Table 2: Worldwide prevalence of *S. typhimurium* in diarrhea

SL No	Country	Species	Total sample	Prevalence	References
01	Egypt	Diarrheic calf, up to 3 months, feces	127	*Salmomella* – 23 (18.1%), *S. typhimurium*- 7 (5.5%)	El-Seedy *et al.* (2016)
02	Burkina Faso, West Africa	Healthy slaughtered cattle, feces	304	*Salmomella* – 159 (52.3%), *S. typhimurium*- 3 (0.98% ~ 1%)	Kagambèga *et al.* (2013)
03	Buea, Cameroon, South Africa	Slaughtered cattle, feces and gall bladder fluid	150 Intestinal sample: 100 and gall fluid: 50	Total (both feces and gall fluid): *S. typhimurium*- 43 (28.7%) Intestinal: 67.5% Gall fluid: 32.6%	Akoachere *et al.* (2009)
04	India	Calf, feces	230	*S. typhimurium* – 36 (14.4%)	Murugkar *et al.* (2005)
05	USA	Cattle	20,089	*S. typhimurium* - 1026 (4.9%)	Fossler *et al.* (2005)
06	Ethiopia	Slaughtered cattle, feces	370	*Salmomella* – 7(1.9%) *S. typhimurium*- 5 (1.4%)	Molla *et al.* (2003)
07	Bangladesh	Human stool	139,279	*S. typhimurium*- 72425 (52%)	Rahman *et al.* (2001)

To identify enteric pathogens, laboratory methods include pathogen isolation and characterization along with histopathology considered as the gold standard for etiologic agent and disease confirmation (Popow-Kraupp and Aberle, 2011). However, many enteric pathogens are difficult to isolate from the gastrointestinal environment (Espy *et al.*, 2006). Direct visualization (e.g., light microscopy or electron microscopy) of pathogens in feces or intestinal contents as well as the detection of antigens (e.g., Ag capture ELISA), nucleic acids (e.g., PCR) in specimens are widely accepted as identification methods. A number of diagnostic tests are currently available for detecting ETEC including: Double-antibody enzyme-linked immunosorbent assay (ELISA) (Holley *et al.*, 1984), DNA gene probes specific for genes encoding toxins and adhesions of ETEC (Woodward and Wray, 1990), multiplex polymerase chain reaction (PCR) for the rapid screening of ETEC toxins (Watterworth *et al.*, 2005), and monoclonal antibody-based co-agglutination test (Varshney *et al.*, 2007). On the other hand, *Salmonella* spp. are typically cultured from fecal samples using Samonella-Shigella agar, bismuth sulfite agar, HE medium, brilliant green agar, and XLD agar. Immunological and Molecular techniques can be employed to detect different strains of *Salmonella* spp. (Voetsch *et al.*, 2004).

Multivariable logistic regression was used by other researcher (Younis *et al.*, 2009) to identify the risk factors associated with calf diarrhea. Age, colostrum feeding practice, rotavirus infection, vaccination of pregnant dams with combined vaccine against rotavirus, coronavirus and E. coli (K99), vitamin E and selenium administration to the pregnant dam were the identified risk factors associated with ETEC borne calf diarrhea (Younis *et al.*, 2009). The most consistently identified factor associated with Salmonella presence on dairy farms has been increased herd size, purchase of cattle from dealers, keeping cattle in housing, lack of isolation facilities for sick animals, the presence of other animals in proximity, handling manure, and feeding brewer's products to lactating cows, exposure to wild geese, rodent activity in housing and feed areas and spreading poultry manure on bordering property and purchase of livestock, liver fluke infection and feeding only grass in the summer (Fossler *et al.*, 2005). On the other hand age, hygiene and region/location were found significantly associated with S. typhimurium infection in neonatal calves (Younis *et al.*, 2009).

Chittagong division of Bangladesh is a livestock enriched area. Most of its population relies on livestock farming. Calves are the future asset of the farm. Calf death plays a major impact on livestock economy. Little attention was undertaken to study the epidemiology and risk factors of specific etiology of neonatal calf diarrhea. To the authors' knowledge, molecular screening of bacterial causes of neonatal calf diarrhea and the associated risk factors have not been studied in Bangladesh. There exists no study on molecular screening and risk factor analysis of ETEC K99 and *Salmonella typhimurium* DT 104 in Chittagong. Present study was intended with following objectives:

a. isolation and molecular detection of *ETEC* and *Salmonella typhimurium* DT104 causing calf diarrhea
b. to study comparative pathology of dead calves due to different etiological agents
c. to identify the risk factors associated with different etiology of calf diarrhea

Chapter-2: Review of Literature

2.1. **Calf Diarrhea**

Neonatal calf diarrhea (also known as calf scour) is a major illness worldwide and constitutes major cost in terms of calf mortality, opportunity costs for labor and capital, veterinary costs and loss in calf value. The term neonatal calf diarrhea generally refers to a disease complex characterized by acute, undifferentiated diarrhea in young calves. Calf diarrhea is attributed to both infectious and non-infectious factors (Bartels *et al.*, 2010; Izzo *et al.*, 2011). Multiple enteric pathogens (e.g., viruses, bacteria, and protozoa) are involved in the development of this disease. Co-infection is frequently observed in diarrheic calves although a single primary pathogen can be the cause in some cases. The prevalence of neonatal calf diarrhea can vary by geographical location of the farms, farm management practices, herd size and other risk factors. It is a multifactorial disease where, besides the causative pathogenic agent, calf age, management and environmental factors, may influence the clinical outcome (Snodgrass *et al.*, 1986). A large number of infectious agents have been incriminated as causes of neonatal calf diarrhea, including *Salmonella* and bovine viral diarrhea virus (BVDV). Commonly reported causative pathogens are rotavirus, coronavirus, *Cryptosporidium* spp and enteropathogenic *Escherichia coli* (*E. coli*) (Cho and Yoon, 2014). Enteric colibacillosis is manifested primarily by varying degrees of diarrhoea and dehydration and the outcome may be fatal. The major virulence factors of enteropathogenic strains of *E. coli* in neonatal calf diarrhea are the F5 (K99) adhesion antigen and also the heat-stable enterotoxin (ST) (Bouckenooghe *et al.*, 2002; Kuhnert *et al.*, 2005).

2.2. Infectious etiologies of calf diarrhea

Numerous infectious agents have been involved in calf diarrhea. Ten different enteric pathogens are recognized as either major (BRV, BCoV, BVDV, *Salmonella* spp, *E. coli*, C. perfringens, and C. parvum) or emerging (bovine caliciviruses and BToV) pathogens (Cho and Yoon, 2014).

Bovine rotavirus is a primary etiological agent of calf diarrhea. The virus belongs to the genus Rotavirus within the family Reoviridae. Rotavirus is a non-enveloped virion

possessing 11 double-stranded RNA segments (16~21 kb) and is very stable over a wide pH range with heat lability (Maclachlan and Dubovi, 2011).

Bovine coronavirus is an enveloped virus with a positive-sense, single-stranded RNA genome (27~32 kb). This pathogen is a member (Betacoronavirus 1) of the genus Betacoronavirus that was formerly classified as group 2a coronaviruses (Decaro *et al.*, 2008). Virus infection can present as three distinct clinical syndromes in cattle: a) calf diarrhea in calves at 1 to 2 weeks of age; b) winter dysentery with hemorrhagic diarrhea in adult animals; and c) respiratory diseases including bovine respiratory disease complex in both young and adult cattle (Liu *et al.*, 2006).

Bovine viral diarrhea virus (BVDV) is an enveloped, positive-sense, single-stranded RNA virus (12.3 kb) and a member of the genus Pestivirus in the family Flaviviridae (Flores *et al.*, 2002). There are three species included in the genus: BVDV, border disease virus, and classical swine fever virus. BVDV can be divided into two types (BVDV1 and BVDV2) based on sequence similarity of the 5' untranslated region (UTR) in the viral genome. In addition to these two types, BVDV3 was recently proposed as tentative species together with other Pestivirus species (e.g., border disease virus type 2, Pronghorn, and Bungowannah) (Giangaspero *et al.*, 2013). Each type can be further divided into two biotypes (cytopathic and noncytopathic) based on their ability to cause lytic cytopathic effects in cell culture. Noncytopathic strains of BVDV are responsible for persistent infection of the virus in cattle (Harding *et al.*, 2002).

Bovine torovirus (BTo-V) is an enveloped, positive-stranded, RNA virus (25~30 kb) belonging to the genus Torovirus in the family of Coronaviridae, order Nidovirales along with equine torovirus, porcine torovirus, and human torovirus. Toroviruses are infectious gastrointestinal agents in cattle, and a predominant cause of acute enteric infection in piglets and children (Lodha *et al.*, 2005). Morphological similarities and antigenic cross-reactivity between human and bovine toroviruses has raised a concern about the potential zoonotic nature of BToV. Bovine toroviruses can produce mild to moderate diarrhea in young calves less than 3 weeks of ages (Hoet and Saif, 2004).

Neboviruses belong to the newly established genus Nebovirus in the family Caliciviridae (Carstens, 2010). The viral genome is approximately 7.4 kb in length and contains two ORFs: ORF1 (encoding nonstructural proteins and capsid protein) and ORF2 (encoding small basic proteins with unknown functions). Newbury agent-1 and Nebraska-like bovine calicivirus form two distinct genotypes that were associated with calf diarrhea cases in the UK (1978) and Nebraska, USA (1980), respectively (Oliver *et al.*, 2006).

Salmonella enterica colonizes the gastrointestinal tract of a wide range of hosts. S. *enterica* serovar Typhimurium (*S. typhimurium*) and serovar Dublin (*S. dublin*) are the most common etiologic agents that cause salmonellosis in cattle (Sojka *et al.*, 1977). *S. typhimurium* is the most common serotype that affects calves (Rothenbacher, 1965).

Escherichia coli can be classified into six pathogroups based on virulence scheme. Among them, neonatal calves are most susceptible to Entero Toxigenic E coli (ETEC) infection during first 4 days after birth and develop watery diarrhea if infected (Foster and Smith, 2009).

Clostridium perfringens is a Gram-positive, spore forming anaerobic bacterium that causes a wide range of diseases in mammals and birds. These microorganisms can be subdivided into five toxin types (A, B, C, D, and E) based on the production of four major toxins. Newborn calves which produce a low level of proteolytic enzymes (e.g., trypsin) in the gastrointestinal tract can be easily infected by *C. perfringens* type C since β toxin is recognized as the main virulence factor responsible for clinical signs seen in animals affected by this bacterium. Intestinal lesions in these infected animals are characterized by diffuse or multifocal hemorrhagic necrotizing enteritis and bloody fluid distension (Barker *et al.*, 1993).

Cryptosporidium parvum is a protozoan parasite that is frequently associated with gastrointestinal tract disease in humans and neonatal cattle. Calves infected with *C. parvum* can be asymptomatic or develop severe diarrhea with dehydration. There are approximately 24 species of *Cryptosporidium*. Cattle are commonly infected by *C. parvum, C. bovis, C. ryanae, and C. andersoni*. *C. parvum* is considered to be primary cause of calf diarrhea and is a potential zoonotic agent (Chalmers *et al.*, 2011).

2.3. Calf diarrhea caused by *Escherichia coli*

Among the known intestinal pathogens there are six well-described categories: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Nataro and Kaper, 1998; Kaper *et al.*, 2004). An additional animal pathotype, known as avian pathogenic *E. coli* (APEC), causes extraintestinal infections - primarily respiratory infections, pericarditis, and septicaemia of poultry. The various pathotypes of *E. coli* tend to be clonal groups that are characterized by shared O (lipopolysaccharide, LPS) and H (flagellar) antigens that define serogroups (O antigen only) or serotypes (O and H antigens) (Nataro and Kaper, 1998). Pathogenic *E. coli* strains use a multi-step scheme of pathogenesis that is similar to that used by other mucosal pathogens, which consists of colonization of a mucosal site, evasion of host defences, multiplication and host damage. Most of the pathogenic *E. coli* strains remain extracellular, but EIEC is a true intracellular pathogen that is capable of invading and replicating within epithelial cells and macrophages.

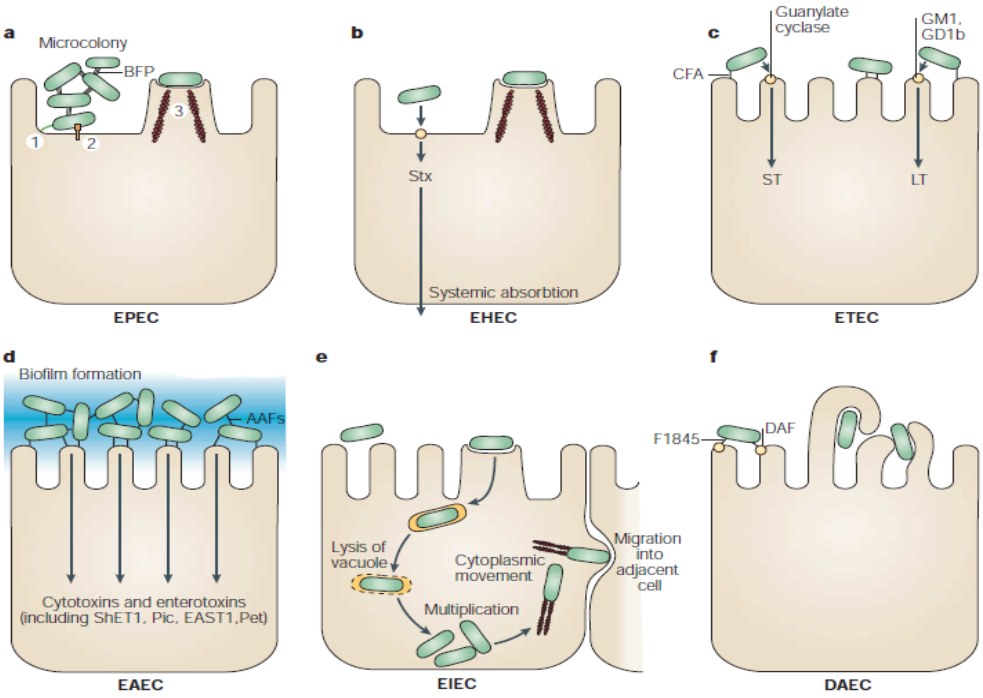

Figure 1: Schematic diagram of diarrhoeagenic *E. coli*

In Figure 1, six recognized categories of diarrhoeagenic *E. coli* each have unique features in their interaction with eukaryotic cells. Here, the interaction of each category with a typical target cell is schematically represented. These descriptions are largely the result of in vitro studies and might not completely reflect the phenomena that occur in infected humans. **a.** EPEC adheres to small bowel enterocytes, but destroy the normal microvillar architecture, inducing the characteristic attaching and effacing lesion. Cytoskeletal derangements are accompanied by an inflammatory response and diarrhoea. i. Initial adhesion, ii. Protein translocation by type III secretion, iii. Pedestal formation. **b.** EHEC also induce the attaching and effacing lesion, but in the colon. The distinguishing feature of EHEC is the elaboration of Shiga toxin (Stx), systemic absorption of which leads to potentially life-threatening complications. **c.** Similarly, ETEC adhere to small bowel enterocytes and induce watery diarrhoea by the secretion of heat-labile (LT) and/or heat-stable (ST) enterotoxins. **d.** EAEC adheres to small and large bowel epithelia in a thick biofilm and elaborates secretory enterotoxins and cytotoxins. **e.** EIEC invades the colonic epithelial cell, lyses the phagosome and moves through the cell by nucleating actin microfilaments. The bacteria might move laterally through the epithelium by direct cell-to-cell spread or might exit and re-enter the baso-lateral plasma membrane. **f.** DAEC elicits a characteristic signal transduction effect in small bowel enterocytes that manifests as the growth of long finger-like cellular projections, which wrap around the bacteria. AAF, aggregative adherence fimbriae; BFP, bundle-forming pilus; CFA, colonization factor antigen; DAF, decay-accelerating factor; EAST1, enteroaggregative *E. coli* ST1; LT, heat-labile enterotoxin; ShET1, Shigella enterotoxin 1; ST, heat-stable enterotoxin. Adapted from Kaper *et al.* (2004).

2.3.1. Virulence genes of ETEC

Epidemiologic studies of both beef and dairy calves have implicated ETEC as the major cause of neonatal diarrhea occurring in the first 4 days of life; however it rarely leads to diarrhea in older calves or adult cattle. Immediately after birth, oral exposure to fecal coliforms leads to colonization of the gut with the normal commensal flora, and these organisms continue to move caudally through the gastrointestinal tract with ingesta. If environmental contamination is high, ETEC organisms are ingested at this same time and are able to produce disease caused by the presence of two virulence factors, K99 fimbria and heat stable toxin. Because nonpathogenic *E. coli* are extremely common, fecal cultures as a diagnostic test are of little value unless the presence of these two virulence factors can be demonstrated (Foster and Smith, 2009).

Like most mucosal pathogens, *E. coli* can be said to follow a requisite strategy of infection: (i) colonization of a mucosal site, (ii) evasion of host defenses, (iii) multiplication, and (iv) host damage. The most highly conserved feature of diarrheagenic *E. coli* strains is their ability to colonize the intestinal mucosal surface despite peristalsis and competition for nutrients by the indigenous flora of the gut (including other *E. coli* strains). The presence of surface adherence fimbriae is a property of virtually all *E. coli* strains, including nonpathogenic varieties. However, diarrheagenic *E. coli* strains possess specific fimbrial antigens that enhance their intestinal colonizing ability and allow adherence to the small bowel mucosa, a site that is not normally colonized (Levine *et al.*, 1984).

ETEC is defined as containing the *E. coli* strains that elaborate at least one member of two defined groups of enterotoxins: ST and LT (Levine, 1987). ETEC strains were first recognized as causes of diarrheal disease in piglets, where the disease continues to cause lethal infection in newborn animals. Studies of ETEC in piglets first elucidated the mechanisms of disease, including the existence of two plasmid encoded enterotoxins. The first descriptions of ETEC in humans reported that certain *E. coli* isolates from the stools of children with diarrhea elicited fluid secretion in ligated rabbit intestinal loops (Nataro and Kaper, 1998).

Bovine enteric colibacillosis is an economically important disease in neonatal calves caused by enterotoxigenic *Escherichia coli* (ETEC), which attach to the intestinal cells using pili. Most ETEC isolated from cattle use the F5 pili (K99) to bind to the enterocytes of the small intestine (Jay *et al.*, 2004). The K99 pili are primarily composed of a repeating 18.2 kDa major subunit, which is responsible for binding to the host glycoprotein receptor, N-glycolyl neuraminic acid-GM3 (NeuGc-GM3). This ganglioside receptor located on the surface of bovine enterocytes is also found on equine red blood cells. A 7.1 kb operon of eight genes (fanA–H) on a plasmid encodes the pilus and other associated proteins responsible for regulation, transport, and assembly of the K99 pili. The pilus, primarily composed of multiples of the fanC gene product, is associated with the fanF product. FanC, a 159 amino acid subunit, is responsible for binding to the ganglioside receptor. Studies with mutated K99 expressed on the *E. coli* surface have shown that the five amino acid region NH2–132-KKDDR-136–COOH, located at the carboxyl terminus of the fanC product, is required for the binding of the pili to the enterocytes (Jacobs *et al.*, 1987).

2.3.2. Pathogenesis and pathology of calf diarrhea caused by ETEC

Infections due to pathogenic *E. coli* may be limited to the mucosal surfaces or can disseminate throughout the body. Three general clinical syndromes result from infection with inherently pathogenic *E. coli* strains: (i) urinary tract infection, (ii) sepsis/meningitis, and (iii) enteric/diarrheal disease. ETEC causes watery diarrhoea, which can range from mild, self-limiting disease to severe purging disease. The organism is an important cause of childhood diarrhoea in the developing world and is the main cause of diarrhoea in travellers to developing countries (Nataro and Kaper, 1998). ETEC colonizes the surface of the small bowel mucosa and elaborates enterotoxins, which give rise to intestinal secretion. Colonization is mediated by one or more proteinaceous fimbrial or fibrillar colonization factors (CFs), which are designated by CFA (colonization factor antigen), CS (coli surface antigen) or PCF (putative colonization factor) followed by a number. More than 20 antigenically diverse CFs have been characterized, yet epidemiological studies indicate that approximately 75% of human ETEC express either CFA/I, CFA/II or CFA/IV51. ETEC enterotoxins belong to one of two groups: the heat-labile enterotoxins

(LTs) and the heat-stable enterotoxins (STs). ETEC strains might express only an LT, only an ST, or both LTs and STs. LTs are a class of enterotoxins that are closely related in structure and function to cholera enterotoxin (CT), which is expressed by Vibrio cholerae (Spangler, 1992). The LT that is found predominantly in human isolates (LT-I; a related protein called LT-II is found in some animal ETEC isolates) has ~80% amino acid identity with CT and, like CT, consists of a single A subunit and five identical B subunits. The B subunits mediate binding of the holotoxin to the cell surface gangliosides GM1 and GD1b, and the A subunit is responsible for the enzymatic activity of the toxin. LT has ADP-ribosyl transferase activity and transfers an ADP-ribosyl moiety from NAD to the α-subunit of the stimulatory G protein - a regulatory protein of the basolateral membrane that regulates adenylate cyclase. The resulting permanent activation of adenylate cyclase leads to increased levels of intracellular cAMP, activation of cAMP-dependent kinases and the eventual activation of the main chloride channel of epithelial cells - the cystic fibrosis transmembrane conductance regulator (CTFR). The net result of CFTR phosphorylation is increased Cl^- secretion from secretory crypt cells, which leads to diarrhea (Sears and Kaper, 1996). LT can also stimulate prostaglandin synthesis and stimulate the enteric nervous system; both of these activities can also lead to stimulation of secretion and inhibition of absorption. LT is also a potent mucosal adjuvant independent of its toxic activity (Pizza *et al.*, 2001).

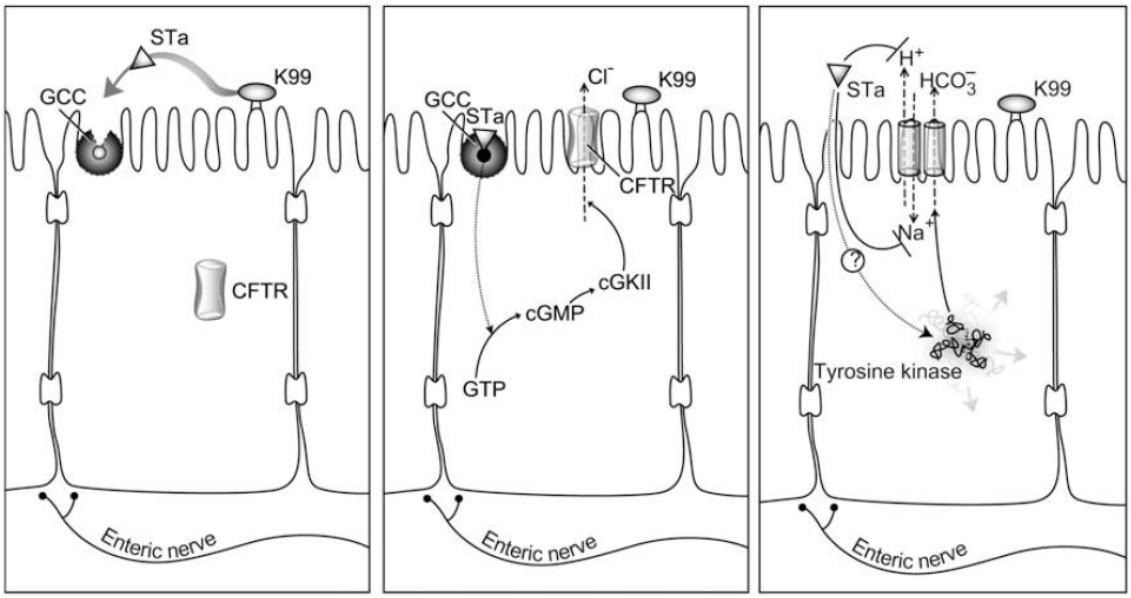

Figure 2: Pathogenesis of diarrhea by K99 ETEC

In Figure 2, Frame 1: K99 ETEC binds to an intestinal epithelial cell, and heat stable toxin (STa) is secreted, which binds to the receptor GCC. The enteric nervous system becomes activated by the secretion of STa, but the mechanism of this activation is unclear. At this point, CFTR is not active. Frame 2: STa binds to GCC, which converts guanylyl triphosphate (GTP) to cGMP. cGMP activates cGKII to phosphorylate the CFTR, and the CFTR moves to the luminal surface and is activated, leading to chloride (Cl) secretion. Frame 3: Secreted STa activates tyrosine kinase through an unknown pathway, which leads to bicarbonate (HCO_3) secretion. STa also directly inhibits the sodium–hydrogen exchanger, decreasing the movement of sodium (Na) and hydrogen (H) across the membrane. Adapted from Kaper *et al.* (2004)

STs are small, single-peptide toxins that include two unrelated classes - STa and STb which differ in both structure and mechanism of action. The main receptor for STa is a membrane-spanning guanylate cyclase; binding of STa to guanylate cyclase stimulates guanylate cyclase activity, leading to increased intracellular cGMP, which, in turn, activates cGMP-dependent and/or cAMP-dependent kinases and, ultimately, increases secretion. Interestingly, intestinal guanylate cyclase is the receptor for an endogenous ligand called guanylin54, which has a similar structure to that of STa. So the ST family seems to represent a case of molecular mimicry. The STb toxin is associated with animal disease and is a 48-amino-acid peptide containing two disulphide bonds. STb can elevate cytosolic Ca^{2+} concentrations, stimulate the release of prostaglandin E_2 and stimulate the

release of serotonin, all of which are mechanisms that could lead to increased ion secretion (Kaper *et al.*, 2004).

2.4. Calf diarrhea caused by *Salmonella* spp.

Salmonella typhimurium is a major cause of calf morbidity and mortality in the United States and in Europe. Furthermore, the infection in calves closely resembles illness caused by *S. typhimurium* in humans and can therefore serve as a model to study diarrheal disease. Calves infected experimentally with *S. typhimurium* develop diarrhea within 48 h. The bacteria invade the intestinal epithelium in the terminal ileum, resulting in exfoliation of epithelial cells and stunting of villi. Bovine enteritis caused by *S. typhimurium* is primarily an enteric infection with mortality resulting from dehydration and intestinal lesions (Tsolis *et al.*, 1999). A second *Salmonella* serotype frequently associated with disease in cattle is *Salmonella dublin* (Tsolis *et al.*, 1999).

However, there are several differences between *S. dublin* and *S. typhimurium* during infection of cattle. While cattle inoculated orally with *S. dublin* develop a systemic infection (Williams Smith and Jones, 1967), *S. typhimurium* causes an infection which is primarily enteric, with only occasional bacteremia. Furthermore, 75% of *S. typhimurium* infections occur in calves less than 2 months of age. In contrast, *S. dublin* is associated at similar frequencies with morbidity in both young and adult cattle. Since differences between *S. dublin* and *S. typhimurium* infections are apparent, it is not clear whether mutations in orthologous genes result in identical virulence defects in both serotypes (Tsolis *et al.*, 1999).

Calves may be infected with a diverse array of *Salmonella* serotypes within hours of birth. The subsequent manifestations of disease are variable, reflecting the balance between host immunity, pathogen dose, and virulence. Neonatal disease outbreaks are frequently observed in calves between 4 and 28 days of age (Mohler *et al.*, 2009).

Among different serotypes, *Salmonella typhimurium* defnitive type 104 (*S. typhimurium* DT104 or DT104) is an increasingly common multiple-antibiotic-resistant strain that has rapidly emerged as a world health problem. Most DT104 strains are characterized by chromosomal resistance to ampicillin (A), chloramphenicol (C), streptomycin (S),

sulfonamides (Su), and tetracycline (T) and are referred to as ACSSuT-type (Khan *et al.*, 2000). Infections, caused by five drug-resistant *S. typhimurium* DT104, were associated with greater morbidity and mortality than other *Salmonella* infections in the UK: 41% of DT104 cases were hospitalized, and 10 (3%) of 295 culture confirmed cases died. An investigator (Glynn *et al.*, 1998) reported a significant increase in the prevalence of multidrug resistant *S. typhimurium* DT104 in the USA (~300,000 cases per year) and described it as a rapidly-emerging pathogen (Rahman *et al.*, 2001).

2.4.1. Virulence factors of Salmonella spp.

The basic mechanism underlying *Salmonella* virulence includes the ability to invade the intestinal mucosa, multiply in lymphoid tissues, and evade host defense systems, leading to systemic disease. For *Salmonella* pathogenesis, the organism should be capable of invading intestinal epithelial cells, surviving within macrophages, and causing enteropathogenicity (Tsolis *et al.*, 1999). *Salmonella* colonizes M-cells, enterocytes, and tonsilar tissues (Reis *et al.*, 2003). Following lymphoid tissue (e.g., tonsilar tissue) infection, *Salmonella* easily spreads throughout the whole body by invading mononuclear cells and phagocytes (Kaper *et al.*, 2004). *Salmonella* pathogenicity island 1 (SPI-1) and SPI-5 are known to influence the type III secretion system, and are mainly responsible for *Salmonella*-induced diarrhea in calves (Kaper *et al.*, 2004). SPI-2 is involved in the second type III secretion system and is responsible for intracellular survival of the organism (Ochman *et al.*, 1996).

The diarrhea associated with salmonellosis is largely believed to be mediated by the inflammatory response to infection. *S. typhimurium* requires a functional type III secretion system encoded by *Salmonella* pathogenicity islands (SPI1 and SPI5) to cause diarrhea. The main function of the invasion-associated type III secretion system is to translocate effector proteins into the cytosol of a host cell. The resulting cell death is associated with a proinflammatory response and influx of neutrophils into the intestinal mucosa. A positive correlation is seen between the severity of histopathological lesions detected in the ileal mucosa and the volume of fluid secretion. Release of endotoxin, prostaglandins, and proinflammatory cytokines (interleukin 1 and tumor necrosis factor a) also promote vascular permeability and hypersecretion. Sloughing off the intestinal

epithelial cells leads to acute hemorrhage, fibrin production, maldigestion, and malabsorption. The resulting hyperosmotic state within the lumen of the intestine draws fluid into the intestinal tract, contributing to a net loss of water, sodium, potassium, and bicarbonate. Mucosal damage also contributes to protein loss and hypoproteinemia. The bovine host-adapted *S. dublin* and some strains of *S. typhimurium* have a virulence plasmid that carries the SpV gene, which promotes the survival of *Salmonella* in macrophages. The ability to survive intracellularly within the reticuloendothelial cells of liver and spleen, lymph nodes, and macrophages contributes to virulence. Other nonadapted serovars may carry the virulence plasmid. However, the virulence plasmid is less common in non-adapted serovars, and their virulence is more variable. Increased virulence has been observed in some antimicrobial resistant strains of *Salmonella*. This is reflected by the finding that calves infected with *S. typhimurium* DT104 are 13 times more likely to die than calves infected with antibiotic-sensitive strains of *S. typhimurium*. The spread of resistance genes by transformation, transduction, and conjugation is well documented in *Salmonella* spp and in the Enterobacteriaceae family (Mohler *et al.*, 2009).

2.4.2. Pathology of calf diarrhea caused by Salmonella spp.

Salmonella infection produces a number of clinical symptoms ranging from asymptomatic to clinical salmonellosis. Acute diarrheal disease is most common with *S. typhimurium* in young calves and systemic disease is associated with *S. dublin* in cattles of all ages. *S. typhimurium* infects the calves usually under 3 months old. Pseudomembrane on the mucosa of the small intestine and enlargement of the mesenteric lymph nodes are frequently observed lesions in affected calves. Infected calf can also function as a potential source of zoonosis through food-borne routes or direct contact. Clinical symptoms of salmonellosis are described by watery and mucoid diarrhea and the presence of fibrin and blood in feces. *Salmonella* can cause diarrhea in both adult cattle and calves. But infection is much more common and fatal in 10-day to 3-month old calves (Fossler *et al.*, 2005). Calves can shed *Salmonella* for variable periods of time and/or intermittently depending on the severity of infection (e.g., clinical or subclinical infection). Acute fibrinopurulent necrotizing enteritis is noticed in both the villi of

terminal ileum and Peyer's patches at necropsy. Histopathological examination discloses destruction of the mucosal epithelium, massive neutrophil infiltration, and depletion of lymphocytes in the germinal centers of intestinal lymphoid follicles (Tsolis *et al.*, 1999).

Peracute salmonellosis often has few or no pathologic findings. Lesions perceived with peracute salmonellosis include pulmonary congestion and submucosal and subserosal petechial hemorrhages of intestines and heart (Bendali *et al.*, 1999). Acute salmonellosis is typically characterized by diffuse catarrhal hemorrhagic enteritis along with diffuse fibrinonecrotic ileotyphlocolitis. The intestinal contents become watery, malodorous. Sometimes feces may contain mucous or whole blood. Inflammation of the gall bladder is common in Salmonellosis. Histopathological evidence of fibrinous cholecystitis is considered pathognomonic for acute enteric salmonellosis in calves. Mesenteric lymph nodes show enlargement, edema, and hemorrhage. Abomasal mucosal erosions may be detected, particularly with *S. dublin* infection. Chronic salmonellosis may result in thickening of the intestinal wall with a yellow-gray necrotic material overlying a red mucosal surface (Mohler *et al.*, 2009).

2.5. Diagnosis of calf diarrhea

Clinical and farm history is not always conclusive in determining the cause of diarrhea. Classical approach includes examining fecal sample are examined by microscopy (for *C. parvum* and Coccidia), bacterial culturing (for *Salmonella* spp., *E. coli*, and *C. perfringens*), and PCR (for BRV and BCoV). In addition, intestinal tissues can be subjected to immunohistochemistry or bacterial culturing. More recently, nucleic acid-based techniques such as PCR and an antigen-capturing enzyme-linked immunosorbent assay (Ag-ELISA) have been used for the rapid detection of various bacterial and viral pathogens in clinical specimens from diarrheic calves (Cho and Yoon, 2014).

Pathogen isolation and characterization along with histopathology for identifying enteric pathogens have typically considered as the gold standard for etiologic agent and disease confirmation (Popow-Kraupp and Aberle, 2011). But a number of enteric pathogens are difficult to recover from the gastrointestinal environment by classical approach (Espy *et al.*, 2006). They can be diagnosed by alternative methods namely direct visualization

(e.g., light microscopy or electron microscopy) of pathogens in feces or intestinal contents and the detection of antigens (e.g., Ag-ELISA) or nucleic acids (e.g., PCR) in specimens. PCR and Ag-ELISA have been widely accepted as reliable diagnostic tools. Diagnostic laboratories usually adopt numerous techniques when testing samples for enteric pathogens. Antigen-capturing ELISA is performed for rapidly detecting a pathogen in a clinical specimen based on antibody (e.g., monoclonal antibody) recognition of the target antigen (Lequin, 2005).

Fecal culturing is a commonly used laboratory method for isolating and identifying bacterial pathogens in fecal swabs and intestinal contents. *Salmonella* spp., *E. coli* K99+, and *C. perfringens* are well known primary bovine enteric pathogens (Larson *et al.*, 2004; Ferrarezi *et al.*, 2008). Several kinds of enrichment medium like nutrient broth, and selective media such as brain heart infusion (BHI) broth (a highly nutritious medium for general bacterial culture) and selenite cysteine broth, Rappaport broth, tetrathionate broth (for *Salmonella* spp.) are employed for growing and identifying different bacterial pathogens. Blood agar is most commonly used to identify fastidious bacteria as well as to get pure growth of any type of bacteria because the majority of bacteria can grow on this medium. MacConkey agar is selectively used to culture Gram-negative bacilli that are commonly present in the gastrointestinal tract and differentiate bacteria that ferment lactose. Large pink colored colony is produced by E. coli on MacConkey agar. Sorbitol-MacConkey agar can help distinguish nonpathogenic *E. coli* from *E. coli* O157:H7 which cannot ferment sorbitol (Farmer and Davis, 1985). E. coli also produces greenish metallic sheen which is indicative of presence. *Salmonella* spp. are typically cultured from fecal samples using Samonell-Shigella agar, bismuth sulfite agar, brilliant green agar, and Xylose Lysine Deoxycholate (XLD) agar (Voetsch *et al.*, 2004). Bacterial culturing is usually done at 37 (*E. coli, Salmonella* etc.) to 45°C (*Camphylobacter* spp.) for overnight (*E. coli, Salmonella* etc.) to 2days (*Camphylobacter* spp.). Incubation can be aerobic (*E. coli, Salmonella etc.*), microaerobic (*Campylobacter* spp.) or anaerobic (*Clostridium perfringens*). Thioglycolate broth growth medium is commonly used for *C. perfringens* culturing. Culturing usually takes 2 days at 36^{o}C under anaerobic conditions. Colony morphology (e.g., color, shape, surface, and elevation of colonies on the agar plates), physical characteristics of the bacteria (e.g., aerobe, anaerobe, or microaerophile),

microscopic features in Gram staining (e.g., rods, cocci, or coccobacilli), and biochemical property tests (e.g., type of fermentation, growth on Triple Suger Iron slant, gelatin or urea utilization; indole, oxidase, or catalase production, etc.) are used to characterize and identify the isolated bacteria in classical techniques. Slow turnaround of the results (growth and identification can take 24∼72 h) is a drawback of bacterial culture tests. This approach cannot always confirms the species and/or strains of bacteria which can be achieved by further immunological testing (e.g., for *E. coli* K99+) (Cho *et al.*, 2010) or serotyping (e.g., for *Salmonella* spp.) of bacteria (Lee *et al.*, 2009), nucleic acid-based assay for typing (e.g., for C. perfringens toxin type) (Gurjar *et al.*, 2008).

For cases of calf diarrhea, the Latex Agglutination test (LAT) has been frequently performed to identify *E. coli* K99+ (Cho *et al.*, 2010). Once the *E. coli* is isolated, the bacterial suspension is mixed with latex beads coated with anti-E coli K99+ antibody and incubated under specific conditions. Agglutination of the latex beads can be clearly visualized when K99 antigen is present in the isolated *E. coli*. It is frequently employed in diagnostic labs because this method can serve as a semi-quantified test and is relatively cheap with rapid turnaround (Golchin *et al.*, 2012). This method is not always appropriate for its lower sensitivity (Polpanich *et al.*, 2007).

Polymerase Chain Reaction (PCR) is a common nucleic acid-based method for detecting enteric pathogens. PCR includes thermocyclic enzymatic amplification of specific DNA sequences of the target pathogen. By using a pair of oligonucleotide primers that represent to DNA/cDNA regions of interest in the genomic sequence PCR is done. Genomic material of the target pathogen is first extracted by following any of the existing techniques. After that the sample is mixed with a heat-stable DNA polymerase (e.g., Taq DNA polymerase), dNTPs, primers, and PCR buffer. Amplification usually proceeds for 25 to 40 cycles in thermal cycler (Erlich and Arnheim, 1992). After completion of the reaction, the PCR products can be visualized on an agarose or acrylamide gel after electrophoresis and staining with ethidium bromide which binds to double-stranded DNA. Successful amplification of the target sequence is determined based on molecular size and/or sequencing of the PCR product. PCR testing is especially useful for detecting viruses that are difficult to isolate in cell culture or bacteria that require a long time to

grow or molecular detection of virulent genes or strains of pathogen (Espy *et al.*, 2006). There are numerous commercial PCR reagents available which provide convenience, high sensitivity, and rapid results. Inadvertent contamination during sampling in the field or processing at the laboratory can be a source of false positive results due to its high sensitivity. Viruses with a high mutation rate, often RNA viruses (e.g., rotavirus and calicivirus), need to be continuously monitored for sequence changes in the target gene otherwise negative results will be obtained due to primer incompatibility. Fecal samples are known to contain factors that inhibit PCR and can lead to false negative results if appropriate reagents or steps to remove such inhibitory substances are not included in the test procedures.

2.6. Public health significance of diarrheic agents of calf

Salmonellosis in humans and other warm-blooded animals is predominantly caused by *Salmonella enterica* subspecies Typhimurium. Over 2600 serovars have been classified based on reactivity of antisera to somatic lipopolysaccharide (O) and flagellar (H) antigens. From a clinical perspective, these may be broadly grouped on the basis of host range and disease presentation. Ubiquitous serovars such as Typhimurium and Enteritidis tend to produce acute but self-limiting enteritis in a wide range of hosts, whereas host-specific serovars are associated with severe systemic disease in healthy outbred adults of a single species that may not involve diarrhoea (e.g. Typhi in humans, Gallinarum in poultry). Host-restricted serovars are primarily associated with systemic disease in one host (e.g. Dublin in cattle, Choleraesuis in pigs), but may cause disease in a limited number of other species. Although it is convenient to classify serovars in this way, the O and H antigens alone do not dictate the outcome of infection. Thus, serovar Typhimurium is typically associated with acute enteritis in humans, cattle and pigs, yet causes typhoid in mice and colonizes the intestines of adult poultry in the absence of clinical signs. Furthermore, while S. *enterica* serovar Typhimurium (*S. typhimurium*) infection of healthy outbred adult birds is largely restricted to the intestinal tract, oral inoculation of newly hatched chicks results in fatal systemic disease (Stevens *et al.*, 2009).

Salmonella is the etiological agent of both human and animal salmonellosis, a very common and widely spread enteric disease. It is a significant cause of acute and chronic

diarrhoea and death in numerous animal species and in human beings (McGavin*et al.*, 2001). Salmonellosis is therefore of significant importance both in animal production and in public health. Although there are *Salmonella* serovars that are strictly host-restricted (such as *Salmonella* Typhi in humans, *S.* Gallinarum and *S.* Pullorum in poultry, and *S. dublin* in cattle), the majority of other *Salmonella* serovars can infect a wide host range. However, faeces of nearly all animal species may be a potential source of *Salmonella*; therefore, the zoonotic transmission of *Salmonella* is not limited to food animals alone. Pets, especially dogs that have close interaction with humans, may be responsible for *Salmonella* transmission (Ojo and Adetosoye, 2009).

Enterotoxigenic *Escherichia coli* (ETEC) were first recognized as causative agents of acute diarrhea in domestic animals. Other investigators have since demonstrated that ETEC are associated with acute diarrhea in man and that ETEC account for a significant proportion of diarrhea cases that were previously undifferentiated with respect to infectious agents. The K88 antigen of swine-specific ETEC, the K99 antigen of cow and sheep-specific ETEC, and the colonization factor antigen (CFA) of human associated ETEC apparently require different receptors for attachment, or adherence, to intestinal epithelial cell surfaces. Nevertheless, K88, K99, and CFA antigens share a number of properties. Each is plasmid mediated and hence can be lost spontaneously upon subculture of clinical isolates in the laboratory (Evans *et al.*, 1977).

2.7. Global Distribution of ETEC and *S. typhimurium*

ETEC is an important cause of calf diarrhea worldwide. There exist a number of studies on prevalence of ETEC in different countries of the world. The prevalence of ETEC K99 in rectal swabs of diarrheic were reported 10.36% in Iran (Younis et al., 2009), 5.3% in China (Wang et al., 2006), 20.3% in France (Bendali et al., 1999). The highest record of ETEC was described 40% in Mozambique (Acha et al., 2004). In neighboring country of Bangladesh, India, reports on ETEC described prevalence as 8.6% and 20% by two investigators (Singh et al., 2007; Manzoor et al., 2015). On the other hand, there exist few reports on *S. typhimurium* in calf diarrhea that isolated from rectal swabs. The highest prevalence was reported as 28.7% in Cameroon (Akoachere et al., 2009) and the lowest as <1% at Burkina Faso (Kagambèga et al., 2013). In Bangladesh, a study on *S.*

typhimurium from human stool reported 52% in hospital case study (Rahman et al., 2001). Unfortunately, there is no existing study on prevalence of ETEC K99 and S. typhuimurium in calf diarrhea in Bangladesh. Most of the researches were centered on detection of toxins liberated by E. coli and detection of Salmonella spp. Table 1 and Table 2 indicates the current pattern of prevalence of ETEC K99 and *Salmonella typhimurium* DT104, respectively throughout the world.

Chapter-3: Materials and Methods

3.1. Study area and period

The study was carried out in selected areas under Chittagong district located in South-Eastern Bangladesh. Its area is 5282.98 sq km, located in between 21°54' and 22°59' north latitudes and in between 91°17' and 92°13' east longitudes. It is bounded by Khagrachhari and Rangamati districts and Tripura state of India on the north, Cox's Bazar district on the south, Bandarban, Rangamati and Khagrachari districts on the east and Noakhali district and the Bay of Bengal on the west. It consists of 14 Upazilla (Administrative locations) and Chittagong Metropolitan Area. This study was conducted in Chittagong Metropolitan Area and Patiya. 200 fecal swabs from below 45 days old calves were collected from June, 2015 to March, 2016.

3.2. Study design and data collection tool

A cross sectional survey design was followed to collect data from the selected farms. A questionnaire (for data collection) was formulated before initiation of the survey. A thorough literature review was done before formulating the questionnaire to gather information about the probable risk factor candidates for *E. coli* and *Salmonella* infection in calves. The draft questionnaire was sent to some selected practitioners working with calf diarrhea and to epidemiologists working with risk factor analysis. The questionnaire was corrected according to the suggestions of the experts. A pilot study was done including a small number of farms around the center of the research station (CVASU) and was modified accordingly. The questionnaire is attached as appendix.

3.3. Selection of study unit

We followed a two stage random sampling procedure was followed to select study subjects. Our study area was Chittagong Metropolitan Area (CMA) and Patiya upazilla and our study unit was individual calf. We used a sampling frame created by others (under Higher Education and Quality Enhancement Project (HEQEP) CP: 3220) for the list of the dairy farms within CMA and Patiya. At initial stage, a random number table was used to draw farms from the sampling frame. In the second stage, all calves within the selected farms were selected for sample collection. Our targeted sample size was 200

randomly from the sampling frame. At the end, we selected 200 calves from 68 randomly selected farms.

3.4. Sample collection

Samples were collected from calves as rectal swab in Chittagong Metropolitan Area and Patiya upazilla. All calves aged less than 45 days were sampled. Rectal swab was with two pre-sterilized cotton swabs and immediately transferred into screw capped test tubes containing Nutrient Broth and Selenite Cystine Broth, respectively. Gloves were changed between calves to reduce the risk of contamination during sampling (Photo attached in Annex-IV: E, F).

3.5. Transportation and preservation of sample

Thermo flask containing ice was used to transport the samples from the collection site to Clinical Pathology Laboratory, CVASU for analysis. Samples were incubated for 24 hours and preserved in 4^0C until prior to culture. Isolated samples, after selective culture and biochemical analysis, were preserved at -20^0C until further analysis.

3.6. Isolation of *Escherichia coli*

The collected samples were brought to the Clinical Pathology Laboratory, CVASU for isolation and identification of bacteria (*E. coli*) using the procedures mentioned below-

Media used

i. Primary enrichment media: nutrient broth (Oxoid Ltd., $P^{H:}$ 7.4±0.2)
ii. Selective media: MacConkey agar (Oxoid Ltd., P^H 7.4±0.2) and EMB agar (Merck, P^H: 7.1±0.2)
iii. Preservation media: Tryptone Soya Broth (Oxoid Ltd., pH 7.3 ± 0.2)

3.6.1. Culture protocol for isolation and identification

Nutrient broth was prepared according to manufacturer's direction (Oxoid, England). The inoculated broths were incubated at 37^oC overnight. Then the broth samples were sub-cultured on MacConkey agar and incubated for another 24 hours at 37^0C. After incubation the bacterial growth was observed. The large pink color colonies were suspected for *E. coli*. Then positive samples were again sub-cultured on EMB agar and

incubated for 24 hours at 37^0C. The growth having characteristic metallic sheen was considered as *E. coli* positive (Photo attached in Annex-IV: G-I). This was further confirmed by Gram's staining and Indole biochemical test as described below -

3.6.2. Gram's staining

Gram's staining was performed as per standard operating procedures (SOP) to determine the size, shape and arrangement of bacteria (Bertu *et al.*, 2010). The suspected colonies were taken over a slide to make a thin smear as achieved by sliding the edge of another glass slide across the glass slide containing the sample prior to air drying. The smear was then heat fixed by quickly passing it two to three times through a flame. After fixation the Gram's staining was done as follows: Crystal violet (primary stain) was used for two minutes, Gram's iodine (mordent) for 1 minute, Acetone (decolorizer) for 5-7 seconds and finally, Safranin (counter stain) for 1 minute. Gently rinsing was done with tape water after every step. The slide was then observed by microscope under 100X with emersion oil and characterization of bacteria was recorded (Photo attached in Annex-IV: J).

3.6.3. Biochemical test

The tube of Tryptone Soya Broth (TSB) was inoculated with a small amount of pure culture and incubated at 37^0C for 24 hours. A positive Indole test is indicated by the formation of a pink to red color ("cherry-red ring") in the reagent layer on top of the medium within seconds of adding the reagent (Photo attached in Annex-IV: K).

3.7. Isolation of *Salmonella* spp.

Media used

I. Primary selective enrichment media: Selenite Cystine Broth (Oxoid Ltd., $P^{H:}$ 7.0±0.2)
II. Selective media: XLD agar (Oxoid Ltd., P^H: 7.4±0.2), Blood Agar (Oxoid Ltd., P^H: 7.3±0.2) and TSI agar (Oxoid Ltd., P^H: 7.2±0.2)

3.7.1. Culture protocol for isolation and identification

The collected samples in Selenite Cystine Broth were brought to the Clinical Pathology laboratory, CVASU for isolation and identification of bacteria (*Salmonella* spp.). For the isolation of *Salmonella*, test tube was incubated for 24 hours at 37^0C. After primary enrichment samples were picked up and streaked on Xylose Lysine Deoxycholate (XLD) agar. The agar plates then were incubated at 37^0C for 24 hours. After development of characteristic black centered colony the positives were selected for biochemical test (TSI stab) to confirm *Salmonella* (Photo attached in Annex-IV: L).

3.7.2. Gram's staining

Gram's staining was performed as described in 3.6.3.

3.7.3. Biochemical test

A straight inoculating needle was used to pick up isolated colony from culture of isolates. The TSI slant was inoculated by stabbing the butt down to the bottom, and then streaked over the surface of the slant (back and forth). The TSI slant was then incubated overnight at temperature of 37^0C. The positive result for *Salmonella* was detected based on the properties provided by (Power and McCuen, 1988) (Photo attached in Annex-IV: M).

3.8. Preservation of the bacterial culture

All positive isolates were inoculated into Tryptone Soya Broth (TSB) (Oxoid, England), incubated overnight at 37^0C and then preserved at -20^0C with 50% glycerol in 1.5 ml eppendorf tubes for future investigation.

3.9. DNA Extraction from bacterial culture for PCR test

The preserved identified isolates using conventional bacteriological culture and biochemical characterization method were removed from the freezer and thawed at room temperature. Then each of the isolates were again streaked onto Blood agar and incubated at 37^0C overnight. For DNA extraction classical boiling method (Queipo-Ortuño *et al.*, 2008) was followed as below:

1) Using a sterile inoculating loop, a loop full of fresh single colony was picked up from each of the plates and transferred to 1.5 ml eppendorf tubes containing 100µl deionized water. The tubes were then vortexed to have a homogenous cell suspension.

2) Using a sterile needle a ventilation hole was made on the lids of each of the eppendorf tubes. The tubes with the cell suspension were then boiled in a water bath at 100^0C for 10 minutes and immediately thereafter cooled by placing them into flaked ice for 10 minutes. This process of boiling and sharp cooling allowed the bacterial cell wall to break down thus releasing DNA.

3) Then the eppendorf tubes with the suspension were centrifuged at 10000 rpm for 2 minutes and the supernatants containing DNA were then collected in the fresh eppendorf tubes and preserved at -20^0C until testing.

3.10. Identification of *E. coli* and K99 by PCR

Primers used to identify *E. coli* and K99 virulent ETEC is enlisted in Table 3. At first the stock solution (100 picomole concentration) of each primer was diluted with molecular grade water to make a 20 picomole concentration to be used for a PCR test. The reaction mixture, with the constituents and their amounts, prepared for detection of *E. coli* and K99 using PCR test is shown in Table 4**.**

Table 3: Oligonucleotide primers used in PCR assay during this study

Primers	Sequence (5´-3´)	G/C content (%)	Melting temp ^{0}C (T_m)	Size of amplified product (bp)	References
ECO-F	GACCTCGGTTTAGTTCACAGA	48	52	585	Wang *et al.* (1996)
ECO-R	CACACGCTGACGCTGACCA	63	50		
K99F	TATTATCTTAGGTGGTATGG	35	45.6	280	Roosendaal *et al.* (1984)
K99R	GGTATCCTTTAGCAGCAGTATTTC	42	54		
InvAF	GTGAAATTATCGCCACGTTCGGGCAA	50	59.5	284	Rahn *et al.* (1992)
InvAR	TCATCGCACCGTCAAAGGAACC	55	56.7		
TyphF	TTGTTCACTTTTTACCCCTGAA	36	49	401	Alvarez *et al.* (2004)
TyphR	CCCTGACAGCCGTTAGATATT	48	53		
104F	ATGCGTTTGGTCTCAACGCC	55	54	164	
104R	GCTGAGGCCACGGATATTTA	50	52		

Table 4: Composition of each reaction mixture for PCR

Ingredients	Amount
Thermo Scientific dream Taq PCR Master Mix (2x) Ready to use	13 µl
Forward Primer	1µl
Reverse Primer	1µl
DNA template	3µl
Deionized water (Nuclease free)	7µl
Total	**25µl**

Amplification (PCR) was performed in a thermal cycler (Applied Biosystem®, 2720). All reactions were carried out in a final volume of 25µl. The cycling conditions used for PCR are shown in Table 5 and 6.

Table 5: PCR cycling conditions used for PCR detection of *ETEC*

Serial No.	Steps	ECO	K99
1	Initial denaturation	94 ^{0}C for 5 minutes	95˚C for 15 minutes
2*	Final denaturation	94 ^{0}C for 30 seconds	95˚C for 30 seconds
	Annealing	53 ^{0}C for 1minute	57˚C for 1 min
	Extension	72 ^{0}C for 1minute	72˚C for 1 min
3	Final extension	72 ^{0}C for 8 minutes	72˚C for 7 min
4	Final holding	4 ^{0}C	4 ^{0}C
5	Cycles of **2***	30	36

Adapted from Roosendaal *et al.* (1984) and Wang *et al.* (1996)

3.11. Identification of S. Typhymurium DT104 by PCR

Primers used to identify *Salmonella* spp., *S. typhimurium* and *S. typhimurium* DT104 is enlisted in Table 3**.** At first the stock solution (100 picomole concentration) of each primers were diluted with molecular grade water to make a 20 picomole concentration to

be used for a PCR run. The reaction mixture, with the constituents and their amounts, prepared for detection of *E. coli* and K99 using PCR test is shown in Table 4.

Amplification (PCR) was performed in a thermal cycler (Applied Biosystem®, 2720) (Photo attached in Annex-IV: N, O). All reactions were carried out in a final volume of 25µl. The cycling conditions used for PCR are shown in Table 6.

Table 6: PCR cycling conditions used for PCR detection of *S. typhimurium* DT104

Serial No.	Steps	InvA	TYPH	DT104
1	Initial denaturation	94°°C for 60sec	95˚C for 15 min	95˚C for 15 min
2*	Final denaturation	94˚C for 60 sec	95˚C for 30 sec	95˚C for 30 sec
	Annealing	64˚C for 30 sec	57˚C for 1 min	57˚C for 1 min
	Extension	72˚C for 30 sec	72˚C for 1 min	72˚C for 1 min
3	Final extension	72 °C for 7 min	72˚C for 7 min	72˚C for 7 min
4	Final holding	4 °C	4 °C	4 °C
5	Cycles of **2***	30	36	36

Adapted from Rahn *et al.* (1992) and Alvarez *et al.* (2004)

3.12. Visualization of PCR Product of *E. coli* through agar gel electrophoresis

To visualize 1.5% agarose gel was made by using 0.75 g agarose powder and 50ml TAE buffer with ethidium bromide. The DNA amplicons were run using 5µl of the final PCR product and 2µl standard 100bp DNA markers (Invitrogen) at 120 V/ 90mA for 30 min. Gels were visualized using a gel documentation system. Positive or negative amplifications were evaluated as presence or absence of visible orange colour bands on agarose gels under UV transilluminator (BDA digital, biometra GmbH, Germany) (Photo attached in Annex-IV: P).

3.12.1. Procedure of agarose gel electrophoresis

1. For 1.5% agarose gel, 500mg of agarose powder and 50 ml of 1X TAE buffer was mixed thoroughly in a conical flask.
2. The mixture was heated in a microwave oven until agarose was completely dissolved.
3. The agarose-TAE buffer solution was then allowed to cool in room temperature.

4. Gel casting tray was prepared by sealing ends of gel chamber with tape or appropriate casting system and placed appropriate number of combs in gel tray.
5. 10µl of ethidium bromide was added to agarose-TAE buffer mixture, shaked well and poured into gel tray.
6. The gel was then allowed to be cool (left for 15-30 minutes at room temperature).
7. The comb(s) were removed and the electrophoresis chamber was filled with 1x TAE buffer untill the casted gel is drowned completely.
8. 4µl of DNA and 2µl of 100bp marker (ladder) were loaded into gel.
9. The electrophoresis was run at 120V and 90mA for 20 minutes.
10. Then the gel was taken to the UV transilluminator for image acquisition and analysis.

3.13. Histopathological investigation

A total of 5 dead calves died from diarrhea were examined at necropsy. Intestinal samples from different region were collected and preserved in Bouin's solution. Histopathology was done for all the samples and stained with routine Haematoxylin and Eosin (H&E) stain. Stained slides were than mounted and examined under light microscope for comparative pathology (Photo attached in Annex-IV: A-D).

3.14. Data Management and Statistical Analysis

Scoring (good, moderate, poor) was applied to some variables; categorized as follows (Details attached in Annex III and photo attached in IV: Q-T):

Hygiene score: Good, Cleaning of floor with disinfectants every day, un-authorized people and vehicle access in farm premises is prohibited, regular washing of udders with antiseptics before milking and washing of milker's hand with antiseptics before milking; moderate, Cleaning of floor with disinfectant once or twice per week (others similar as previous); poor, Cleaning of floor with only water regularly, un-authorized people and vehicle access is not prohibited, regular washing of udders with water before milking, washing of milker's hand with antiseptics before milking.

Drainage system: Good, water runs out perfectly from the pen within 15 minutes during cleaning and rainy season and leaves the pen dry; moderate, water runs out perfectly from the pen within 15 minutes during cleaning but during heavy rain water stays for more

than 15 minutes; poor, water does not run out perfectly within 15 minutes during cleaning or rain and the floor remains wet most the time.

Feed storage: good, locked separate feed store room with proper ventilation; moderate, locked separate feed store room without proper ventilation; poor, no separate feed store room, feed are stored within the burn.

Surrounding environment: Good, at least fifty meter distance from roads and highways/industrial areas and residential areas; moderate, at least fifty meter distance from roads and highways/industrial areas but closed to residential areas; poor, less than fifty meter distance to roads and highways/industrial areas as well as residential areas.

3.15. Statistical analysis

Data from questionnaire and test results from laboratory were entered, collated, coded and stored in Microsoft Excel spreadsheet. For statistical analysis, statistical software STATA version 12.1 (StataCorp LP, College Station, Texas, USA) was used.

Logistic regression analyses with random effects using a generalized linear mixed model were performed. The hierarchy of the data was farm/calf, where the infection (*E. coli* and *Salmonella*) status (positive/negative) was measured for the calf. An initial empty random effect model was set up, including the random effect of farm. Subsequently, univariable analyses were conducted for each of the independent variables (potential risk factors collected in the questionnaires) against the dependent variable (infection status of the calf). This was followed by a multivariable analysis, where the variables that were significant at the level of 30% in the univariable model were included into the multivariable model. We followed a backward selection approach, with excluding variables with the highest P-values first. All variables with a P-value <0.05 were included in the final model based on an F test. With each variable included in the model, the changes in the coefficients of the significant variables were checked, and, if this resulted in more than 20% change in parameter estimates, the variable was retained in the model to account for its confounding effect. All variables in the final model were checked for possible interactions.

Chapter-4: Results

The study was carried out in selected farms under Chittagong Metropolitan area and Patiya upazila of Chittagong to know the prevalence of *E. coli* K99 and *S. typhimurium* DT104 in the fecal swabs of calves. A total of 200 samples were collected as rectal swab from calves aged less than 40 days of 68 randomly selected farms. Both farm data and individual calf data were collected by developing standard questionnaire (Annex-II).

4.1. Prevalence of *Escherichia coli* and ETEC K99+

Among 200 samples 126 (63%) were found positive for *E. coli* and 19 (9.5%) were found ETEC carrying K99+ gene by PCR. Highest prevalence of *E. coli* was found in Chawkbazar (90.91%) followed by Karnafuly (81.82%), Doublemooring (75%), Bakalia (69.57%), Halishahar (66.67%), Bayezid (50%), Kotwali (50%), Patenga (38.89%) and Khulshi (0%). In case of ETEC K99+ serotype, highest prevalence was found in Bakalia (30.43%) followed by Halishahar (16.67%), Kotwali (16.67%), Patiya (12.12%), Chawkbazar (9.09%), Karnafuly (9.09%), Bayezid (5.56%) and Chandgaon (4.69%). No isolates were found positive for K99 in Patenga, Khulshi and Doublemooring. There was no significant difference ($p>0.05$) was found among different locations for the prevalence of *E. coli* and ETEC K99+ serotype (Table 7).

Prevalence of E. coli and ETEC K99 is presented in Table 8. It states that 62.24% and 13.27% male calves were carrying *E. coli* and ETEC K99+, respectively whereas it was 63.73% and 5.88% in female calves. No significant difference between two sexes was found for both factors.

For herd size, prevalence of *E. coli* showed no significant difference but there was found statistically significant difference for prevalence of ETEC K99+. Prevalence of *E. coli* was found highest (82.14%) in small farms (11-20 heads) and lowest (54.43%) in large farms (>50 heads). But for K99+ serotype highest (42.86%) prevalence was found in very small farms (<10heads) and lowest (2.53%) in large farms (>50heads) which was demonstrated in Table 9.

4.2. Prevalence of *Salmonella typhimurium* DT104

Table 7 shows that prevalence of *Salmonella* spp., *S. typhimurium* and *S. typhimurium* DT104 were 20 (10%), 8 (4%) and 2 (1%), respectively. Highest prevalence of *Salmonella* spp. was found in Chawkbazar (63.64%) followed by Kotwali (33.33%), Patiya (15.15%), Bakalia (8.7%), Bayezid (5.56%), Chandgaon (4.69%). No samples were found positive in Khulshi, Doublemooring, Patenga, Halishahar, Karnafuly. *S. typhimurium* was found positive in the samples from Bayezid (5.56%), Chawkbazar (27.27%) and Patiya (12.12%) only. There was found statistically significant difference ($p<0.05$) in prevalence of *Salmonella* spp. and *S. typhimurium* for different locations in Chittagong Metropolitan Area and Patiya. *S. typhimurium* DT104 was prevalent only in Patiya (6.06%).

Prevalence of *Salmonella* spp., *S. typhimurium* and *S. typhimurium* DT104 in male calves were 11.22, 5.10 and 2.04%, respectively which were 8.82, 2.94 and 0%, respectively in female calves. The prevalence was found statistically insignificant in different sex as presented in Table 8.

Prevalence of *S*almonella spp., *S. typhimurium* and *S. typhimurium* DT104 very small farm (<10 heads) was 0% but it was 10.71, 3.57 and 3.57%, respectively in small farms (11-20 heads). The prevalence of *Salmonella* spp., *S. typhimurium* and *S. typhimurium* DT104 were found 17.44, 5.81and 0%, respectively in medium farms (21-50 heads) which were 2.53, 2.53 and 1.27%, respectively in large farms (>50 heads). Statistically significant difference was found for the prevalence of *S.* spp. in different farm sizes only (Table 9).

Table 7: Prevalence of virulent gene carrying *Salmonella* spp. and *E. coli* in different locations

Location	n	*Salmonella* spp.	*S. typhimurium*	*S. typhimurium* DT 104	*E. coli*	*E. coli* K99
		No (%)	No (%)	No (%)	No (%)	No (%)
Bakalia	23	2 (8.70)	0 (0)	0 (0)	16 (69.57)	7 (30.43)
Bayezid	18	1 (5.56)	1 (5.56)	0 (0)	9 (50)	1 (5.56)
Chandgaon	64	3 (4.69)	0 (0)	0 (0)	43 (67.19)	3 (4.69)
Chawkbazar	11	7 (63.64)	3 (27.27)	0 (0)	10 (90.91)	1 (9.09)
Doublemooring	4	0 (0)	0 (0)	0 (0)	3 (75)	0 (0)
Halishohor	6	0 (0)	0 (0)	0 (0)	4 (66.67)	1 (16.67)
Khulshi	6	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Karnafuly	11	0 (0)	0 (0)	0 (0)	9 (81.82)	1 (9.09)
Kotowali	6	2 (33.33)	0 (0)	0 (0)	3 (50)	1 (16.67)
Patenga	18	0 (0)	0 (0)	0 (0)	7 (38.89)	0 (0)
Patiya	33	5 (15.15)	4 (12.12)	2 (6.06)	22 (66.67)	4 (12.12)
Total	200	20 (10)	8 (4)	2 (1)	126 (63)	19 (9.5)
p-value		0.00*	0.003*	0.421	0.01*	0.06

S. = Salmonella, $p<0.05$ = Significant

Table 8: Prevalence of virulent gene carrying *Salmonella* spp. and *E. coli* in different sex

Prevalence of Salmonella spp. S. typhimurium DT104, ETEC K99 in both sexes is presented. There was found no statistically significant variation in prevalence of Salmonella spp., S. typhimurium DT104, ETEC K99 in both sexes.

Sex	**N**	***Salmonella* spp.**	***S. typhimurium***	***S. typhimurium* DT 104**	***E. coli***	***E. coli* K99+**
		No (%)	No (%)	No (%)	No (%)	No (%)
Male	98	11 (11.22)	5 (5.10)	2 (2.04)	61 (62.24)	13 (13.27)
Female	102	9 (8.82)	3 (2.94)	0 (0)	65 (63.73)	6 (5.88)
Total	200	20 (10)	8 (4)	2 (1)	126 (63)	19 (9.5)
p-value		0.572	0.436	0.147	0.828	0.075

S. = Salmonella, p<0.05 = Significant

Table 9: Prevalence of virulent gene carrying *Salmonella* spp. and *E. coli* according to herd size

Prevalence of Salmonella spp. S. typhimurium DT104, ETEC K99 in different herd sizes is shown. There was found statistically significant variation in prevalence of Salmonella spp. and ETEC K99 for herd sizes.

Herd size	**N=200**	***Salmonella* spp.**	***S. typhimurium***	***S. typhimurium* DT 104**	***E. coli***	***E. coli* K99+**
		No (%)	No (%)	No (%)	No (%)	No (%)
<10	7	0 (0)	0 (0)	0 (0)	5 (71.43)	3 (42.86)
11-20	28	3 (10.71)	1 (3.57)	1 (3.57)	23 (82.14)	7 (25)
21-50	86	15 (17.44)	5 (5.81)	0 (0)	55 (63.95)	7 (8.14)
>50	79	2 (2.53)	2 (2.53)	1 (1.27)	43 (54.43)	2 (2.53)
Total	200	20 (10)	8 (4)	2 (1)	126 (63)	19 (9.5)
p-value		0.012*	0.686	0.413	0.068	0.0*

S. = Salmonella, p<0.05 = Significant, <10 = Very small farm, 11-20=Small farm, 21-50= Medium farm, >50= large farm

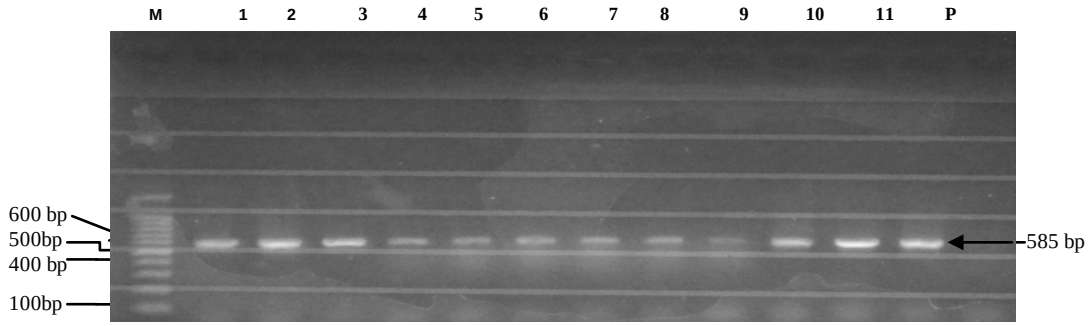

Figure 3: PCR amplification of ECO gene

The PCR amplification of *ECO* gene of *E. coli* showing positive amplicons at 585 bp on 1.5% agarose gel with ethidium bromide; M: DNA size marker (100–1000 bp), Lane P: Positive control, Lane (1-11): Conventional bacterial culture positive isolates

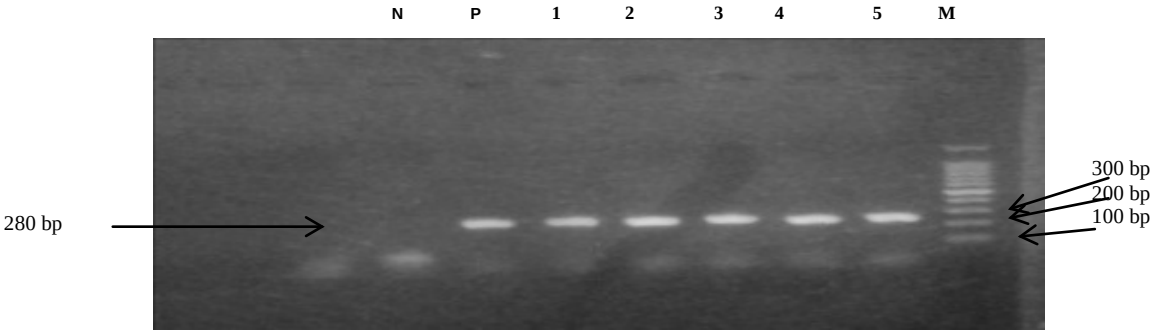

Figure 4: PCR amplification of K99 gene

The PCR amplification of *K99 virulent* gene of *E. coli* showing positive amplicons at 280 bp on 1.5% agarose gel with ethidium bromide; M: DNA size marker (100–1000 bp), Lane P: Positive control, Lane N: Negative control, Lane (1-5): *ECO* gene positive isolates

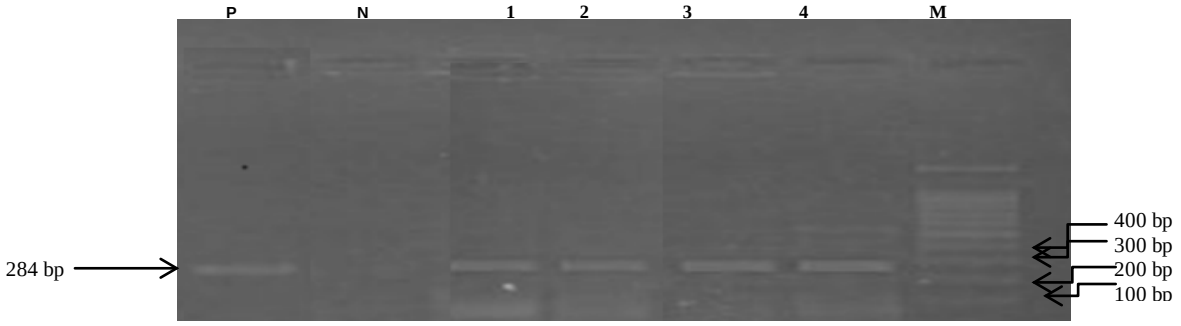

Figure 5: PCR amplification of InvA gene

The PCR amplification of *InvA* gene of *Salmonella* showing positive amplicons at 284 bp on 1.5% agarose gel with ethidium bromide; M: DNA size marker (100–1000 bp), Lane P: Positive control, Lane N: Negative control, Lane (1-4): Conventional bacterial culture positive isolates

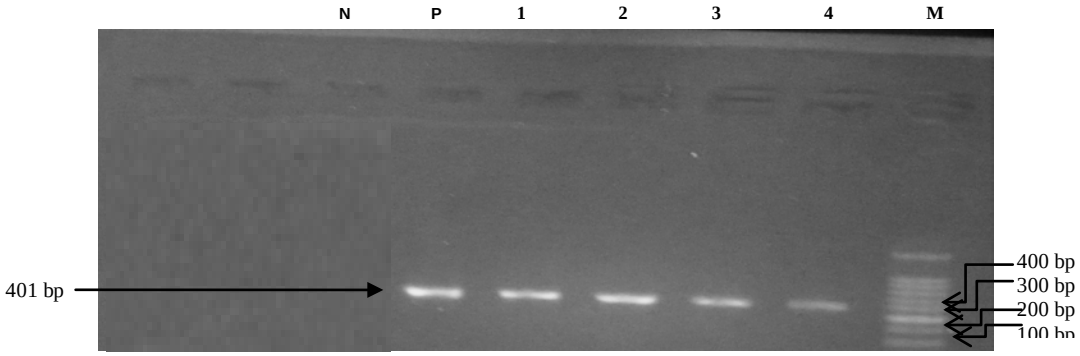

Figure 6: PCR amplification of TYPH gene

The PCR amplification of *TYPH virulent* gene of *Salmonella typhimurium* showing positive amplicons at 401 bp on 1.5% agarose gel with ethidium bromide; M: DNA size marker (100–1000 bp), Lane P: Positive control, Lane N: Negative control, Lane (1-4): *InvA* gene positive isolates

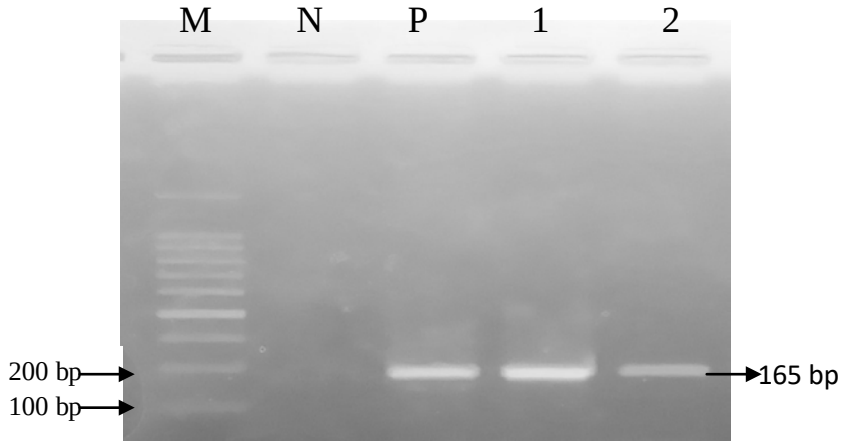

Figure 7: PCR amplification of 104 gene

The PCR amplification of *104* resistance gene of *Salmonella typhimurium DT104* showing positive amplicons at 165 bp on 1.5% agarose gel with ethidium bromide; M: DNA size marker (100–1000 bp), Lane P: Positive control, Lane N: Negative control, Lane (1-2): *TYPH* gene positive isolates

4.3. Comparative pathology of *E. coli* K99 and *S. typhimurium* induced diarrhea

Five dead calves with history of diarrhea aged less than 30 days were examined at necropsy and samples were collected for histopathology. Two of them were found positive for ETEC K99 and one was isolated for mixed infection with *E. coli* and rotavirus in confirmatory molecular identification. No dead animal was found positive for *Salmonella typhimurium*. Villous atrophy was found in all five cases. Gross changes observed in all cases were watery intestinal contents with bad odor, mucus and blood (in two cases), dilatation of intestinal lumen (Photo attached in Annex-IV: A-C). No gross

abnormality was observed in other organs. Thickening of intestinal epithelium, sloughing off epithelium, congestion in lamina propria, infiltration of reactive cells were the findings of bacterial diarrhea (Figure 8). In case of rotaviral diarrhea there was fragmentation of lymphocytes in the pear's patches, increased lymphoblasts in germinal centers, intracytoplasmic inclusion bodies in lymphocytes and enterocytes, syncytia formation in mesenteric lympnodes and Pyer's patches was recorded. In the case of mixed infection there was seen congestion and haemorrhage in submucosa along with intracytoplasmic inclusion bodies.

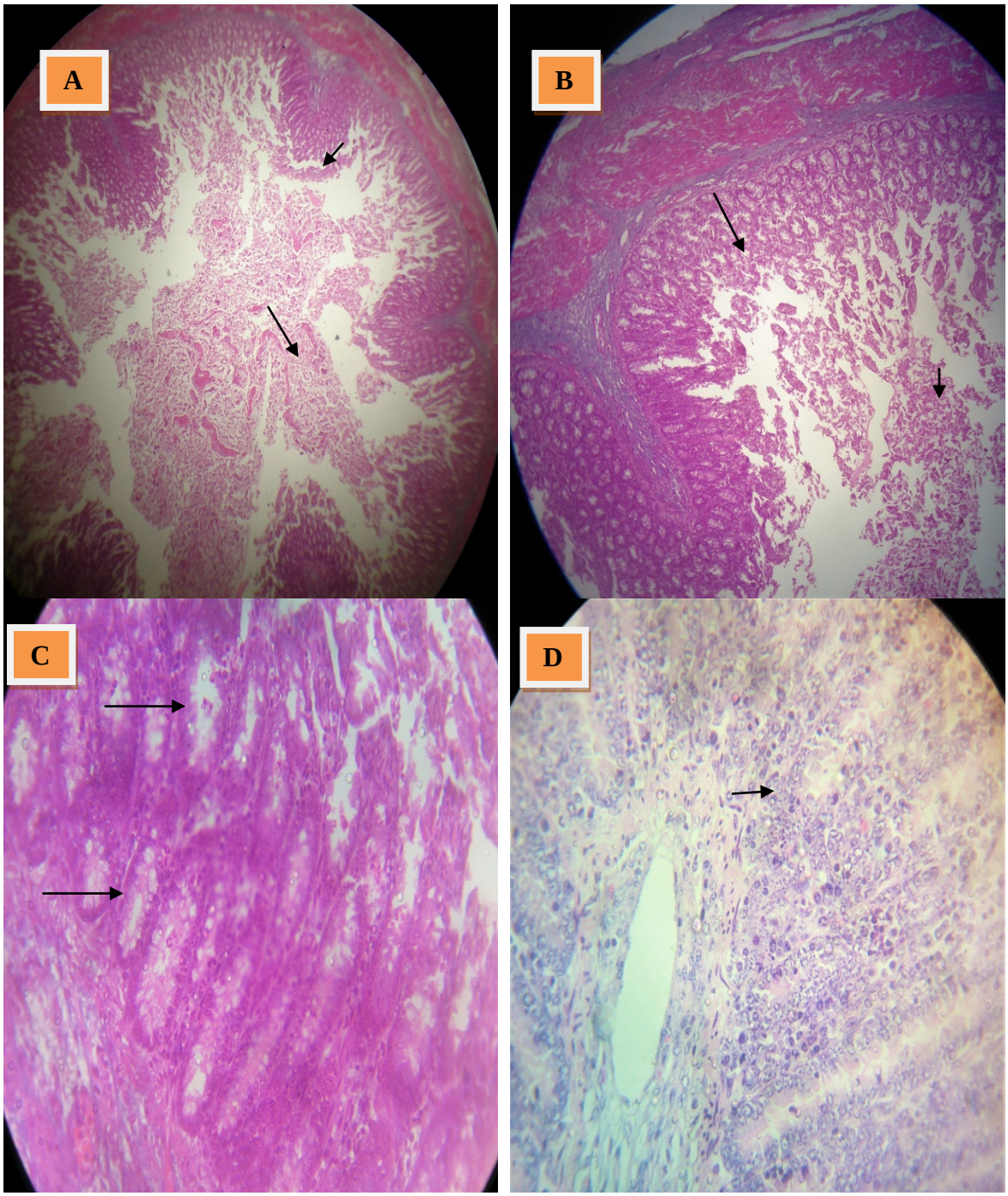

Figure 8: Pathological findings in diarrheic calves

A & B. Atrophy of intestinal villi (large arrow) and sloughing off mucosa (small arrow) (4X & 10X); C & D. Increased goblet cells (large arrow), mononuclear cellular infiltration (small arrow) (40X & 100X)

4.4. Multilevel logistic regression model with presence of *E. coli*

To know the association of different farm and animal level factors with the presence of *E. coli*, a multilevel univariable regression model was performed keeping farm as random effect. In univariate analysis, the following factors were significantly ($p<0.3$) associated with presence of *E. coli*: herd size, population density, maximum age difference ($p <0.05$), fecal consistency ($p = 0.03$), degree of dehydration ($p = 0.05$), body weight, introduction of new calf, sharing same barn with other animals, sucking as feeding regime, farmers' education.

Table 10: Multilevel univariable logistic regression model (farm as random effect) showing the significant ($p<0.3$) association of different farm and animal level factors with the presence of *E. coli* in the sample

Variable	Level	OR	CI	P-value
Herd size	>50	1		0.15
	21-50	1.19	0.41-3.47	
	5-20	3.73	0.94-14.77	
Population density	Continuous variable	1.01	0.99-1.03	0.17
Maximum age difference	<4 wks	1		0.002
	4-8 wks	0.55	0.19-1.57	
	>8 wks	3.48	1.03-11.75	
Fecal consistency	Solid	1		0.03
	Semi-solid	1.30	0.45-3.73	
	Liquid	0.33	0.12-0.91	
Dehydration	Within 2 sec	1		0.05
	2-6 sec	0.65	0.28-1.49	
	>6 sec	0.07	0.007-0.64	
Body weight (kg)	41-55	1		0.16
	26-40	2.27	0.88-5.84	
	15-25	1.18	0.35-3.91	
New calf	No	1		0.16
	Yes	2.20	0.71-6.77	

Other animal	No	1		0.10
	Yes	2.92	0.81-2.34	
Sucking as feeding regime	No	1		0.23
	Yes	2.1	0.61-7.60	
Farmer's education	No to primary	1		0.22
	Eight to HSC	1.83	0.38-8.65	
	Graduate	0.76	0.15-3.68	

Results of the full and reduced (only including factors identified as significant using the backwards stepwise univariable regression) models is presented in Table 10. In the reduced model, several factors were associated with presence of *E. coli*: having 21-50 heads in the farm (OR = 1.19, 95% CI: 0.41, 3.47) or 5-20 heads (OR = 3.73, 95% CI: 0.94, 14.77) versus >50 heads in the farm; population density (OR = 1.01, 95% CI: 0.99, 1.03); 4-8 weeks age (OR = 0.55, 95% CI: 0.19, 1.57) or >8 weeks age (OR = 3.48, 95% CI: 1.03, 11.75) versus <4 weeks age; Body weight (BW) 26-40 Kg (OR = 2.27, 95% CI: 0.88, 5.84) or BW 15-25 Kg (OR = 1.18, 95% CI: 0.35, 3.91) versus BW 41-55 Kg; introduction of new calf (OR = 2.20, 95% CI: 0.71, 6.77) versus no new introduction; coexisting of other species (OR = 2.92, 95% CI: 0.81, 2.34) versus no coexistence of other animal in same house; sucking as feeding regime (OR = 2.10, 95% CI: 0.61, 7.60) versus other systems as feeding with sucking; class eight to HSC educated farmers (OR = 1.83, 95% CI: 0.38, 8.65) versus illiterate farmers. Surprisingly, semisolid feces (OR = 1.30, 95% CI: 0.45, 3.73) had more odd of having *E. coli* than liquid feces; skin fold retention time within 2 sec had more odd of getting *E. coli* than time 2-6 sec or >6 sec.

Table 11: Final multilevel multivariable logistic regression model (farm as random effect) showing the significant (p<0.05) association of different farm and animal level factors with the presence of *E. coli* in the sample

Variable	Level	OR	95% CI	P-value
Herd size	>50	1		
	21-50	0.68	0.26-1.76	0.43
	5-20	3.54	1.02-12.78	**0.04**
Population density	Continuous variable	1.02	1.00-1.05	**0.01**
Maximum age difference in a pen	<4 wks	1		
	4-8 wks	0.51	0.18-1.45	0.21
	>8 wks	5.51	1.59-19.04	**0.007**
Dehydration	Within 2 sec	1		
	2-6 sec	0.51	0.23-1.19	**0.09**
	>6 sec	0.07	0.01-0.52	**0.009**
Other animal	No	1		
	Yes	3.24	1.12-9.34	**0.02**
Sucking as feeding regime	No	1		
	Yes	5.07	1.43-17.16	**0.01**

In multilevel multivariable reduced model, several factors were associated with presence of E. coli (Table 11). In this model significantly associated factors include: having 21-50 heads in the farm (OR = 0.68, 95% CI: 0.26, 1.76) or 5-20 heads (OR = 3.54, 95% CI: 1.02, 12.78) versus >50 heads in the farm; population density (OR = 1.02, 95% CI: 1.00, 1.05); 4-8 weeks age (OR = 0.51, 95% CI: 0.18, 1.45) or >8 weeks age (OR = 5.51, 95% CI: 1.59, 19.04) versus <4 weeks age; coexistence of other species (OR = 3.24, 95% CI: 1.12, 9.34) versus no coexistence of other animal in same house; sucking as feeding regime (OR = 5.07, 95% CI: 1.43, 17.16) versus other systems as feeding with sucking. Cutaneous fold reduction time within 2 sec had more odds of having E. coli than time 2-6 or >6 sec.

4.5. Multilevel logistic regression model with presence of *Salmonella* spp.

A multilevel regression model was performed keeping farm as random effect to know the association of different farm and animal level factors with the presence of *Salmonella* spp. In multilevel univariable analysis, the following factors were significantly ($p<0.3$) associated with presence of *Salmonella* spp.: herd size, age, drinking water supply, Therapy, body weight, introduction of new calf, sucking as feeding regime, farmers' education.

Table 12: Multilevel univariable logistic regression model (farm as random effect) showing the significant ($p<0.3$) association of different farm and animal level factors with the presence of *Salmonella* in the sample.

Variable	Level	OR	CI	P-value
Herd size	>50	1		0.18
	21-50	8.84	0.85-91.31	
	5-20	4.73	0.33-66.70	
Age (days)	Continuous variable	0.95	0.90-1.01	0.11
Drinking water supply	Deep well	1		0.07
	Surface water	2.19	0.18-26.32	
	Municipal supply	21.41	1.49-307-387	
Therapy	Antibiotics	1		0.08
	Other	2.14	0.09-49.83	
	None	6.96	1.17-41.16	
Body weight (kg)	41-55	1		0.19
	26-40	1.05	0.19-5.83	
	15-25	4.08	0.55-29.83	
New calf	No	1		0.08
	Yes	0.06	0.002-1.43	
Sucking as feeding regime	No	1		0.05
	Yes	0.11	0.01-1.08	

Farmer's education	No to primary	1		0.16
	Eight to HSC	0.08	0.004-1.63	
	Graduate	0.05	0.002-1.12	

Results of the full and reduced (only including factors identified as significant using the backwards stepwise univariable regression) models is presented in Table 12. In the univariable regression model, several factors were associated with presence of *Salmonella* spp.: having 21-50 heads in the farm (OR = 8.84, 95% CI: 0.85, 91.31) or 5-20 heads (OR = 4.73, 95% CI: 0.33, 66.70) versus >50 heads in the farm; age (OR = 0.95, 95% CI: 0.90, 1.01); Surface water (OR = 2.19, 95% CI: 0.18-26.32) or municipal supply (OR = 21.41, 95% CI: 1.49, 307-387) versus tubewell water supply; other therapies except antibiotics (OR = 2.14, 95% CI: 0.09, 49.83) or no therapy (OR = 6.96, 95% CI: 1.17, 41.16) versus antibiotic therapy; Body weight (BW) 26-40 Kg (OR = 1.05, 95% CI: 0.19, 5.83) or BW 15-25 Kg (OR = 4.08, 95% CI: 0.55, 29.83) versus BW 41-55 Kg; introduction of new calf (OR = 0.06, 95% CI: 0.002, 1.43) versus no new introduction; sucking as feeding regime (OR = 0.11, 95% CI: 0.01, 1.08) versus other systems as feeding with sucking; class eight to HSC educated farmers (OR = 0.08, 95% CI: 0.004-1.63) versus illiterate farmers.

Table 13: Final multilevel multivariable logistic regression model (farm as random effect) showing the significant (p<0.05) association of different farm and animal level factors with the presence of *Salmonella* in the sample

Variable	Level	OR	95% CI	P-value
Farmer's education	No to primary	1		
	Eight to HSC	0.16	0.03-0.81	**0.02**
	Graduate	0.14	0.03-0.70	**0.01**
Drinking water supply	Deep well	1		
	Surface water	6.00	0.78-46.09	0.08
	Municipal supply	32.28	5.76-180.94	**0.0001**
Therapy	Antibiotics	1		
	Other	2.35	0.10-54.30	0.59
	None	7.03	1.30-37.81	**0.02**
Introduction of new calf	No	1		
	Yes	0.09	0.01-0.90	**0.04**

In multilevel multivariable regression model, several factors were associated with presence of *Salmonella* spp. (Table 13). In this model significantly associated factors include: class eight to HSC educated farmers (OR = 0.16, 95% CI: 0.03, 0.81) or graduate farmers (OR = 0.14, 95% CI: 0.03-0.70) versus illiterate farmers; Surface water (OR = 6.00, 95% CI: 0.78-46.09) or municipal supply (OR = 32.28, 95% CI: 5.76-180.94) versus tubewell water supply; other therapies except antibiotics (OR = 2.35, 95% CI: 0.10, 54.30) or no therapy (OR = 7.03, 95% CI: 1.30, 37.81) versus antibiotic therapy; introduction of new calf (OR = 0.09, 95% CI: 0.01, 0.90) versus no new introduction.

Chapter-5: Discussion

Neonatal calf diarrhea remains as one of the most important problems faced by livestock, causing great economic losses. Calves are at greatest risk of diarrhea within the first month of life and the incidence of diarrhea decreases with age (Garcia *et al.*, 2000). Intent of this study was to describe the prevalence and risk factors associated with ETEC K99 and *Salmonella* spp. infections in neonatal diarrheic calves. Particular strengths of this study included the large number of herds, the diversity of farm sizes sampled, and the longitudinal sampling approach spanning all months of the year. Conventional herds were randomly selected among those eligible for inclusion so that participating herds represented a diversity of management characteristics of herds within the sampling frame. A greater percentage of operations with over 30 cows and a lesser percentage of operations with fewer than 30 cows participated in this study compared to percentages of like-sized operations present within the respective area. Thus, the farms selected for this study cannot be seen as representative of the underlying distribution of operations by herd sizes in the respective states. This study used the generalized estimating equations approach for analysis of risk factors, which is increasingly being used in epidemiology and clinical science for analysis of correlated data. This approach allows for multi-variable analysis of longitudinal or other correlated data with binary outcomes and provides robust standard errors while adjusting for herd effects.

5.1. Prevalence of *E. coli* K99

Since ETEC infection is the most common type of colibacillosis in young animals such as calves (Nataro and Kaper, 1998), detailed studies of the virulence and risk factors of ETEC in calves are needed. Although colibacillosis in calves is a common infection, any molecular screening of virulence factors and the associated risk factors have not been done, to date, in Chittagong, nor in the south east Bangladesh. The prevalence of K99 gene was 19%. A similar result (20.3%) was reported by Bendali *et al.* (1999); however, a higher prevalence was reported by Acha *et al.* (2004) who reported a prevalence rate of 40% (Acha *et al.*, 2004), on the contrary, a lower prevalence (0.57, 16 and 10.3%) was recorded by other investigators (Salvadori *et al.*, 2003; Wang *et al.*, 2006; Zhang *et al.*, 2007), respectively.

In India, PCR could identify similar prevalence (20%) in buffalo calves (Singh *et al.*, 2007). On the contrary, lower prevalence of ETEC K99 4.7%, 3.86% and 5.8% were recorded by Akam *et al.* (2004), Kanwar *et al.* (2007) and Oliveira Filho *et al.* (2007), respectively. In a similar study carried out in Egypt and Israel, higher prevalence (23%) was also recorded (Perk *et al.*, 2000). The differences in the prevalence from those previously recorded may be due to variations in region, management conditions and hygienic measures. Present study revealed two cases have mixed infection by S. *enterica* serovar Typhimurium and ETEC K99, and one case has mixed with rotavirus (from other study) and ETEC K99. Diarrhea caused by ETEC is considered the main infectious disease of newborn calves (Martín *et al.*, 2003); however, bovine *E. coli* F5 (K99) seemed to be of minor importance in the investigated population compared with cryptosporidiosis and rotavirus infection (Younis *et al.*, 2009). The similarity in the prevalence from those previously (Acha *et al.*, 2004) indicated that enterotoxin STa was not detected in any *E. coli* K99 isolates from the diarrheal calves. Pili expression provides the ETEC strains a capacity to attach to the intestinal epithelium, which leads to colonization, multiplication and toxin production to cause diarrhea observed in colibacillosis. In animals, although this disease is preventable if sufficient anti-pili antibodies are taken orally in the colostrum by the animals within the first 24 h of birth, it has been suggested that competitive inhibition of attachment of bacteria to the enterocytes can cause a similar effect. Oral inoculation of a non-ETEC but possessing the attachment pili K88 decreased the intestinal fluid accumulation on challenge with the K88 ETEC strain (Jay *et al.*, 2004). Similarly, other investigator (Mouricout *et al.*, 1990) demonstrated that a receptor mimicking blood plasma derived glycan inhibits the adhesion of K99 ETEC strain to entero enterocytes at least two-fold and thus may use to prevent colibacillosis. K99 pili is a filamentous structure composed of multiple ~18 kDa subunits that allow some ETEC strains to attach to the small intestine epithelium of calves, lambs and piglets. Purified K99 pili proteins have been used in inhibition assays to demonstrate the presence of pili specific receptors on enterocytes (Jay *et al.*, 2004).

5.2. Prevalence of *Salmonella typhimurium*

Salmonella spp. was isolated from twenty calves (10%); eight of them were typed as S. *enterica* serovar Typhimurium and two of them were S. *enterica* serovar Typhimurium DT104, whereas 12 cases were non- *S. enterica* serovar Typhimurium. Two cases have mixed infection by *S. enterica* serovar Typhimurium and ETEC K99. During this study, the prevalence of *Salmonella* spp. was found to be higher than that previously reported by other investigators includes 2% (Acha *et al.*, 2004) and 4.05% (Younis *et al.*, 2009). Moreover, Akam *et al.* (2004) reported that the susceptibility was higher during the end of the first month to *Salmonella* (66.6%). In this study, *Salmonella* spp. were examined only in diarrheic calves; however, subclinical fecal *Salmonella* shedding can persist in dairy herds for up to 18 months with no measurable effects on health or production of individual cows (Younis *et al.*, 2009). Prevalence of *Salmonella typhimurium* was found 4% which is higher (2.73%) than the record of Younis *et al.* (2009) in neonatal calves.

5.3. Comparative pathology of ETEC K99 and *Salmonella typhimurium*

Five dead calves aged less than 45 days were examined at necropsy and intestinal samples were collected for histopathology. Besides feces was collected for confirmatory molecular identification of etiological agent. Villous atrophy was found in all five cases. Gross changes observed in all cases were watery intestinal contents with bad odor, mucus and blood (in two cases), dilatation of intestinal lumen. No gross abnormality was observed in other organs. Thickening of intestinal epithelium, sloughing off epithelium, congestion in lamina propria, infiltration of reactive cells were the findings of bacterial diarrhea. Findings of this study are in agreement with the findings of Alvarez *et al.* (2004) and Koh *et al.* (2008) who found same changes in pigs. Mohler *et al.* (2009) reported acute salmonellosis is typically characterized by diffuse catarrhal hemorrhagic enteritis with diffuse fibrinonecrotic ileotyphlocolitis. The lesions observed in histopathology are in agreement with lesions described by Jones *et al.* (1997).

5.4. Risk factors associated with prevalence of ETEC K99

Multivariate logistic regression model enabled to identify the significant risk factors associated with examined *E. coli*. Herd size, population density, age difference in a pen,

Dehydration, presence of other animal, sucking as feeding regime were found significantly associated with prevalence of calf diarrhea caused by *E. coli*. Calf age significantly affects the prevalence (P<0.05, OR = 5.51, 95% CI: 1.59, 19.04). Thus first week of life is the main age for occurrence. This finding came in accordance with those previously recorded by other investigators (Bendali *et al.*, 1999; Akam *et al.*, 2004; Radostits *et al.*, 2006; Wochenschr, 2007; Younis *et al.*, 2009). It is suggested that the age-dependent shedding dynamic of the ETEC has to be considered regarding prophylaxis as well as planning intervention studies for calves.

Having 21-50 heads in the farm (OR = 0.68, 95% CI: 0.26, 1.76) or 5-20 heads (OR = 3.54, 95% CI: 1.02, 12.78) versus >50 heads in the farm was found significantly associated with prevalence of *E. coli*. This finding is in agreement with (Acha et al., 2004; Akam et al., 2004; Younis et al., 2009). It was evident that calves in farms, which received colostrum directly from their dames were less frequent to be infected with ETEC (P < 0.01; OR: 5.525; CI 95%: 2.025–15.076) than those hand fed calves. This result coincided with that previously recorded by other investigator (Barrington et al., 2002) who reported that passively acquired immunity through colostrum is the major risk factor related to the calf and the occurrence of diarrhea. It was also found that colostral leukocytes obviously contribute to the passive immunity and resistance of the newborn calf against experimental infection by ETEC (Younis *et al.*, 2009). Level of dehydration was found significantly associated with prevalence of *E. coli* (P=0.05, OR= 1.07, 95% CI: 0.007-0.64). Coexistence of other animal species in same pen (OR = 3.24, 95% CI: 1.12, 9.34) showed higher risk than non-coexistence of other animal in same house which was also reported by Younis *et al.* (2009).

5.5. Risk factors associated with prevalence of *Salmonella* Spp.

Final multivariate logistic regression model showed that educational status of owner, source of drinking water, antibiotic therapies, recent introduction of new calf and hygiene were significantly associated with *Salmonella* spp. Shedding. Illiterate farmers experienced more prevalence of *Salmonella* spp. in herd than educated farmers (OR = 0.14, 95% CI: 0.03-0.70). Hygiene recorded significant association with *Salmonella* infections in diarrheic calves (P < 0.05; OR: 0.628; CI 95%: 1.729–5.612). No therapies

against diarrhea (P< 0.05, OR = 7.03, 95% CI: 1.30, 37.81) showed higher prevalence over antibiotic therapies. Surface water supply (OR = 6.00, 95% CI: 0.78-46.09) and municipal supply (OR = 32.28, 95% CI: 5.76-180.94) had higher load of *Salmonella* spp. over tubewell water. These findings are supported by the findings of (Bendali *et al.*, 1999; Acha *et al.*, 2004; Akam *et al.*, 2004; Younis *et al.*, 2009). But Akam *et al.* (2004) found no association of educational status of farmer and antibiotic therapies with risk of *Salmonella* spp. infection in neonatal calves.

In the present study, PCR enabled identification of ETEC K99 and *Salmonella* spp. in neonatal calves. Although there are available many diagnostic tests, PCR was found specific and convenient for large-scale screening of ETEC K99. To the best of our knowledge, this is the first report for molecular screening of ETEC K99 and *Salmonella* spp. not only in Chittagong but also in Bangladesh. The results of the present study indicate that molecular screening with PCR would be helpful for rapid and accurate tool for identification of ETEC K99 and *Salmonella* spp. in diarrheic calves. Moreover, identification of the risk factors associated with the spreading of bacteria causing diarrhea, may be helpful for construction of suitable methods for prevention and control.

Chapter-6: Conclusion

The intent of this study was to describe the comparative pathology and molecular detection of ETEC K99 and *Salmonella* spp. infections in neonatal calves. Particular strengths of this study included the large number of herds, the diversity of farm sizes sampled, and the longitudinal sampling approach spanning all months of the year. Multivariable logistic regression model was framed to find the risk factors behind the prevalence and distribution of virulent ETEC, and *S. typhimurium*. The prevalence of K99 gene was 19%. *Salmonella* spp. was isolated from twenty calves (10%); eight of them were typed as S. *enterica* serovar Typhimurium and two of them were S. *enterica* serovar Typhimurium DT104, whereas 12 cases were non-S. *enterica* serovar Typhimurium. Two cases have mixed infection by S. *enterica* serovar Typhimurium and ETEC K99. In the present study, PCR enabled identification of ETEC K99 and *Salmonella typhimurium* DT104 in neonatal calves below 45 days of age. PCR was found specific and convenient for large-scale screening among available diagnostic tests. Herd size, population density, age difference in a pen, Dehydration, presence of other animal, sucking as feeding regime were found significantly associated with prevalence of calf diarrhea caused by *E. coli*. On the other hand educational status of owner, source of drinking water, antibiotic therapies, history of introduction of new calf and hygiene were significantly associated with *Salmonella* spp. shedding. The results of the present study indicate that molecular screening with PCR can be used as a rapid and reliable tool for identification of ETEC K99 and *Salmonella* spp. in calves. Identification of the risk factors associated with the spreading of bacteria causing diarrhea will be helpful for developing suitable approach to prevent and control etiology of calf diarrhea.

Chapter-7: Recommendation

This study was done for molecular identification and characterization of *E. coli* K99 and *S. typhimurium* DT104 as well as for finding the farm and animal level factors associated with calf diarrhea in Chittagong Metropolitan area and Patyia. There are remarkable other etiological agents who can cause neonatal calf diarrhea in correlation with or without *Salmonella* and *E. coli*. Those factors were not identified in this study. The virulence factors of the organisms were not identified in present study. Based on above limitations future approaches can be as below:

a. Molecular identification and prevalence of other etiological agents (rotavirus, coronavirus, *Campylobacter jejuni*, *Cryptosporidium* spp etc.) that can cause neonatal calf diarrhea in Chittagong

b. Sequencing of identified genes and phylogenetic analysis of sequenced data through bioinformatics analysis

c. Animal model experimentation by virulent and mutant genes to know the pathology produced by prevalent microorganisms in Chittagong

d. Developing multiplex PCR protocol to identify etiology of neonatal calf diarrhea.

e. Developing disease model based on identified risk factors.

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Annex-I

Composition of different media used in bacterial isolation

Selenite Cystine Broth

Composition	gm/liter
Tryptone	5.0
Lactose	5.0
Disodium phosphate	10.0
L-cystine	0.01

Nutrient broth

Composition	gm/liter
`Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0

MacConkey Agar

Composition	gm/liter
Peptone	20.0
Bile salts	5.0
Lactose	10.0
Sodium chloride	5.0
Nutral red	0.075
Agar	12.0

Eosin Methylene Blue Agar

Composition	gm/liter
Peptone	10.0
Lactose	10.0
Dipotassium hydrogen phosphate	2.0
Eosin Y	0.4
Methylene blue	0.065
Agar	15.0

Blood Agar Base

Composition	gm/liter
Tryptone	14.0
Peptone Neutralised	4.5
Yeast Extract	4.5
Sodium Chloride	5.0
Agar	12.0

Xylose-Lysine-Desoxycholate Agar

Composition	gm/liter
Yeast Extract	3.0
L-Lysine HCl	5.0
Xylose	3.75
Lactose	7.5
Sucrose	7.5
Sodium Desoxycholate	1.0
Sodium Chloride	5.0
Sodium thiosulphate	6.8
Sodium ammonium citrate	0.8
Phenol red	0.08
Agar	12.5

TSI (Triple Sugar Iron) Agar

Composition	gm/liter
`Lab-Lemco' powder	3.0
Yeast extract	3.0
Peptone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Glucose	1.0
Ferric citrate	0.3
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0

Annex-II

Questionnaire for factors associated to bacterial agents in calf diarrhea

1. Serial no. : ……. Date: …….
2. Name of the farm and owner: ……
3. Educational Status……..
4. Upazilla/Thana: ……...District: ……..…
5. Location: ……..Latitude: ……Longitude: ……..
6. Region of location: ☐Plain☐Hilly☐Coastal
7. Herd size: ☐ 5-10 ☐11-20 ☐21-50 ☐>50 (Numbers……..)
8. Number of Calves: ……
9. Population density: no. of animals..../sft
10. Age:
11. Sex: ☐ Male ☐ Female
12. Breed of dam: ☐ Local ☐ cross
13. Type of barn: ☐Closed ☐ Partialy open ☐ Open barn
14. Flooring type in calving area: ☐ Concrete ☐ Sletted ☐ Brick ☐ Grass
15. Type of litter in calf pen: ☐ Straw ☐ Rubber pad ☐Litter less
16. Source of drinking water: ☐ Ponds ☐ River ☐ Deep tubewell ☐ Supply ☐ More than one type
17. Bedding cleaning method: ☐ Water cleaning ☐ water cleaning with disinfectant
18. Calving month:☐J ☐F ☐M ☐A ☐M ☐J ☐J ☐A ☐S ☐O ☐N ☐D
19. Separation of calf from dam: ☐ Immediately ☐ <24 hr ☐ >24hr ☐ No information
20. First feeding of colostrum after birth: ☐ Within 30 min ☐within 2 hr ☐ within 2-6hr ☐ no information
21. Feeding calf with waste milk: ☐Yes ☐No (☐from mastitis ☐Contain antibiotics)
22. Sucking as feeding regimen: ☐Yes ☐No (☐Restricted ☐No restricted)
23. Confinement from birth: ☐ Single ☐ Group
24. Maximum age difference between youngest and adult calf housed in same pen: ☐ <4wk ☐ 4-8wk ☐ >8 wk
25. Diarrhoeic calves in farm: ☐ Yes ☐ No
26. History of calf scour: ☐Yes and dead ☐Still Diarrhoeic ☐Recovered ☐ No
27. Therapy: ☐ Antibiotics ☐ Antiparasitics ☐ Others ☐None
28. Feces consistency: ☐ Liquid ☐Semi-liquidor semi-solid ☐Solid or formed
29. Dehydration (Skin fold test): ☐ Within 2 second ☐ Within 2-6 second ☐ > 6 sec
30. Body weight(kg):

31. Newly introduced calves from other farms within----days/months:
    ☐ Yes ☐ No
32. Dystocia during delivery of this calf: ☐ Yes ☐ No
33. Parity: ☐1st ☐2nd ☐3rd ☐4th ☐5th ☐ More
34. Hygiene of calf feeding untensils: ☐ Not shared ☐ Shared & rinsed with water ☐ Shared and disinfected
35. Navel treatment: ☐Yes ☐ No
36. Physical contact with other: ☐ None ☐ unweaned ☐ weaned ☐>6 month ☐ adult
37. Surrounding environment of the farm: ☐ Good ☐ Moderate ☐ Bad
38. Drainaige system: ☐ Good ☐ Moderate ☐ Bad
39. Number of other animals on the farm and if any have diarrhoea: Yes/No
    Bull: Sheep: Goat: Poultry:
40. History of calf death within -----days/months: ☐ Yes ☐ No; If yes, mention number:
    Clinical signs: ☐ Respiratory ☐ Digestive ☐ Stillbirth
41. Floor disinfection system: ☐ Yes ☐No If yes, Frequency: ------/month; Name of agent using now:
42. Grazing system: ☐Zero ☐Community grazing ☐Tethering; If zero grazing, Practice of washing before offering: ☐ Yes ☐ No
43. Storage system of feed: ☐ Good ☐ Fair ☐Poor
44. Is calf experiencing concurrrent condition: ☐ Respiratory ☐ Umbilical ☐ Others……..
45. If diarrhea calves present, any of your family members has loose motion?
    ☐ Yes ☐ No

Signature of Interviewer

Annex-III

Definition for grading qualitative variables

1. Surrounding environment:

Good: Clearly separated from household, away from road, and clean

Moderate: Near to household but away from road, and clean

Poor: Near to household, road and unclean

2. Drainage system

Good: Clearly circulated all the time and cleaned at least twice/week

Moderate: Clearly circulated only at the time of washing and cleaned at least once/week

Poor: Stagnant of wastage all the time and irregular cleaning

3. Food storage

Good: Separated from the farm as well as good light and ventilation in storage room

Moderate: Adjacent to the farm as well as good light and ventilation in storage room

Poor: Adjacent to the farm as well as poor light and air circulation/ don't have any separate storage room

4. Hygiene Score

Good: Farms having at least 2 good score without any poor for above 3, pre and post dipping during milking, disinfection of floor at least once/week

Moderate: Farms with at least 1 good score for above 3, pre/post dipping during milking, disinfection of floor at least once/15 days

Fair: Farms with at least 2 moderate/fair marks for above 3, no dipping during milking, disinfection of floor at least once/month

Poor: Farms with score below than above, no dipping, water cleaning only / no disinfection practice

Annex -IV

Activities during the research work

Plate: A, B: Necropsy of dead calves; C: Haemorrhage in intestinal wall; D: Microscopic Examination of histopathological slides

Plate: E: Collecting fecal swab, F: Transferring swab to broth G: Inoculating samples on agar medium; H: Large pink colored colony on MacConkey agar by *E. coli*

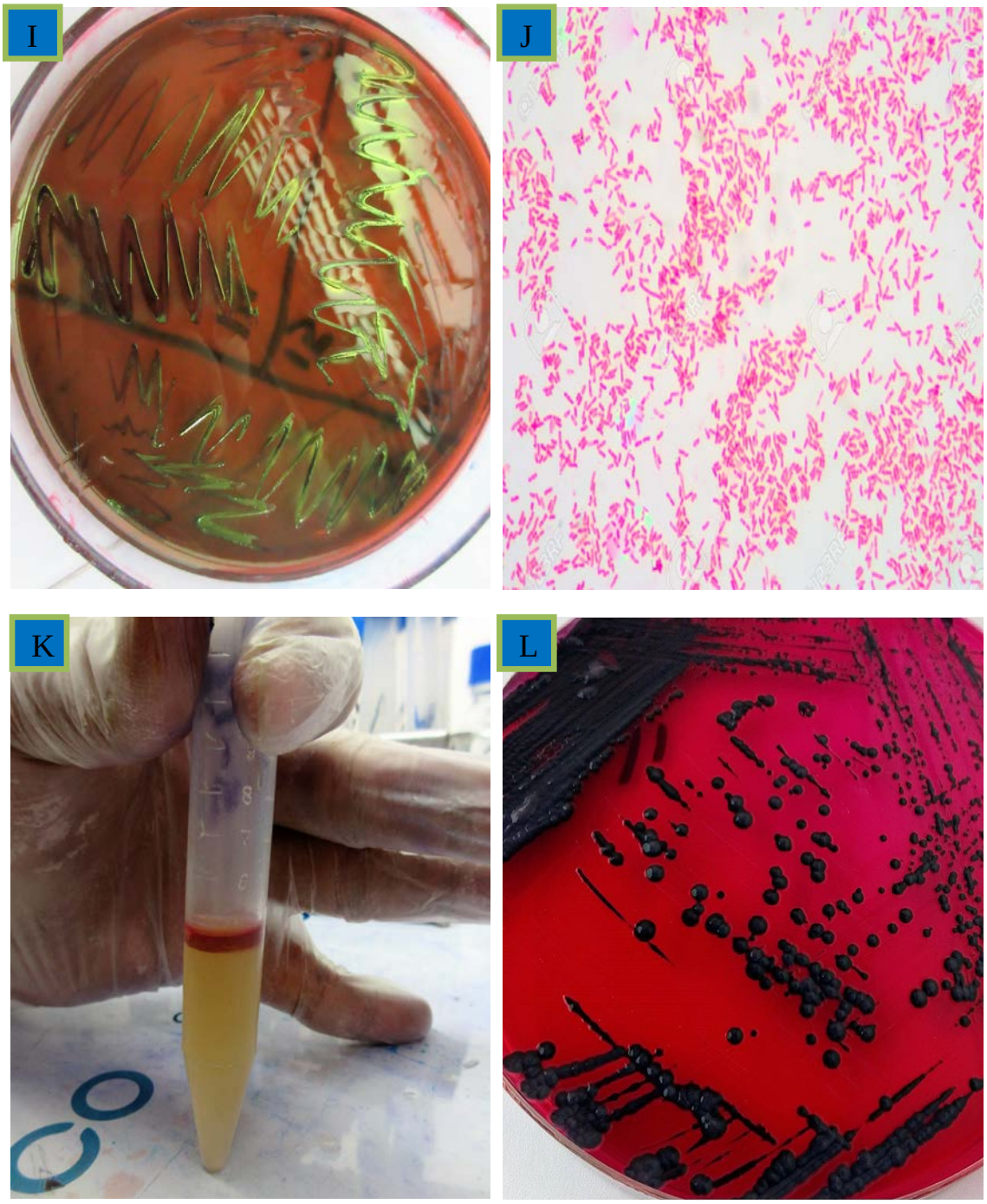

Plate: I: Metallic sheen on EMB agar by *E. coli* J: Gram negative bacilli under light microscope (100X); K: Positive indole reaction by *E. coli*; L: Black centered colony on XLD agar by *Salmonella* spp.

Plate: M: Growth of *Salmonella* on TSI slant; N: Preparation of PCR mixture on clean bench; O: Placing PCR mixture in thermal cycler; P: Gel electrophoresis of PCR products

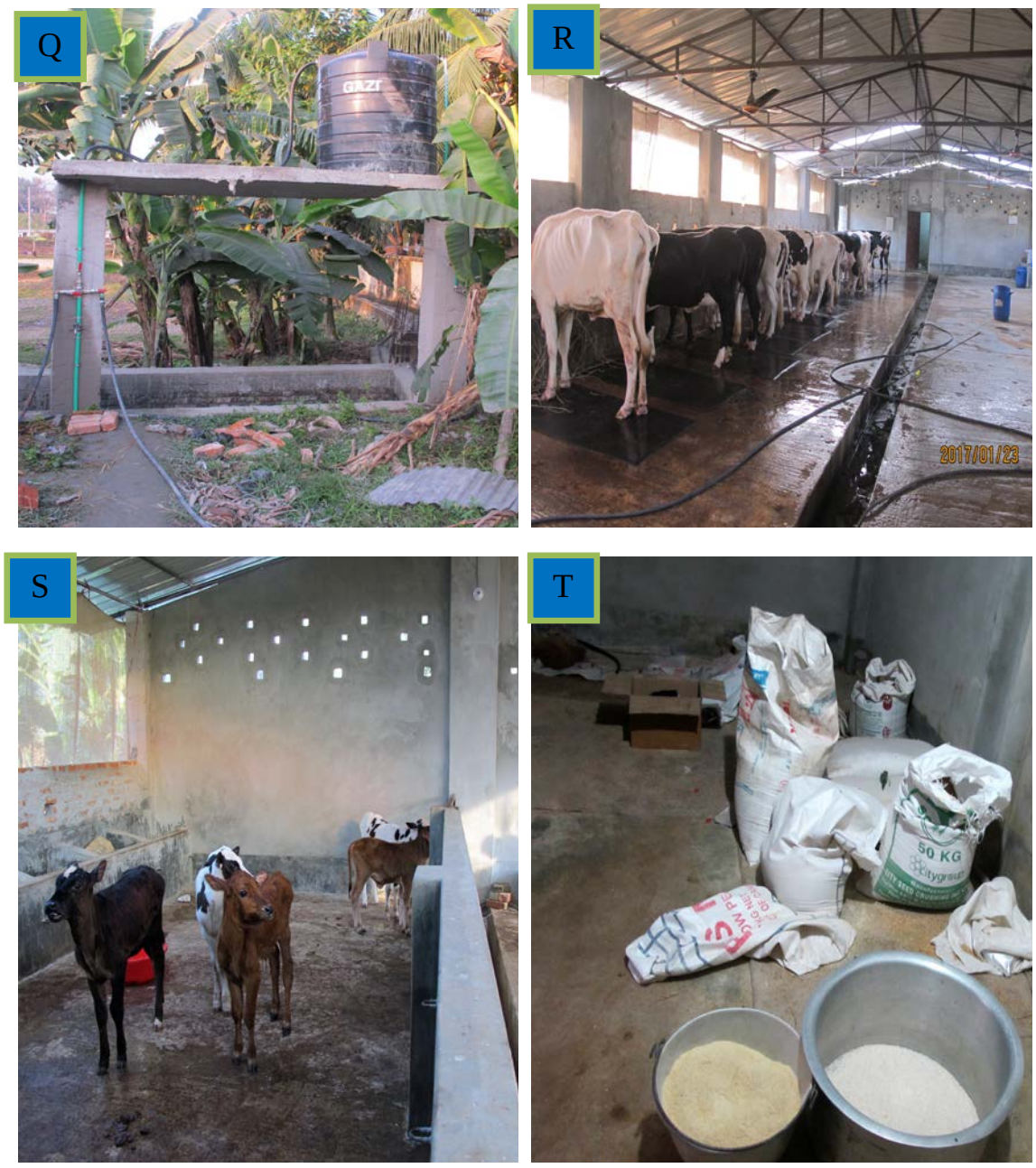

Plate (Q-T): Observation of different farm factors for risk analysis

Brief Biography of the Student

This is Tofazzal Md. Rakib; son of Md. Nurul Amin and Hasina Begum from Pekua Upazila under Cox's Bazar district of Bangladesh. He passed the Secondary School Certificate Examination in 2004 followed by Higher Secondary Certificate Examination in 2006. He obtained his Doctor of Veterinary Medicine Degree in 2013 (held in 2015) from Chittagong Veterinary and Animal Sciences University (CVASU), Bangladesh with CGPA 3.95 (out of 4.00) that hold him to the first position among 53 students of his batch. Now, he is a candidate for the degree of MS in Pathology under the Department of Pathology and Parasitology, Faculty of Veterinary Medicine, CVASU. He has been working as a lecturer in the Department of Pathology and Parasitology, Faculty of Veterinary Medicine, CVASU since August 03, 2016. He published 11 (eleven) scientific articles in international journals and 4 (four) poster and/or oral presentations in national and international proceedings. He has immense interest to work on infectious diseases of livestock and their molecular pathobiology in the host.