Isolation & molecular characterization of *Campylobacter jejuni* and virulent gene associated avian fecal *Escherichia coli* in broiler, Bangladesh

Md. Sirazul Islam

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Department of Pathology and Parasitology
Faculty of Veterinary Medicine

Chattogram Veterinary And Animal Sciences University
Chattogram-4225, Bangladesh

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Authorization

I hereby declare that I am the sole author of the thesis submitted in fulfillment of the requirements for the Degree of Masters of Science (MS) in the Department of Pathology and Parasitology, Faculty of Veterinary Medicine, Chattogram Veterinary and Animal Sciences University (CVASU). I authorize CVASU to lend this thesis or to reproduce the thesis by photocopying or by other means, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research.

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

…………………………………....
Prof. Dr. Sharmin Chowdhury
Supervisor
Department of Pathology and Parasitology

……………………….………..
Prof. Dr. Sharmin Chowdhury
Chairman of the Examination Committee
&
Head
Department of Pathology and Parasitology

Chattogram Veterinary and Animal Sciences University
Chattogram-4225, Bangladesh
JUNE 2020

Dedicated
to my
parents and maternal
grandfather

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List of Acronyms and Symbols Used

Abbreviation and symbols	Elaboration
AFEC	Avian fecal *Escheria coli*
APEC	Avian pathogenic *Escheria coli*
%	Percent
>	Greater than
<	Less than
≥	Greater than equal
≤	Less than equal
=	Equal to
°C	Degree Celsius
BHI	Brain Heart Infusion
bp	Base Pair
BPW	Buffered Peptone Water
C. jejuni	*Campylobacter jejuni*
C. coli	*Campylobacter coli*
C. hepaticus	*Campylobacter hepaticus*
CDC	Center for Disease Control and Prevention
CFU	Colony Forming Unit
CI	Confidence Interval
CVASU	Chattogram Veterinary and Animal Sciences University
DAEC	Diffusely Adherent *E. coli*
DPP	Department of Pathology and Parasitology
DNA	De-oxy Ribonucleic Acid
EaggEC	Enteroaggregating *E. coli*
E. coli	*Escherichia coli*
EHEC	Enterohemorrhagic *E. coli*
EMB	Eosin Methylene Blue
ETEC	Enterotoxigenic *E. coli*
Hrs	Hours
Kb	Kilo Base
LBMs	Live Bird Markets
Ltd.	Limited
LT	Heat Labile Toxin
µg	Microgram
µL	Microliter
mA	Milliampere
mL	Milliliter
OR	Odds Ratio
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
spp	Species
TAE	Tris Acetate EDTA
UTI	Urinary Tract Infection
UV	Ultra Violet
VAGs	Virulent associated genes
WHO	World Health Organization

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Summary

The intestinal environment of broilers is a potential *Campylobacter jejuni* and avian fecal *Escherichia coli* (AFEC) reservoir. Understanding the intestinal microbiota in broilers and their subsequent human transmission is a key public health concern here, where *C. jejuni* and intestinal virulent associated gene (VAG) carriage *E. coli* are simultaneously studied in apparently healthy birds from broiler farms, live bird markets (LBMs) and supershops. In the period from June 2019 to February 2020, pooled cloacal samples were collected from broiler farms located in 6 districts of Bangladesh and pooled meat samples were collected from LBMs and super shops in Chattogram. Data on farm management, biosecurity, and hygiene practices were collected through a face-to-face interview during sampling using a structured questionnaire. To examine the occurrence of *C. jejuni* and VAG AFEC in broiler chickens, microbial culture and PCR-based methods were applied. Positive PCR amplicon was confirmed by gene sequencing and phylogenetic analysis was subsequently performed. To evaluate the risk factors, epidemiological data were analyzed using univariable logistic regression models followed by multivariable logistic regression. Among the 216 farms, 27 were positive to *C. jejuni*; thus, the farm level prevalence was estimated to be 12.5% (95% CI: 8.5%–17.7%). Remarkably, at LBMs and super shops, a higher prevalence of 27.08% (95% CI: 15.28%–41.84%; N=48) of *C. jejuni* was recorded in broiler meat. In risk factor analysis, downtime of less than 14 days, no separate footwear for shed entry, increased number of flock rotations per shed per year, and entry of more than 1 person to sheds were found to be significantly associated with *C. jejuni* infection. Phylogenetic analysis showed a close connection between *C. jejuni* strains isolated from Bangladesh and other strains isolated from humans, pigs and bats of India, South Africa and Grenada. VAGs AFEC was isolated from the apparently healthy chickens with a prevalence of 55.6% (95% CI: 48.7%–62.3%). Virulence Associated Genes; *astA*, *iucD*, *iss*, *irp2* and *cva/cvi* were detected in a rate of 46.3%, 25.5%, 17.6%, 12.9% and 2.8%, respectively. Geographical locations and flock age of less than 21 days were significantly associated with the VAGs AFEC positive status in broilers. The results of this study showed a high level of microbial contamination of zoonotic importance and the presence of pathogenic bacteria in the intestine and meat of Bangladeshi broilers. Potential sources of contamination and anthropogenic factors associated with the alarming occurrence of *C. jejuni* and VAGs AFEC noted in this study would assist in developing interventions under the 'One Health' banner that includes chickens, humans and environmental perspectives to minimize the increasing risks of broiler-associated pathogens.

Chapter-1: Introduction

Foodborne infections are a rising public health concern worldwide (Elmi, 2004). Among these Campylobacteriosis is a well-characterized bacterial foodborne infections which occurred due to consumption of poultry and poultry products (Wieczorek et al., 2012). Several species within the genus *Campylobacter* are mostly responsible for this illness where *Campylobacter jejuni* are most frequently isolated species cause severe foodborne illness which accounts for 77.3% of illness (Doyle & Erickson, 2006).

Campylobacter is commonly found in intestinal tract of poultry as a commensal due to their thermotolerant properties, especially *C. jejuni* which favors poultry owing to their high body temperature (Verwoerd, 2000). *Campylobacter* has no detrimental effect on chicken, known to be harmless chicken intestinal flora, which stays healthy but serves as the major carrier of human infection. Nevertheless, in some flocks of broiler chicken in the intensive poultry production system, *C. jejuni* infection can induce inflammatory response, damage to gut mucosa and diarrhea (Humphrey et al., 2014). However, broiler meat consumption and direct contact through broiler handling and meat processing were considered the main route of transmission in humans (Boysen et al., 2014). The risk of transmission is greater from broiler chickens because of high level of meat consumption (Neogi et al., 2020). During meat processing vast number of organisms are transferred from chicken intestinal tract to meat, since cecum and colon are considered as region of tropism for *Campylobacter* species, particularly if intestinal tract of poultry is ruptured and contents are mixed with skin prompt to further pollute the carcass (Vinueza-Burgos et al., 2017).

The major clinical sign caused by *Campylobacter* in human is acute diarrhea (Acheson & Allos, 2011). The pathogen mainly causes not only severe gastroenteritis in humans but also causes several clinical condition such as reactive arthritis, pancreatitis, enterocolitis and bacteremia (Linton et al. 1996) and also a main health burden for both developing and developed countries (Abd El-Baky et al., 2014). This clinical condition is occurred due to consumption of undercooked or poultry, liver or grilled chicken meat (Edwards et al., 2014). A complication known as guillain-barré syndrome is observed as a result of campylobacteriosis in humans (Nachamkin et al., 2003). Poor sanitation and hygiene practice are responsible for campylobacteriosis in community people including children in developing countries like Bangladesh (Pollett

et al., 2012; Taniuchi et al., 2013). In Bangladesh the burden of *Campylobacter* colonization in broilers is mostly unknown and therefore it seeks proper attention from the public health point of view.

In various ecological niches, including the intestines of animals and humans, *Escherichia coli* is a ubiquitous organism with a fabulous adaptive capacity (Jang et al., 2017). Most strains of *E. coli* in poultry are considered non-pathogenic and are known as commensal or non-pathogenic or avian fecal *E. coli* (AFEC) (Kunert et al., 2015). A group of these bacteria developed the ability to survive in different species (Human, Poultry etc.) by acquiring particular virulence traits and causing clinical features related to intestinal and extra-intestinal diseases (Kaper et al., 2004 and Kaper et al., 2005).

Avian pathogenic *E. coli* (APEC) infection leads to colibacillosis represents critical morbidity and mortality in the poultry business worldwide (Barnes et al., 2003). APEC's pathogenic capacity is facilitated by a wide range of virulence factors encoded by virulence-associated genes (VAGs) (Subedi et al., 2018). Past analysis (Ewers et al., 2004) showed that the prevalence of different virulence genes in isolates was a valuable marker for both APEC and AFEC detection and characterization (Schouler et al., 2011; Kemmett et al., 2013). VAG AFEC are not specifically characterized, and no specific definition of these microorganisms has been identified so far (Fagan et al., 1999; Van Bost et al., 2003).

Risk factors for *Campylobacter* colonization and VAG AFEC infection in broiler chickens vary depending on farming practices, geographical locations and climatic conditions (Chowdhury et al., 2013; Ibrahim et al., 2019; Hasan et al., 2020). Risk factors identified for industrial chicken production in developed countries, however, are highly context-specific and cannot be directly deployed to small-scale commercial chicken farms in some low- and middle-income countries, such as Bangladesh, where bio-security is compromised. In addition, the lack of documentation on *C. jejuni* and VAGs AFEC risk factors in chicken meat production systems in Bangladesh, as far as authors' knowledge, represents a major gap where rearing systems may differ significantly from western settings. Therefore, taking into account the public health significance of *C. jeuni* and VAG AFEC, this study was conducted to isolate, identify and molecular characterization together with prevalence estimation and identification of possible risk factors in commercial broiler farms and live bird markets (LBMs) infection. However, the present study also invoked the molecular characterization of

AFEC isolates, based on the identification of virulence factors that could be used for diagnosis of colibacillosis in chickens.

With this background, the present study was conducted to achieve to following objectives:

Objectives:

1. Isolation, identification, molecular and phylogenetic characterization of *C. jejuni* from cloacal swab and meat of broiler chicken.
2. Isolation, identification and molecular characterization of VAG AFEC from cloacal swab of broiler chicken.
3. Estimation of farm level prevalence of *C. jejuni* and VAG AFEC in broiler chicken.
4. Estimation of prevalence of *C. jejuni* in broiler meat collected from super shops and live bird markets.
5. Identification of risk factors associated with colonization of *C. jejuni* and VAG AFEC in broiler chicken.

Chapter-2: Review of Literature

The aim of the chapter is to provide up-to date information on previous research works on the subject matter and to identify the gaps and justify the rationale of this thesis works. Relevant literature on molecular characterization of *C. jejuni* and VAG *E. coli* in broiler farms and meat, prevalence, diagnostic methods, associated risk factors etc. have thoroughly been reviewed in this chapter.

2.1 Broiler industry in Bangladesh:

Bangladesh has recently joined the middle-income group of countries, and it is impossible to ignore the contribution of livestock to this achievement (World Bank, 2020). Not only is livestock a source of animal protein, but it is also an inevitable component of Bangladesh's complex farming system and a source of employment (Rahman et al., 2014). Agricultural Gross Domestic Product's share of livestock is 13.5 percent and livestock contribute 1.5 percent of GDP to the national economy, employing 20-50 percent of Bangladesh's people (BBS, 2018-19). Poultry farming plays a significant role in the livestock sector. Our country's total poultry population is roughly 347 million (DLS, 2018-19). Broiler chickens are widely reared for meat purposes. Poultry meat alone accounts for 35.3 percent of Bangladesh's overall meat production (Hamid et al., 2017). A significant part of the global economy, especially in the developing world, is the broiler sector. In a country like Bangladesh, where most people are landless, disadvantaged and devoid of formal education or skills to participate in income-generating activities, broiler rearing can play a vital role.

Broiler can not only be an important tool for this group of people to fight poverty, but also for distressed women, because poultry requires minimum land, short capital and skills. Saleque (2006) stated that currently a total of 5 million people are working in this sector of different farm size. Broiler plays a very important role for humanity through the supply of food, income and job creation, the supply of raw materials to certain industries, the facilitation of research work, etc. Broilers are therefore an integral part of the farming system in Bangladesh.

2.2 Live Bird Markets (LBMs) in Bangladesh

Poultry-based businesses are now one of the top rated businesses among agribusinesses in Bangladesh. In the financial year 2018-2019, the contribution of the

livestock sector to GDP was 1.5% (BBS, 2018-19), most of which was earned from agro-business in the poultry industry. The majority of people in Bangladesh go to small stalls where live birds are sold for poultry meat. These stalls are located in practically every region of Bangladesh. There are generally 5-30 such stalls in big markets. For sale, 30-100 birds, mostly broilers, are displayed in each stall. And, when all or more birds of a batch are sold, shop owners go to clean up. But they are not aware of hygiene enough, and they dump waste materials most of the time into drain water, which can eventually be mixed with ground water. Therefore, when the stalls are contaminated with food borne infections, these live bird markets may pose a great risk.

2.3 *Campylobacter*

2.3.1 An overview on *Campylobacter*

Campylobacters were originally listed in the *Vibrio* genus. Campylobacter's first documentation was found in 1886 when Theodor Escherich noted the presence of a spiral bacterium from children who died of what he called '*Cholera infantum*' in stool. The first well documented "Vibrio-related" case of human infection occurred during a diarrheal outbreak caused by contaminated milk (Levy, 1946) where organisms resembling “*Vibrio jejuni*” were isolated. In 1913, two veterinarians, McFadyean and Stockman isolated “*Vibrio fetus*” from the stomach contents of an aborted lamb (McFadyean and Stockman, 1913). A new '*Vibrio*' that caused dysentery in calves during the winter was reported in 1931, and its name was proposed as *V. jejuni* (Jones et al., 1931). Doyle isolated a similar Vibrio from swine dysentery in 1944, and he named it *Vibrio coli* (Doyle, 1944). The first to successfully culture microaerophilic isolates of *V. fetus* at 42°C was King (1957). However, this temperature was higher than the optimal growth temperature of traditional vibrios and isolates was referred to as “related vibrio”. Later on, these organisms were renamed *Campylobacter* by Sebald and Veron due to differences in the DNA base composition, growth requirements, and metabolism between Vibrios and Campylobacters (Veron and Chatelain, 1973). *Campylobacter* was finally recognized as a human pathogen after successful isolation from human feces in 1972 (Dekeyser et al., 1972). *Campylobacter* isolation from the stool was made possible by the development of the filtration technique (Dekeyser et al., 1972), the selective media-Skirrow medium

(Skirrow, 1977) and Butzler's medium (Butzler et al., 1983), a crucial step in the re-evaluation of the epidemiology of *Campylobacter*.

2.3.2 Characteristics of *Campylobacter*

The *Campylobacter* genus belongs to the Campylobacteriaceae family in the phylum Proteobacteria class of Epsilonproteobacteria. Campylobacters are Gram-negative, curved or spiral rods 0.2–0.4 μm wide, 0.5–5μm long and non-spore forming bacteria. Having a single polar unsheathed flagellum at one or both ends of the cell, all Campylobacters are oxidase-positive, catalase-positive, urease negative and motile (Ursing et al.,1994), with the exception of *Campylobacter gracilis* (oxidase-negative and aflagellate). Campylobacters are 'microaerophilic' and generally require a 3–5 percent concentration of carbon dioxide, 3–15 percent concentration of oxygen and a temperature of 42°C for optimum growth. They become coccoid-shaped when *Campylobacter* cells start to age. There are currently 34 species and 14 subspecies in the genus *Campylobacter*.

2.3.3 Campylobacteriosis due to *Campylobacter* species

Campylobacter is generally recognized as the leading cause of human bacterial gastroenteritis worldwide (Skarp et al., 2016), with *C. jejuni* and *C. coli* representing the main sources of infection (Adak et al., 2005 and Fhogartaigh & Edgeworth, 2009). Infectious administered doses of *C. jejuni* as low as 500-800 bacteria have been reported to be sufficient to cause illness in healthy adults (Robinson, 1981 and Black et al., 1988).

2.3.4 Risk factors of *Campylobacter*

Biosecurity is an important measure for *Campylobacter* control because horizontal transmission can be rapid once colonization occurs in a poultry flock (Battersby et al., 2016). A study conducted by Gibbens et al. (2001) showed that well-implemented disinfection protocols could reduce the prevalence of *Campylobacter* in broilers from 80 to less than 40% (Gibbens et al., 2001).It has been shown to be effective in installing hygienic barriers between internal and external environments, controlling staff entry, strict hygiene rules (hand washing and sanitizing hands), changing boots and overalls before entering (Silva et al., 2011). From 1980 to 2008, a literature review concluded that high standards in biosafety measures should contribute to the

reduction of *Campylobacter* flock colonization (Newell and Fearnley, 2003). Risk factors for increased colonization of *Campylobacter* include: poor farm hygiene, reduced flock replacement time, the presence of other farm animals, rodents and insects, seasonal changes and partial depopulation (Russa et al., 2005). In a Danish study, the use of dedicated footwear proved to be the most significant risk factor (Newell and Fearnley, 2003). The greater number of people working on the farm increased the likelihood of biosecurity violations by other factors affecting biosecurity measures (Newell et al., 2011). Farm staff hygiene measures include hand washing, use of separate boots for each house, use of footbath disinfectants, limited access to key staff only, control of pests and staff training (Sahin et al., 2015). The presence of boot baths at the entrance of the broiler house is considered a risk factor (Hog et al., 2016) and for these to be efficient, high maintenance with disinfection replenishment being done at regular intervals. If not well-maintained the footbaths will increase the risk rather than act as a defensive barrier. Disinfectants should be replaced weekly or if the dilution is reduced (due to rain water) or if there is organic matter build up at the bottom of the foot bath, they must be replenished (Evans and Sayers, 2000). Van Wagenberg et al. (2016) concluded that the most cost effective procedure was to apply barriers in each house and to utilize dedicated tools for each house to minimize the cross-contamination risk; however, the most cost effective intervention was the ban of partial depopulation and introducing the all-in/all-out system at approximately day 35. Hygiene barriers proved effective to some extent in preventing *Campylobacter* infection in broiler flocks. These results suggested that expanding the hygiene barriers to include gates, vehicle disinfection, respecting biosecurity measures during catching would increase *Campylobacter* reduction, however the value (cost effectiveness) of these additional efforts would not be detectable in the final results (Hald et al., 2000). Preventing farm staff from direct contact with the broilers has been shown to protect the broilers from *Campylobacter* infection (Battersby et al., 2016).

A study conducted on Danish farms concluded that factors like the oldness of the poultry house, rodent control, the age of broiler at slaughter, storage of whole wheat, number of chimneys on the broiler house, the location of the broiler farm in relation to cattle density are very important in controlling *Campylobacter* presence. However, it must also be taken in account that these observations are specific to Danish farm

practices and may differ from one country to the next, however, they are a valuable place to begin understanding the value of proper biosecurity appreciation and implementation, as well as some of the challenges that underpin implementation on every farm world-wide (Sommer et al., 2013).

Results of a questionnaire study by Hald et al. (2000) indicated that other animals located in the intermediate vicinity of the broiler house posed a significant risk to broiler flocks in terms of *Campylobacter* colonization. It is strongly suggested that a farmer tending both cattle and poultry on the same farm transmitted *Campylobacter* from cattle to poultry (and vice versa) on his/her boots (van de Giessen et al., 1998) Farm personnel and equipment (e.g., feed trucks) can carry *Campylobacter* between broiler houses and onto subsequent or neighboring farms (Newell et al., 2011). In the absence of infected neighbors in 2 km radius of susceptible farm, in the same month, showed a significant protective effect in comparison with presence of infected neighbors in the same distance and time (Chowdhury et al., 2012). A study conducted in Ireland revealed that 85% of flocks were positive at depopulation, and their results identified thinning as a significant risk factor for *Campylobacter* introduction and the authors provided the suggestion was that partial depopulation should be discontinued (Smith et al., 2016). Age of birds and depopulation are closely associated, making it difficult to be certain which of these two factors affects *Campylobacter* prevalence most significantly, but more recently it has been shown that seasonality is also an important factor on the prevalence that *Campylobacter* in broiler flocks that have not been thinned (Jorgensen et al., 2011). Russa et al. (2005) suggested that there was a link between age at depopulation and *Campylobacter* prevalence. Although the method used in their investigation is not clear, the results show that the proportion of *Campylobacter* positive flocks increased with increasing age. They also detected a link between the proportion of *Campylobacter* positive flocks to weather where higher numbers were seen in the autumn (Russa et al., 2005). Live bird crates that were contaminated with *Campylobacter* from previous (or other) flocks are reintroduced on the farm during catching, and quite often these crates undergo inadequate washing at the slaughter house (Newell et al., 2011). Crates can carry identical genotypes of microorganisms which originate from broiler flocks and abattoirs, which suggests that transport crates are responsible for contamination during transport to slaughter or they could contribute to the *Campylobacter*

colonization of broiler houses (Hastings et al., 2011). Research has shown that *Campylobacter* can survive on crates post-sanitization (Allen et al., 2008). Results from the survey by Powell et al. (2012) showed company specific risk factors or probable recurrence of strains within a company, this warrants further investigation.

Due to the intensive cleaning and disinfection that is often between flocks it is difficult to predict *Campylobacter* infection from the status of previous flocks. When farms remove litter between grow-out periods, it is often found that negative flocks follow positive ones. The presence of colonized flocks was linked to the turnaround time in a house. Periods of over 14 days can decrease the possibility of residual bacterial contamination (Newell et al., 2011). The benefit of longer turnaround periods is also supported by Battersby et al. (2016) who state that rapid flock turnover contributes to *Campylobacter* carry over with increased risk being reported if houses are restocked within 9 days of depopulation. A study by Jonsson et al. (2012) also investigated the effect of the length of time the house was empty. Based on a small data set, the study showed that keeping the broiler house empty for less than 9 days would increase the risk for *Campylobacter spp*. Also, if the empty time is extended the risk of introducing *Campylobacter* into the houses is kept low only if the biosecurity and hygiene levels are maintained optimal (Hog et al., 2016). It is well-known that an external reservoir can host multiple *Campylobacter* strains, during the empty period, which will allow colonization of the new flock (Ellis-Iversen et al., 2012). Ellis-Iversen et al. (2012) found that contaminated shed entrances, anterooms and drinkers and shedding of *Campylobacter* by other animals (e.g., cattle, dogs, rodents) have been found to be linked to positive flocks. In order to reduce the risk of *Campylobacter* introduction into the shed they have suggested disinfecting the surroundings of the poultry shed around day 25 of the cycle. Other reservoirs of contamination include, vehicles, equipment used by catchers and catching crews (Ellis-Iversen et al., 2012). The prevalence of *Campylobacter* in chickens has been found to be associated with seasonality (Taylor et al., 2013; Friedrich et al., 2016). In western countries with temperate climates seasonal peaks of human campylobacteriosis are observed between July and August. The summer peaks in human infection are consistent with higher *Campylobacter* isolation levels from chickens in the summer period, compared to winter, with the human infection peaks preceding the chicken one suggesting a link between the two (Skarp et al., 2016).

There is a clear risk level of acquiring campylobacteriosis between rural and urban regions and this risk must be taken in consideration (Deckert et al., 2014; Williams et al., 2015). Research has also shown that the sources of environmental exposure are season dependent with flies being a common vehicle of transmission between the environment and food (Ekdahl et al., 2005). The use of fly screens ventilation openings was recently described as an efficient method to reduce the number of *Campylobacter* positive flocks (Sahin et al., 2015). These findings confirm that flies serve as a vector particularly during the summer months when temperatures are high (Sahin et al., 2015). Temperature is correlated with *Campylobacter spp*, colonization of broilers in a study by Jonsson et al. (2012). *Campylobacter* has a high survival rate in water and thus can contaminate water reservoirs following translocation from pastures of grazing animals. Water chlorination appears to be very effective against *Campylobacter* (Newell and Fearnley, 2003; Hutchison et al., 2004). Contamination of water in the broiler house usually follows colonization of a flock indicating that this is caused by contamination of water lines with microorganisms excreted from the birds.

2.3.5 Prevalence of *Campylobacter* in foods

The species of *Campylobacter* are ubiquitous and isolated from a wide variety of foods, including chicken meat, milk, beef, chevon and water, but the primary source of *Campylobacter spp.* to humans remains poultry meat. It has been estimated that chickens were attributed to 71% of human campylobacteriosis cases in Switzerland between 2001 and 2012 (Kittl et al., 2013 and Wei et al., 2015). The UK Food Standards Agency reported that 72.9% of all fresh retail chickens surveyed were infected with *Campylobacter* from 2014 to 2015 (Food Standards Agency, 2014). The literature on the contamination of retail poultry meats and by-products in the world was surveyed by Suzuki and Yamamoto (2009). Despite their sanitary conditions, most retail poultry meat and products have been contaminated with *Campylobacter spp*. in most countries (both developed and developing countries). In different countries, the observed prevalence of *Campylobacter* varied in poultry. Australia (100%), Argentina (92.9%), the Czech Republic (100%), New Zealand (89.1%), Oceanica (90.4%) and Bangladesh (40.5%) have a much higher prevalence, while Belgium (17%), Estonia (8.1%), the Former Soviet Union and Eastern Europe (19.1%), Switzerland (25.1%) and Vietnam (30%) have a much lower prevalence of

Campylobacter in poultry (Suzuki and Yamamoto, 2009, Hasan et al., 2020). The presence of *C. jejuni* in broilers at retail outlets in Bangladesh was studied by Neogi et al., 2020. 29% meat samples and 43% of cloacal swabs were positive for *C. jejuni*. 23.4% meat surface swabs and 30.9% cloacal swabs found positive to *C. jejuni* in broilers from retail outlets in India (Sharma et al., 2007). The prevalence of *Campylobacter* among broilers in the region of Bareilly was investigated by Malik et al. (2014) and 32% of samples were identified positive for *Campylobacter* species. Tayde and Brahmbhatt (2014) investigated the prevalence of thermophilic *Campylobacter spp* isolated from poultry in and around Anand city, Gujarat, India. For the species of *Campylobacter*, 6.66% chicken and 76.66% caecal samples were found positive. Raw milk has also been identified as a vehicle of human gastroenteritis by *Campylobacter spp* (Blaser et al., 1979; Robinson et al., 1979; Porter and Reid, 1980; Potter et al., 1983). Due to faecal cross-contamination during milking or as a result of udder infection, *C. jejuni* may be present in milk (Doyle and Roman, 1982, Orr et al., 1995). The prevalence of *Campylobacter spp* was investigated by Elango et al., (2009) isolated from local vendors' raw milk samples in Chennai, India. With a prevalence of 1.36%, a total of 42 *C. jejuni* isolates were obtained. The prevalence of *Campylobacter spp* in milk and milk products in and around Anand, Gujarat, was investigated by Modi et al., (2015). In 2.91% of raw milk samples, *Campylobacter* species were detected, whereas none of the milk products were positive. Monika et al., (2016) in Uttarakhand, India, studied 759 samples containing human stool (50) and poultry meat (251), chevon (183), pork (127), fish (106) and carabeef (42). They reported an overall prevalence of *Campylobacter spp* 6.58% in these samples. In poultry meat, the highest prevalence rate was 13.54%, followed by 7.6% in chevon, 0.78% in pork and 2% from the samples of human stool.

Water is an effective means of transmitting *Campylobacter* species to humans and animals, and several outbreaks in different countries have been caused by contaminated water (Taylor et al., 1982, Rogol et al., 1983, Hanninen et al., 2003, Richardson et al., 2007.) Arvanitidou et al. (1994) isolated *C. jejuni* from 1.0% of Northern Greek drinking water samples (5/500 samples). In 70% of the water samples from rivers or lakes in the Warsaw region of Poland, Popowski and colleagues detected *C. jejuni, C. coli, or C. lari* (Popowski et al., 1997).

2.3.6 Detection methods of *Campylobacter*

There is a growing need for fast and sensitive methods for specific detection and identification of zoonotic microorganisms as food safety has become an increasing concern for consumers. The use of culture-dependent and/or culture-independent methodologies requires laboratory diagnosis of *Campylobacter* infection. Several new approaches to the detection of different bacteria from food have been used recently. These methods include ELISA, IFT, nucleic acid probes, PCR etc. With respect to the detection limit, speed, and the potential for automation, the PCR technique has several advantages over classical bacteriology and has been successfully applied to the detection of *Campylobacter spp* (Linton et al., 1997; Lawson et al., 1999; Metherell et al., 1999; Vanniasinkam et al., 1999 and Lubeck et al., 2003).

2.3.6.1 Culture methods

There is a lack of consensus on the issue of the standard culture medium for *Campylobacter* growth in the laboratory. Special requirements for growth temperature, gaseous environment and nutrient-rich basal medium are major obstacles to the development of the optimum medium for this fastidious organism. Another difficulty is the over-growth of coliform bacteria, *Proteus spp.*, yeasts and molds in the *Campylobacter* culture (Goossens and Butzler, 1992, Stern et al., 1992 and Jeffrey et al., 2000).

A selective medium for the isolation of *Campylobacter* from stool samples was developed by Skirrow (1977). This medium allowed *Campylobacter* to be successfully recovered and therefore provided evidence linking illness to food contamination, especially chicken. Enriched brucella medium was developed by Wang et al. (1980) for the storage and transport of *Campylobacter fetus* subsp. *jejuni* cultures.

A selective medium (Preston medium) for the isolation of *Campylobacter* from faeces as well as environmental samples was developed by Bolton & Robertson (1982). The inclusion of sodium pyruvate, sodium metabisulphite and ferrous sulphate (FBP) to improve the quenching of toxic oxygen derivatives (Bolton et al., 1984) was a modification to the original Preston formulation.

The comparison of selective media for isolation of *Campylobacter jejuni / coli* was studied by Bolton et al., (1983). They compared five different selective media for the

isolation of *Campylobacter*: Skirrow's, Butzler's, Blaser's, Campy-BAP and Preston's medium. Preston medium was found to be the most selective medium for *Campylobacter*, preceded by enrichment on modified Preston Enrichment Broth, while Butzler was the least efficient. Several selective agars were tested for their effectiveness in isolating *Campylobacters* by Zanetti et al., (1996). Equally effective are Preston, Charcoal Cefoperazone Deoxycholate (CCDA) and Butzler agars. The use of CCDA and incubation at 42^0 C instead of 37^0C is usually the methodology of choice because it allows more strains of *Campylobacter* to be isolated.

The performance of pre-enrichment media for the recovery of *Campylobacters* from food using both artificially and naturally contaminated samples was compared by Baylis et al. (2000). All pre-enrichments included an initial resuscitation period, 4 hours at 37° C, and all were subcultured to mCCDA agar after pre-enrichment. The broths for pre-enrichment were: Bolton broth, *Campylobacter* broth for enrichment (CEB) and Preston broth. The growth of the most significant number of *Campylobacter* strains was supported by Preston broth, but failed to inhibit some competitor organisms. CEB, on the other hand, inhibited all competitors but failed to support all of the strains of *Campylobacter*. The standard method for detection and isolation (ISO, 2006a) and the direct plating method for *Campylobacter* enumeration (ISO, 2006b) both use mCCDA as selective agar. In these techniques, Bolton broth is used for the enrichment phase with incubation for 4-6 hours at 37 ^{0} C in a microaerophilic atmosphere and with further incubation for 40-48 hours at 41.5 ^{0}C and plating on mCCDA and another operator's own choice of agar medium.

2.3.6.2 Molecular methods

Campylobacter isolation from clinical, food and environmental samples is laborious and takes an incubation period of up to 4-5 days. Moreover, methods dependent on culture can misidentify species. Numbers of techniques are in use for detection of Campylobacter spp. with precision, such as random amplification of polymorphic DNA (RAPD), pulsed field gel electrophoresis (PFGE), DNA hybridization, latex agglutination, polymerase chain reaction. PCR is usually accepted for detection of *Campylobacter* in food and is the most sensitive, specific and reliable test.

Many PCR-based assays have been developed by researchers in recent years for the detection of *Campylobacter* in food. Most of these PCR assays developed *16S rRNA*

targets for rapid detection and identification of *Campylobacter* (Kulkarni et al., 2002, Maher et al., 2003), *23S rRNA* gene (containing strain-specific intervening sequences) and the area of internal transcribed spacer (ITS) containing species-dependent sequence composition (Eyers et al., 1993, Fermer and Engvall, 1999 and Man et al., 2010). The resulting tree had the highest resolution in differentiating between members of the *Campylobacter* genus when all three regions (16S rRNA, ITS-region and 23S rRNA) were combined to create a phylogenetic tree (Man et al., 2010). More recently, real-time PCR techniques have been developed that demonstrate the potential for detection in chicken samples of as little as 1 CFU and in less than 2 hours (Debretsion et al., 2007).

A method for PCR detection, species level identification, and fingerprinting of *C. jejuni* and *C. coli* directly from diarrheic samples was developed by Linton et al. (1997). For PCR detection and differentiation of *C. jejuni* and *C. coli*, three sets of primers were designed. On the basis of their 16S rRNA gene sequences, the first PCR assay was designed to co-identify *C. jejuni* and *C. coli*. The second PCR assay, based on the sequence of the hippuricase gene and the outer protein membrane (*mapA* gene), identified all *C. jejuni* reference strains tested and also strains of that species that lack detectable activity of hippuricase and MAPA protein presence. All tested reference strains of *C. coli* were identified by the third PCR assay, based on the sequence of a cloned (putative) aspartokinase gene and the downstream open reading frame.

A technique for identifying *Campylobacter jejuni* based on a species-specific gene that encodes a membrane protein (MAPA protein) has been developed (Stucki et al., 1995). In all the *C. jejuni* strains tested, the MAPA protein was present and was absent in *C. coli* and related *Campylobacters.*

A rapid and sensitive assay for the detection of small numbers of *C. jejuni* and *C. coli* cells in environmental water, sewage and food samples was developed by Waage et al., (1999). A semi-nested PCR was performed based on specific amplification of the intergenic sequence between the two genes of *Campylobacter* flagellin, flaA and flaB, and agarose gel electrophoresis was visualized for the PCR products. In water samples containing background flora consisting of up to 8,700 heterotrophic organisms per ml and 10,000 CFU of coliform bacteria per 100 ml, the assay detected 3 to 15 CFU of *C. jejuni* per 100 ml. Assay 10 was also carried out with food samples analyzed with or without overnight enrichment, with samples subjected to overnight

enrichment being able to detect as little as <3 CFU per g of food, while variable results were obtained for samples analyzed without prior enrichment.

A multiplex PCR assay to identify and discriminate between *C. coli*, *C. jejuni*, *C. lari*, and *C. upsaliensis* isolates was developed by Klena et al. (2004). With 105 genetically defined isolates of *C. coli*, *C. jejuni*, *C. lari*, and *C. upsaliensis*, 34 strains representing 12 additional *Campylobacter* species, and 24 strains representing 19 non-*Campylobacter* species, the multiplex PCR assay has been validated. Application to whole-cell lysates of the multiplex PCR method obtained from 108 clinical and environmental thermotolerant *Campylobacter* isolates resulted in a 100% correlation with methods of biochemical typing. For the detection of *C. coli* and *C. jejuni*, Persson and Olsen (2005) developed a multiplex-PCR method specifically designed for application in routine diagnostic laboratories. The primers were directed towards the following loci: *C. jejuni* characteristic of the hippuricase gene (hipO), a sequence partially covering the *C. coli* characteristic of the aspartokinase gene, and a universal sequence of 16S rDNA genes serving as the PCR's internal positive control. The method was tested on strains of 47 *C. coli* and 88 *C. jejuni* and found to be almost 100% consistent with biochemical analysis (all except for one strain of *C. coli*), irrespective of whether the DNA was prepared from colonies by simple boiling or Dneasy Tissue Kit. At 10–100 cells per PCR, pure cultures of *C. coli* and *C. jejuni* were identified. Asakura et al. (2008) developed and evaluated a cytolethal distending toxin (cdt) gene-based multiplex PCR assay for the detection of *C. jejuni*, *C. coli* and *C. fetus* cdtA, cdtB or cdtC genes with 76 *Campylobacter* strains belonging to seven different species and 131 other bacterial strains of eight different genera, respectively. A specific primer set for a specific species 'cdtA, cdtB or cdtC gene could amplify the desired gene from a mixture of either two or all three species' DNA templates. The detection limit of the *C. jejuni*, *C. coli* or *C. fetus* was 10-100CFU / tube based on the presence of the cdtA, cdtB or cdtC gene in the multiplex PCR assay.

2.3.7 Public health significance of broiler origin *C. jejuni*

As one of the most significant causes of foodborne gastroenteritis in humans, *Campylobacter* species have gained global notoriety and this is further complicated by the rise in the number of multiple drug-resistant *Campylobacter* species and their presence in a number of animal reservoirs. In the poultry gastrointestinal tract, *Campylobacter* is commonly found and is considered a commensal microorganism

(Newell and Fearnley 2003). Some researchers have considered poultry to be a natural *Campylobacter* reservoir, and its body temperature is appropriate for *Campylobacter* growth. Well-known major sources of human foodborne diseases attributed to *Campylobacter* are poultry meat (Corry and Atabay 2001). *C. jejuni*, followed by *C. coli* are the most prevalent species isolated from chickens (Newell and Fearnley 2003; Wainø et al. 2003 and Salihu et al. 2008). The higher occurrence of *Campylobacter* in chicken meat retailed in hypermarkets (91.4 %) was also reported in an earlier study, while those sold in wet markets were lower (70.7 %) (Kottawatta et al. 2017).

2.3.8 Status of *C. jejuni* in broiler of Bangladesh

Very few studies have been conducted to evaluate the campylobacter status in broilers of Bangladesh. Poultry intestines are a positive environment for the colonization of Campylobacter; therefore, the risk of human campylobacteriosis caused by the consumption of its contaminated meat is of great concern to human health (Mirzaie et al., 2011). The results of a recent study in Bangladesh's Mymensingh and Gazipur districts showed that the prevalence of Campylobacter was 40.5% in broiler and cockerel flocks (Hasan et al., 2020). However, 78% prevalence in broiler flocks was recorded by Kabir et al., 2014a, which is the only study in Bangladesh on Campylobacter prevalence at flock level. Another study was designed to identify and characterize Campylobacter species from broiler meat samples collected from the KR market at Bangladesh Agricultural University, Mymensingh (leg muscle, breast muscle, and cloacal skin). A total of 50 samples were subjected, using cultural and biochemical techniques, to bacterial isolation and identification. 70.97% (n = 22) of the 31 positive Campylobacter isolates were Campylobacter jejuni, and the remaining 29.04% (n = 09) were Campylobacter coli (Kabir et al., 2014b).

2.4 *Escherichia coli*

2.4.1 An overview on *E. coli*

E. coli is a Gram-negative, oxidase negative, facultatively anaerobic, straight cylindrical rod-shaped measuring 1.1-1.5 x 2.0-6.0 coliform bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms (Tenaillon *et al*., 2010). The genera *Escherichia* diverged around 102 million years ago, which conforms with the divergence of their hosts (Battistuzzi *et al*., 2004). Though most of the *E. coli* strains are harmless, some serotypes can cause

severe food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination (Vogt and Dippold, 2005). The harmless strains are part of the normal microbiota of the gut, which can benefit their hosts by producing vitamin K2 (Bentley and Meganathan, 1982).

A Bavarian paediatrician named Escherich had performed studies on the intestinal flora of infants and discovered a typical microbial inhabitant in healthy individuals, which he named *Bacterium coli*. In 1885, *Escherichia coli* was first described by Theodor Escherich (Escherich, 1988) And the bacterium was renamed in his honour to *Escherichia coli* in 1919 (Kaper et al., 2004).

The taxonomy of *E. coli* is summarized below:

Table 2.1-Taxonomy of *E. coli*

Domain: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Enterobacteriales
Family: Enterobacteriaceae
Genus: *Escherichia*
Species: *Escherichia coli*

E. coli has been designated as a model organism for biological research for decades because of its well-known biology, small and less complicated genome, quickly grow in a chemically defined culture media, short doubling time and has also taken the position as one of the most used host organisms in the bioprocess industry. There are versatile strains of *E. coli* exist including pathogens causing diarrheal diseases, urinary tract infections and sepsis (Kaper *et al*., 2004). The most used strains for biotechnological applications are derivate from *E. coli* K-12 (e.g. W3110 strain) and B families (e.g. BL21).

Serotyping is a method for characterization of *E. coli* based on differences in the antigenic structure on the bacterial surface. These are bacterium's O-antigen, a polysaccharide act on the bacterium's lipopolysaccharide (LPS) in the outer membrane, and the H-antigen consists of flagella protein. Serotyping may also include the K antigen and the F-antigen. The strain of *E. coli* is classified based on those known antigens though geographical variation also have observed. Serotyping is

one of the critical tools which can be used in combination with other methods to distinguish pathogenic *E. coli* strains as specific pathogenicity attributes are often linked to certain serotypes (Gyles, 2007).

2.4.2 Diseases caused by avian pathogenic *Escherichia coli* (APEC)

Avian pathogenic *E. coli* strains are known as APEC (Dho-Moulin & Fairbrother 1999) and are associated with diverse diseases, mainly extraintestinal, being responsible for great losses in the avian industry (Gross 1994). These diseases include: inflamation of the oviduct that results in decreased egg production and sporadic mortality in laying chickens and breeders, salpingitis that occurs when *E. coli* ascends the oviduct from the cloaca and extension into the body cavity through the compromised oviduct wall that leads to concurrent peritonitis (Bisgaard et al. 1995).

Colisepticemia is the most important disease caused by APEC strains. This infectious disease is considered to be initiated in the avian upper respiratory tract after a primary infection caused by different virus such as the Newcasttle virus, Infectious Bronchitis virus or Mycoplasma (Gross 1994). These primary infections would increase the avian susceptibility to APEC strains due to the decilliation of the upper respiratory cells and exposition to ammonia and contaminated dust existing in the growth animal environment would also favor the infectious process (Oyentude et al. 1978, Nagaraja et al. 1984). The respiratory infection caused by APEC strains, further to the virus infection, is considered to be the initial step for colisepticemia development in birds (Gross 1994). This infection is also referred to as aero sac disease and usually occurs among birds with 2 to 12 weeks of age, with the majority of the cases occurring among birds with 4 to 9 weeks of age with mortality reaching rates as high as 20% (Dho-Moulin & Fairbrother 1999). Death is the usual outcome of colisepticemia, but some birds may completely recover or recover with residual sequelae, as meningitis, panophthalmitis (swollen eye), osteoarthritis, synovitis and coligranuloma (Hjarres's Diseases) that is characterized by multiple granulomas in liver, cecum, duodenum and mesentery (Bisgaard et al. 1995).

In broilers and hatchers chickens, APEC strains also cause a syndrome named "swollen head syndrome". Lesions observed in this syndrome include gelatinous edema on the skin head and peri orbital tissues, and, in some cases, fibrous exudates

in the subcutaneous head tissues and in the lachrymal glands (Pattison et al. 1989, Nunoya et al. 1991). Swollen head syndrome was first described in the South America (Morley & Thomson 1984) and is considered to be an important avian disease in various countries including Brazil (Arns & Hafez 1992). This syndrome has caused considerable losses in the avian industry because it is responsible for mortality of 3 to 4% of the birds and for reduction of 2 to 3 % at the egg production (Morley & Thomson 1984). Swollen head syndrome usually begins after an acute rhinitis caused by pneumovirus being followed by the invasion of the subcutaneous skin tissues by *E. coli* what causes the characteristic edema (Picault et al. 1987, Hafez & Loehren 1990).

In broilers, APEC strains are also associated with cellulitis that is characterized by a necrotic dermatitis of the abdomen and thighs (Dho-Moulin & Fairbrother 1999). Epidemiological data about this syndrome are not known but the lesions associated with cellulitis causes losses in the avian industry due to carcasses condemnation (Elfadil et al. 1996).

Some studies have showed positive relation among APEC and human extraintestinal pathogenic *E. coli* (ExPEC), mainly uropathogenic *E. coli* (UPEC) and newborn meningitis-causing *E. coli* (NMEC), sugesting that some APEC strains could be considered potential zoonotic agents (Ewers et al. 2007, Moulin-Schouleur et al. 2007, Johnson et al. 2008).

2.4.3 Virulence factors of APEC strains

Several investigations have added knowledge about the pathogenic mechanisms expressed by APEC strains (Dho-Moulin & Fairbrother 1999). The virulence factors that have been described to be expressed by these strains include adhesins, toxins, iron uptake systems, and resistance to the host serum.

2.4.3.1 Adhesins

The bacterial adhesion to epithelium tissues is considered to be an important step for the establishment of the *E. coli* infection since it permits the bacterial linkage and maintenance in close contact to the host epithelial tissues (Moon 1990). Evidences that the adherence capacity of *E. coli* could be a virulence factor were initially proposed by Arp et al. (1980) which observed that a fimbriated and virulent strain was more persistent in the turkey trachea than an avirulent and afimbriated strain.

Among APEC strains, Type 1 fimbriae are related to the adhesion to the avian upper respiratory tract (Wooley et al. 1998). The adhesive properties of Type 1 fimbriae are inhibited by specific antiserum and by D-mannose, a carbohydrate that is its cellular receptor on the eurocariotic cell membrande. These characteristics are used for its characterization (Gyimah & Panigraphy 1988). Pourbaskhsh et al. (1997) suggested that while Type 1 fimbriae is associated with the upper respiratory tract initial colonization P fimbrial adhesin may be involved in the bacterial establishment in deeper avian organs. Wooley et al. (1998) also suggested that while Type 1 is necessary to initial colonization of the respiratory epithelium, additional factors like motility and colicin V production would be responsible by the persistence of the colonization and by the observed trachea lesions development. Marc et al. (1998) demonstrated, with the utilization of a fim- APEC mutant, that Type 1 fimbriae is not strictly required as a colonization factor during the avian colibacilosis development.

P fimbrial adhesins were first described among *E. coli* strains associated with human urinary tract infections (UTI) (Kallenius et al. 1980) being also found among APEC strains (Achtman et al. 1983, Dozois et al. 1992). P fimbriae is encoded by the pap operon that is located in the bacterial chromosome (Latham & Stamm 1994). papA gene encodes for the major structural protein (PapA), papI and papB are regulatory genes responsible for the phase variation process (Mol & Oudega 1996). *papE* gene encodes for the fimbrial structural extremity, *papG* gene encodes for the adhesin, and *papD*, *papH*, *papJ*, *papF* and *papK* genes are responsible for the expression of proteins related to the hole integrity of the complex fimbrial assemblage (Mol & Oudega 1996). The adhesive property of P fimbriae is conferred by the terminal adhesin *PapG* that presents three molecular variants (I, II and III) (Hoschützky et al. 1989) that recognize different isoreceptors containing a-D-galactosyl-(1-4)-b-galactopyranosil carbo-hydrate.

The role of P fimbriae in the APEC pathogenicity has not been completed elucidated yet. Pourbakhsh et al. (1997) using in vivo studies, verified that P fimbriae presented phase variation and suggested that these adhesins would not be important for the initial colonization of the upper respiratory tract, but it would be in the latter infection stages. Recently, Kariyawasam et al. (2006) demonstrated that the pap operon is present in a pathogenicity island of APEC strain APEC-O1. The authors suggested

that the pap occurrence in horizontaly acquired genomic regions might be involved in the transformation of avirulent strains into virulent ones.

Curli fimbriae are thin and curly appendices found on the cell surface of *Salmonella enterica* and *E. coli* (Olsén et al. 1989), and are responsible for the bacterial linkage to proteins of the extracellular matrix (Collinson et al. 1993) and for bacterial survival in the external environment (Olsén et al. 1993). Curli fimbriaes are optimally expressed at 26ºC during the growth in the stationary phase and in low osmolarity medium (Olsén et al. 1993). The genes responsible for curli fimbriae expression are encoded by two operons: csgBAC and csgDEFG. *csgA* gene encodes for the monomers structural subunits, named curlina while *csgB* gene encodes for a protein needed for curlina complex stability. The operon csgDEGF has it expression controlled by environmental factors like temperature, osmolarity, pH, and other stress factors (La Ragione & Woodward 2002). The curli fimbriae expression is also dependent of RpoS factor, an ó factor that controls the expression of several genes during the stationary phase of bacterial growth (Olsén et al. 1993). Curli related sequences have been widely found among APEC strains. Maurer et al. (1998) detected *csgA* gene in all APEC strains analyzed. The same curli-related sequence was found among 90% of the strains isolated by Knöbl (2001) from ostriches with respiratory disease and among 70% of strains analyzed by Campos et al. (2005). In this latter study, the authors detected *csgA* sequence in all APEC isolated from chickens with septicemia and in none of the *E. coli* strains isolated from the intestinal microbiota of chickens. However a study made by McPeake et al. (2005) demonstrated that the *csgA* gene was presented among all strains analyzed, isolated from chickens with septicemia and from healthy chickens.

Other adhesins identified among APEC strains comprised the AC/1 fimbriae and type 1-like fimbriae (La Ragione & Woodward 2002). The presence of fimbriae F17, Afa, Sfa, and Eae DNA-related sequences among APEC strains may indicate that these adhesins can be found on the cell surface of APEC (Stordeur et al. 2002, Campos et al. 2005, McPeake et al. 2005) and could also be implicated in the pathogenesis presented by these strains.

2.4.3.2 Temperature-sensitive hemagglutinin

The tempe-rature-sensitive hemagglutinin (TSH) is a protein expressed by APEC strains that present chicken erythrocytes hemagglutination activity at 26ºC and have this activity repressed at 42ºC (Provence & Curtiss 1994). TSH is a serine-protease autotransporter protein that is synthesized as a 140 KDa precursor, and is cleaved in two subunits in the bacterial periplasm: one subunit, with 33 KDa, that remains inserted in the outer membrane and functions like a passenger domain; and other subunit with 106 KDa that is secreted to extracellular environment. The 106 KDa subunit remains temporally in the outer membrane and mediates the bacterial adhesion during the initial stages of the infection (Stathopoulos et al. 1999). After its secretion, the 106 KDa sub-unit probably presents a characteristic proteolytic activity, that renders the TSH protein a bifunctional protein, with adhesive and proteolytic activities (Kostakioti & Stathopoulos 2004).

TSH is encoded by the *tsh* gene (Provence & Curtiss 1994). This gene is located in high molecular weigh plasmids (Dozois et al. 2000, Stehling et al. 2003, Johnson et al. 2006), mainly in ColV plasmids and is frequently found among APEC strains. Maurer et al. (1998) detected the *tsh* gene among 46% of the studied APEC strains and in none of the commensal isolates. Campos et al. (2005) demonstrated that the *tsh* gene was found among 25 and 50% APEC strains isolated from chickens with septicemia and swollen head syndrome, respectively, and in only 6% of the commensal strains. Because the association of the tsh gene with APEC pathogenicity, Ewers et al (2004) proposed its utilization as a molecular marker to detect APEC strains.

2.4.3.3 Iron acquisition systems

APEC strains survive and growth in environments with low iron availability, mainly inside the host, because the expression of iron acquisition systems (Dho & Lafont 1984). The bacterial iron acquisition mechanisms include the production of siderophores that act as ion chelants in the host (Williams & Griffiths 1992). Two types of siderophores are known: fenolates and hidroxamate. Aerobactin is a hidroxamate siderophore that is encoded by a plasmid operon (Gibson & Magrath 1969, Williams 1979). This siderophore is also found among fungi, enteroinvasive *E. coli* and APEC strains (Dho & Lafont 1984, Waters & Crosa 1991). Dho & Lafont (1984) observed a positive correlation between the low iron concentration, APEC

growth ability and the lethality capacity to one-day old chickens observed in these strains. Also, Linggood et al. (1987), Dozois et al. (1992), Emery et al. (1992) and Silveira et al. (2002a) demonstrated that pathogenic APEC strains expressed iron uptake systems while non pathogenic strains did not express. The yersiniabactin system (*fyuA* and *irp-2* genes) (Pelludat et al. 1998, Schubert et al. 1998, Karch et al. 1999) were found in higher frequencies among APEC strains (Gophna et al. 2001, JanBen et al. 2001). Genes related with another iron acquisition systems, like *iucA* and *fepC* genes, were also found among APEC strains (Okeke et al. 2004). A recent work realized by research group (Campos et al. 2005) showed a high frequency of iron uptake related genes among pathogenic avian *E. coli* strains.

Recently, a sitABCDE *Salmonella enterica* homologue system were identified in an APEC strain (Sabri et al. 2006, Sabri et al. 2008). In this strain, the sitABCDE is an iron and manganese transporter system that, in combination with other iron uptake systems, can contributes to iron acquisition and to the oxidative stress bacterial survival (Sabri et al. 2006). Among APEC strains, iron acquisition systems can be encoded by plasmid genes (Johnson et al. 2006, Sabri et al. 2006) or by chromosomal patho-genicity islands (Kariyawasam et al. 2006).

2.4.3.4 Colicins

These are proteins expressed by *E. coli* that inhibit the bacterial growth from the same or related species. Colicins are compounded by two subunits: one that provokes bacterial cell lesions and other that protects the bacterium against their own colicins (Skyberg et al. 2008). Colicins can be encoded by genes located in plasmids. Because of this, they are frequently called Col plasmids. Works establishing colicin expression by APEC strains indicated that colicins Ia, Ib, E1, E2, E3, I, K, B and V are the most prevalent ones among these bacterial strains (Fantinatti et al. 1994, Silveira et al. 2002a).

The majority of APEC strains have colicin V plasmids (Wray & Woodward 1997). Theses plasmids also contain other pathogenicity related genes (Valvano et al. 1992, Johnson et al. 2003). Mutations at ColV plasmids demons-trated decreasing of the virulence, suggesting that some genes linked to ColV plasmids are involved in the establishment of avian infection (Skyberg et al. 2008).

2.4.3.5 Capsule

Some *E. coli* strains have an N-acetyl muramic acid capsule on their cell surface that interacts with the classical complement pathway conferring immune resistance to the bacteria and that induces the immune resistance (Jann & Jann 1977).

K1 capsular antigen is frequently associated to APEC strains belonging to serogroups O1, O2 and to non typable strains (Gross 1994). Pourbakhsh et al. (1997) demonstrated the three APEC strains expressing capsule K1 were more resistance to the serum bactericidal effects than APEC strains that expressed other K antigens.

2.4.3.6 Serum resistance

The bacterial resistance to the complement, mediated by bacterial surface structures like LPS, capsule, Col V colicin, and outer membrane proteins, have been associated with APEC strains (Gross 1994, Fantinatti et al. 1994, Ngeleka et al. 1996, Lynne et a. 2007).

Pfaff-McDonough et al. (2000) suggested that the Iss factor (increased serum survival) is associated with APEC pathogenicity since the *iss* gene have been found more frequently among pathogenic than non pathogenic strains, despite of serotype, avian species and lesion origin. The occurrence of iss gene in conjugative Col V plasmid (Johnson et al. 2002, Mellata et al. 2003) can suggest the relationship of Iss factor to the APEC pathogenicity. Besides the role of Iss, Mellata et al. (2003) suggested that the O78 polysaccharide and the K1 capsule are virulence factors that increase the bacterial serum survival.

2.4.3.7 Toxins

Some APEC strains are able to produce toxins like labile temperature (LT) and stable temperature (ST) enterotoxins (Smith & Gyles 1969), and verotoxins known as Shiga-toxins (Stx) (O'Brien et al. 1977; O'Brien et al. 1982, Emery et al. 1992, Parreira & Yano 1998, Fantinatti et al. 1994, Blanco et al. 1997).

APEC strains cytotoxic activity to Vero cells was observed by Fantinatti et al. (1994) and by Parreira & Yano (1998). Parreira & Gyles (2002) identified a Stx- gene among *E. coli* strains isolated from chickens suffering from cellulitis, septicemia, and swollen head syndrome and from sick turkeys. These same authors (Parreira & Gyles 2003) described a vacuolating toxin to be expressed by an APEC strain. This toxin is

encoded by the *vat gene* that belongs to a pathogenicity island and was latter found to be expressed by another APEC strains (Ewers et al. 2004, Ewers et al. 2005).

2.4.3.8 Other virulence factors

Other virulence factors found among APEC strains include pathogenicity islands (Parreira & Gyles 2003, Kariyawasam et al. 2006) and the locus of enterocyte effacement (LEE) (Foster et al. 1998, Pennycott et al. 1998, La Ragione et al. 2002). Rodriguez-Siek et al. (2005) showed that *E. coli* strains isolates obtained from human UTI and avian colibacilosis could have substantial overlap in terms of serogroups, phylogenetic groups and virulence genotypes, including plasmid-DNA-related sequences, adhesion, iron uptake, protectins and toxins-related sequences.

The Congo red linkage capacity in agar medium has been observed among APEC strains. Some authors have purposed the utilization of this characteristic as virulence marker to APEC strains (Berkhoff & Vinal 1986, Corbett et al. 1987).

2.4.4 Status of virulence associated genes in *E. coli* in poultry of Bangladesh

Very few studies to evaluate the virulent gene status in Bangladeshi broilers have been conducted. Poultry intestines are a positive environment for the *E. coli* and therefore containting virulent factors can posses colibacillosis in chickens. The results of a recent study in Bangladesh showed that the prevalence of *E.coli* virulent genes in poultry farms was 75-100% (Saha et al., 2020). However, Ivey et al. (2020) recorded a 36.36% prevalence in layer flocks of Bangladesh. Another study (Ahmed et al., 2020) was designed to identify virulent genes in commensal *E. coli* from broiler samples collected from Chattogram, Bangladesh, and 13 virulence genes were identified in the 32 commensal *E. coli* genomes. *astA* (EAST-1 heat-stable toxin) and *iss* (Increased Serum Survival) were the most common virulence determinants, found in 50% and 44% of isolates, respectively. Multiple virulence genes were harbored by a major proportion of isolates (53%). Notably, at least 4-6 virulence determinants were present in seven of the 32 isolates, but none of the strains carried virulence gene combinations known to be characteristic of pathogenic subtypes.

Chapter-3: Materials and Methods

3.1 Study area, design and sample size

The reference population of the present study was commercially reared meat type chicken (broilers). A cross-sectional survey was conducted between June 2019 to February 2020 in six districts of Bangladesh (Chattogram, Dhaka, Narsingdi, Narayanganja, Munshigonj and Khagrachori). First five districts are plain land and were selected based on their poultry and human population density and the later to catch up the situation in hills. List of all broiler farms (sampling frame) of the five selected districts were collected from the District Livestock Office (DLO) and a total of 216 commercial broiler farms of the study area was selected using simple random sampling. Further, raw and processed meat samples from live-bird markets and supermarkets, respectively was collected from the Chattogram region. We randomly selected eight supermarkets in the Chattogram metropolitan area, 20 live-bird markets (LBM) in the Chattogram metropolitan area, and 20 live-bird markets from five peripheral Upazila of Chattogram.

3.2 Data collection

Necessary verbal permission was taken from the individual farmer before sampling the birds and epidemiological information was recorded on questionnaire. The respondents were priorly informed about the purpose of the study and the procedure of sample collection. A farm was included in the study only upon affirmative response, otherwise excluded. A pre-designed structured questionnaire was used to record epidemiological data at farm, live bird market, and super shop level through face-to-face interviews and physical observation. Focal points in the questionnaire were – 1. Farm management: number of houses, establishment year, type of floor, water supply, litter materials, amount of litter materials used, number of flocks per year, number of employees; 2. Biosecurity and hygiene: use of footwear and distinct cloth, foot bath facility, all in all-out system, disinfection of farm before restock, house empty for >14 days before restock; 3. Flock attributes: flock size, age of birds, number of dead birds per flock; 4. Use of vaccine and drugs: vaccination and age of vaccination, usage of antibiotics and duration of usages; and 5. Farmers demographic information. In case of LBM and super shop – data on personal hygiene, use of disinfectants, water source, slaughtering area, etc. were collected.

3.3 Sample collection procedure

3.3.1 Samples from broiler flocks

From each farm, five birds were randomly sampled and cloacal swabs were collected using sterile cotton swab by inserting into the bird's cloaca. Later the cloacal swabs were pooled by placing in a falcon tube containing buffered peptone water (BPW) (Oxoid Ltd, UK) and transported to the clinical pathology laboratory (CPL) of Chattogram Veterinary and Animal Sciences University (CVASU) using the same transport medium maintaining cool chain (4°C).

3.3.2 Samples from live bird markets (LBMs) & super shops

Five random bird-stall per LBM were selected randomly for sampling. One dressed-broiler carcass from each of the five bird-stalls and one dressed-broiler carcass from each supermarket was collected. The dressed-broiler carcasses were transported in an ice-box to CPL of CVASU. At the laboratory, 5 gm of pooled homogenized sterile samples (breast muscle, thigh muscle, liver, and gizzard) were placed in separate falcon tube containing 45 ml BPW (Oxoid Ltd, UK). All the samples were kept immediately after processing at 4ºC for bacteriological investigation. In total, 176 meat samples were collected from 40 LBMs and 8 supermarkets.

3.4 Sample evaluation

Samples were analyzed for identification of *Campylobacter spp* and *E. coli* following the methods described by Lund et al. (2003) and Lund et al. (2004).

3.4.1 Isolation and identification of *C. jejuni* from the collected samples

Standard bacteriological approaches followed by molecular techniques were applied for isolation and identification of *C. jejuni* from the cloacal swabs and retail chicken meat of broiler chicken. Briefly, all samples were directly inoculated on selective campylobacter base agar (Oxoid Ltd, UK) containing antibiotics and 5-7% sheep blood (Vanderzant & Splittstroesser, 2001). The plates were incubated in an anaerobic jar (Oxoid™ AnaeroJar™ 2.5L) under the microaerophilic conditions with a CO_2 sachet (Thermo Scientific™ Oxoid AnaeroGen 2.5L sachet) (10% CO_2, 95% humidity) in 42° C for three days.

After 72 hours, single characteristic (small, round, creamy-gray, or whitish) colonies from each plate were selected and inoculated in tryptic soy broth (Oxoid Ltd, UK) and

incubated 37°C for three days under microaerophilic condition. The presumptive *Campylobacter* isolates were subjected to microscopic examination to observe the seagull appearance of *C. jejuni* with Gram staining (Vandamme et al., 2008). The isolates were then stored at -80°C in brain heart infusion broth (Oxoid Ltd, UK) containing 50% glycerol for further validation using molecular methods.

3.4.2 Isolation and identification of avian fecal *E. coli* (AFEC) from the collected samples

For the isolation of AFEC from the collected samples, the sample was at first inoculated into a test tube containing buffer peptone water (BPW) (Oxoid Ltd, P^H : 6.2±0.0, Basingstoke, Hampshire, UK) and incubated at 37°C overnight for primary enrichment. In the case of a liver sample, 2-3 grams of the sample after primary enrichment, the culture was streaked on MacConkey agar medium (Oxoid Ltd, P^H : 7.4±0.2, Basingstoke, Hampshire, UK) and incubated at 37°C for 24 hours. Bright pink-colored large colonies yielded on a MacConkey agar plate were suspected as the growth of *E. coli*. Such colonies were streaked on to EMB agar plate (Merck, P^H : 7.1±0.2) and incubated at 37°C for 24 hours. Based on the "green metallic sheen" colony morphology yielded on this medium was taken as the growth of *E. coli*, which was later confirmed by applying Grams staining.

3.4.3 Preservation of the isolates

Both *C. jejuni* and AFEC isolates were cultured in brain heart infusion (BHI) broth, incubated overnight at 37°C. For each isolate 700 µl BHI broth culture was added to 300 µl 15% glycerol in an eppendorf tube. Tubes were properly leveled and stored at -80°C for further investigation.

3.4.4 Sub-culturing on blood agar

The preserved isolates were removed from the freezer and thawed at room temperature. After that, the isolates were inoculated on blood agar and incubated at 37°C for 24 hours. After completing the incubation period, colonies from blood agar were used for DNA extraction to be used for polymerase chain reaction (PCR).

3.4.5 DNA extraction from the isolates

For extraction of DNA from the recovered isolates, boiling method (Englen and Kelley, 2000) was used. Briefly, the procedure was as below:

(i) A loop full of new colonies (about 3-4) was picked from blood agar and transferred to 1.5 ml Eppendorf tubes containing 100µl de-ionized water. The tubes were then vortexed to make a homogenous cell suspension. A ventilation hole was made on the lid of each tube.

(ii) Then, the tubes were boiled at 99°C for 15 minutes in a heat block (Major science company). Immediately after boiling, the tubes were placed into the ice pack for 5 minutes. The process of high-temperature boiling and immediate cooling allowed the cell wall to break down to release DNA from the bacterial cell.

(iii) Finally, the tubes with the suspension were centrifuged at 15000 rpm for 5 minutes. Then 50 µl of supernatant containing bacterial DNA from each tube was collected in another sterile Eppendorf tube and preserved at-20°C until used.

3.4.6 Molecular identification of *Campylobacter*

3.4.6.1 Polymerase chain reaction (PCR) to test for the presence of *Campylobacter spp and C. jejuni*

Polymerase chain reaction (PCR) assay was conducted for the final confirmation of the suspected isolates by conventional PCR using genus-specific primer *16S rRNA* gene and species-specific primer *mapA* gene listed in **Table 3.1**. The amplification of the *mapA* gene was carried out for the detection of *C. jejuni*. The detailed procedure followed is given below.

Table 3.1-Primer and oligonucleotide sequence used for the identification of *Campylobacter spp* and *C. jejuni*

Gene	Primer Sequence	Amplification (bp)	Reference
16S rRNA	Forward primer 5’ ATC TAA TGG CTT AAC CAT TAA AC 3’ Reverse primer 5’ GGA CGG TAA CTA GTT TAG TAT T 3’	857	(Linton et al. 1997)
mapA	Forward primer 5’ CTA TTT TAT TTT TGA GTG CTT GTG 3’ Reverse primer 5’ GCT TTA TTT GCC ATT TGT TTT ATTA 3’	589	(Stucki et al. 1995)

3.4.6.2 PCR reactions:

Molecular investigations on all the isolates were conducted in the molecular pathology laboratory, CVASU. The reagents used for the PCR are shown in **Table 3.2.**

Table 3.2-Reagents used for PCR amplification of the *Campylobacter spp* and AFEC

Serial No	Name	Manufacturer
1	Master Mix	Thermo Scientific
2	Molecular marker	Thermo Scientific O‟
3	Ethidium bromide solution (1%)	Sigma- Aldrich
4	Electrophoresis buffer 50x TAE	Fermantas
5	Agarose powder	Seakem® Le agarose-Lonza
6	Nuclease free water	Thermo Scientific

PCR reactions were conducted with a final volume of 15 µl using 20 picomoles of each primer concentration. Proportions of different reagents used for PCR for two different genes are given in **Table 3.3**. *Campylobacter jejuni* subsp. *jejuni* ATCC 33560 strain and Nuclease-free water were used as positive and negative control, respectively.

Table 3.3- Contents of each reaction mixture of PCR assay

Serial no	Name of the contents	Amount
1	Thermo Scientific PCR Master Mix (2x)	7.5µl
2	Forward primer	1µl
3	Reverse primer	1µl
4	DNA template	2µl
5	Nuclease free water	3.5µl
	Total	**15µl**

PCR was run on a thermocycler (Applied Biosystem, 2720 thermal cycler, Singapore) following the cycling conditions mentioned in **Table 3.4 & 3.5.**

Table 3.4-Cycling conditions used during PCR for detection of the *16S rRNA* gene of *Campylobacter spp*

Serial no	Steps	Temperature and time
1	Initial denaturation	95°C for 10 minutes
2	Final denaturation(35 cycles)	95°C for 30 seconds
3	Annealing	59°C for 1 minute 30 seconds
4	Initial extension	72°C for 1 minutes
5	Final extension	72°C for 10 minutes
6	Final holding	4°C

Table 3.5- Cycling conditions used during PCR for detection of *mapA* gene of *C. jejuni*

Serial no	Steps	Temperature and time
1	Initial denaturation	95°C for 10 minutes
2	Final denaturation (35 cycles)	95°C for 30 seconds
3	Annealing	59°C for 1 minute 30 seconds
4	Initial extension	72°C for 1 minute
5	Final extension	72°C for 10 minutes
6	Final holding	4°C

3.4.6.3 Visualization of PCR products by Agar Gel Electrophoresis

1.5 % agarose gel (W/V) was used to visualize the PCR product. Briefly, the procedure followed as follows:

1. 0.75 gm of agarose powder and 50 ml of 1X TAE buffer was mixed thoroughly in a conical flask and boiled in a microwave oven until agarose was dissolved.
2. Then the agarose mixture was cooled at 50°C in a water bath, and one drop of ethidium bromide was added to the mixture.
3. The gel casting tray was assembled by sealing the ends of the gel chamber with tape and placed an appropriate number of combs in the gel tray.
4. The agarose-TAE buffer mixture was poured into the gel tray and kept for 20 minutes at room temperature for solidification; then combs were removed and the gel was shifted into an electrophoresis tank filled with 1X TAE buffer and kept until the gel is drowned completely.
5. An amount of 5 µl of PCR product for a gene was loaded into a gel hole.
6. 3 µl of 100bp plus DNA marker (Addbio INC, Korea) was used to compare the size of a gene product's amplicon, and the electrophoresis was run at 110 volts and 80 mA for 40 minutes.
7. Finally, the gel was examined using a gel documentation system (UVP UVsolo touch - Analytik Jena AG).

3.4.7 Phylogenetic analysis of *C. jejuni*

3.4.7.1 Gene sequencing

A total of six samples were randomly selected from the *mapA* gene positive samples consisting three meat and three cloacal swabs. Selected *mapA* gene PCR amplicons were purified using a DNA purification kit (Favorgen Biotech Corp, Taiwan). The purified PCR products were Sanger-sequenced with BigDye terminator v3.1 sequencing kit and a 3730xl automated sequencer (Applied Biosystems, Foster City, CA). Nucleotide sequences were determined on both strands (forward and reverse) of PCR amplification products at the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea). The forward and reverse sequences of each of the strains were assembled by CAP3 Sequence Assembly Program (Huang and Madan, 1999). All the sequences were submitted to GenBank of the National Center for Biotechnology Information (NCBI).

3.4.7.2 Evolutionary analysis by Maximum Likelihood method

BLASTn was performed to compare the *mapA* gene sequences of the strains sequenced and those available in GenBank. Based on the findings of the search, *mapA* gene sequence data of another 19 strains from different hosts and geographical locations were included in the phylogenetic analysis. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model (Tamura, 1992). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 25 nucleotide sequences. There were a total of 627 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

3.4.8 Molecular Identification of AFEC

3.4.8.1 Polymerase chain reaction (PCR) to test for the presence of AFEC

Polymerase chain reaction (PCR) assay was conducted for the final confirmation of the suspected isolates by conventional PCR using genus-specific primer *16S rRNA* gene listed in **Table 3.6**. The detailed procedure that was followed is given below.

Table 3.6- Primer and oligonucleotide sequence used for the identification of AFEC

Gene	Primer sequence	Amplification (bp)	Reference
16S rRNA	Forward primer 5–GACCTCGGTTTAGTTCACAGA–3 Reverse primer 5 –CACACGCTGACGCTGACCA–3	585	Schippa et al., 2010

PCR reactions were conducted with a final volume of 15 µl using 20 picomoles of each primer concentration. Proportions of different reagents used for PCR for *16S rRNA* gene is given in **Table 3.7**. *Escherichia coli* ATCC 33560 strain and Nuclease-free water were used as positive and negative control, respectively.

Table 3.7- Contents of each reaction mixture of PCR assay

Serial no	Name of the contents	Amount
1	Thermo Scientific PCR Master Mix (2x)	7.5µl
2	Forward primer (each gene 0.5µl*8)	1µl
3	Reverse primer (each gene 0.5µl*8)	1µl
4	DNA template	2µl
5	Nuclease free water	3.5µl
	Total	**15µl**

PCR was run on a thermocycler (Applied Biosystem, 2720 thermal cycler, Singapore) following the cycling conditions mentioned in **Table 3.8**.

Table 3.8- Cycling conditions used during PCR for detection of *16S rRNA* gene of AFEC

Serial no	Steps	Temperature and time
1	Initial denaturation	95°C for 5 minutes
2	Final denaturation (35 cycles)	94°C for 1 minute
3	Annealing	58°C for 1 minute
4	Initial extension	72°C for 1 minute
5	Final extension	72°C for 7 minute
6	Final holding	4°C

The PCR products were visualized on a Gel documentation system (UVP UVsolo touch- Analytik Jena AG) after electrophoresis with 1.5 % agarose gel (SeaKem® LE Agarose from Lonza) (according to previous details).

3.4.8.2 Multiplex Polymerase chain reaction (PCR) to test for the presence of virulent genes (VAGs) in *AFEC*

All *E. coli* isolates were further investigated for different virulent genes by multiplex polymerase chain reactions (PCR) (Ewers et al., 2005). The detailed procedure that was followed is given below.

The primer sequences used for the PCR are shown in **Table 3.9.**

Table 3.9- Sequences and specificity of PCR primers, and product sizes

Gene	Primer sequence(59–39)	Size (bp)	Primer reference
astA	TGCCATCAACACAGTATATCC TCAGGTCGCGAGTGACGGC	116	(Sanger et al., 1977)
iss	ATCACATAGGATTCTGCCG CAGCGGAGTATAGATGCCA	309	(Dozois et al., 1992)
irp2	AAGGATTCGCTGTTACCGGAC AACTCCTGATACAGGTGGC	413	(Dozois et al., 1992; Janßen et al., 2001)
papC	TGATATCACGCAGTCAGTAGC CCGGCCATATTCACATAA	501	(Franck et al., 1998)
iucD	ACAAAAAGTTCTATCGCTTCC CCTGATCCAGATGATGCTC	714	(Franck et al., 1998)
tsh	ACTATTCTCTGCAGGAAGTC CTTCCGATGTTCTGAACGT	824	(Dozois et al., 1992)
vat	TCCTGGGACATAATGGTCAG GTGTCAGAACGGAATTGT	981	(Dozois et al., 1992)
cva A/B cvi cvaC	TGGTAGAATGTGCCAGAGCAAG GAGCTGTTTGTAGCGAAGCC	1181	(Dozois et al., 1992)

PCR reactions were conducted with a final volume of 25 µl using 20 picomoles of each primer concentration. Proportions of different reagents used for PCR for different virulent genes are given in **Table 3.10**. *Escherichia coli* ATCC 33560 strain and Nuclease-free water were used as positive and negative control, respectively.

Table 3.10- Contents of each reaction mixture of multiplex PCR assay

Serial no	Name of the contents	Amount
1	Thermo Scientific PCR Master Mix (2x)	12.5µl
2	Forward primer (each gene 0.5µl*8)	4µl
3	Reverse primer (each gene 0.5µl*8)	4µl
4	DNA template	2µl
5	Nuclease free water	2.5µl
	Total	**25µl**

PCR was run on a thermocycler (Applied Biosystem, 2720 thermal cycler, Singapore) following the cycling conditions mentioned in **Table 3.11**.

Table 3.11- Cycling conditions used during multiplex PCR for detection of virulent genes

Serial no	Steps	Temperature and time
1	Initial denaturation	94°C for 3 minutes
2	Final denaturation (25 cycles)	94°C for 30 seconds
3	Annealing	58°C for 30 seconds
4	Initial extension	68°C for 3 minutes
5	Final extension	72°C for 10 minutes
6	Final holding	4°C

The PCR products were visualized on a Gel documentation system (UVP UVsolo touch - Analytik Jena AG) after electrophoresis with 1.5 % agarose gel (SeaKem® LE Agarose from Lonza) (according to previous details).

3.5 Data analysis

3.5.1 Statistical analysis

The study unit of the analysis was farm in case of fecal samples and market/super shop in case of meat samples. A farm/market/super shop was considered positive if a pooled farm/market/super shop sample was tested positive in PCR. Therefore, the dependent variable in our study was dichotomous as positive and negative. Several continuous variables (e.g.: No of chicken production, person entry, litter amount, flock size, flock age etc.) have been transformed into categorical variables to perform the analysis. All data from the broiler farms, retail chicken shops (urban and rural), and super shops were inserted and coded in Microsoft office Excel 2016 Excel sheet.

3.5.2 Univariable analysis

The prevalence and 95% confidence intervals were calculated using the modified Wald method in GraphPad software QuickCalcs. The map along with the location of the farm was created using QGIS 3.12.0.

To evaluate the association between independent variables (risk factors/determinants) with dependent variable (sample positive/negative), univariable analysis was performed using χ^2 test and univariable logistic regression models in STATA-IC 13. Independent variables with a P-value of ≤ 0.10 in univariable analysis was considered for inclusion in the multivariable logistic regression model.

3.5.3 Multivariable analysis

A model of multivariable logistic regression was built using independent variables found significant in the univariable analysis. Backward elimination process was followed to reach the final model. A p-value ≤ 0.05 was considered significant in the multivariable model.

Chapter-4: Result

4.1: Broiler fecal sample

4.1.1 Descriptive analysis

4.1.1.1 Prevalence of different organism isolated from broiler fecal samples at farm level

A total of 216 fecal pooled samples were collected for the present study. Among them 98 (45.37%-95% CI 38.7%-52.3%) were confirmed by PCR as *Campylobacter spp*, 27 (12.5%; 95% CI 8.5%-17.7%) as *Campylobacter jejuni*, 177 (81.94 %; 95% CI 76.2%-86.9%) as AFEC and 120 (55.6%; CI 48.7%-62.3%) were confirmed as virulent genes associated (VAGs) AFEC **(Table 4.1)**. All the *Campylobacter* positive isolates produced characteristic round convex dew-drops like non-haemolytic colonies on Campylobacter agar base **(Figure 4.1)**. The colonies of these isolates stained with Gram's stain showed a typical gram-negative spiral, S-shaped or sea-gull shaped rods **(Figure 4.2)**. None of the *Campylobacter* isolates showed any growth following aerobic incubation at 42°C. A total of 98 culture positive isolates were confirmed as *Campylobacter spp* (*16S rRNA* gene- 857bp) by PCR. Among them 27 isolates were identified as *C. jejuni* (*mapA* gene- 589bp) by molecular test **(Figure 4.3 and Figure 4.4)**. Characteristic growth of *E. coli* isolates on MacConkey agar plate, EMB agar plate and Blood agar plate are shown in **Figure 4.5, 4.6 and 4.7,** respectively and the result of Gram's staining property is displayed in **Figure 4.8.** A total of 177 Culture positive isolates were confirmed as *Escherichia coli* (*16S rRNA* gene- 585 bp) using molecular test **(Figure 4.9).**

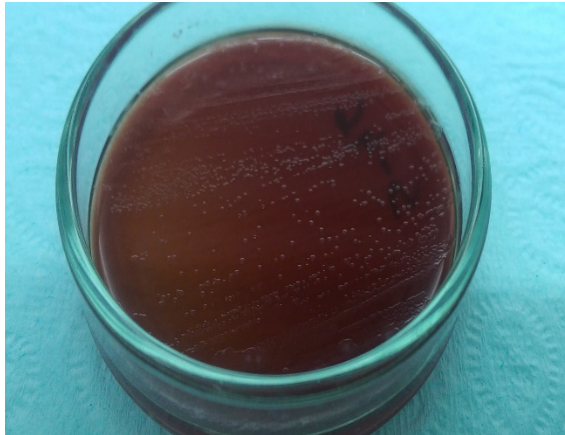

Figure 4.1-Characteristic “round convex dew-drop like non-haemolytic” colonies of *Campylobacter* on Campylobacter agar base

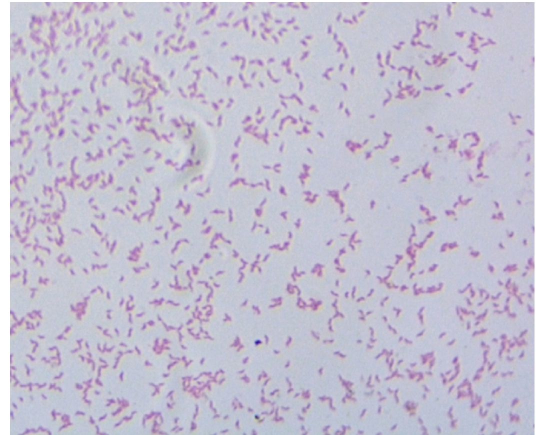

Figure 4.2-Gram’s staining of *Campylobacter* isolate showing characteristic spiral, S-shaped bacteria

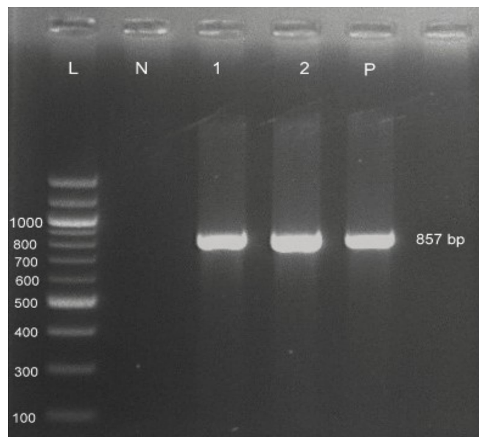

Figure 4.3-Result of PCR assay for *16S rRNA* gene of *Campylobacter spp* isolates; Lane L: 1kb plus DNA ladder; Lane P: Positive control; Lane N: Negative control; Lane 1 and 2: *16S rRNA* gene-sized (857 bp) amplicon

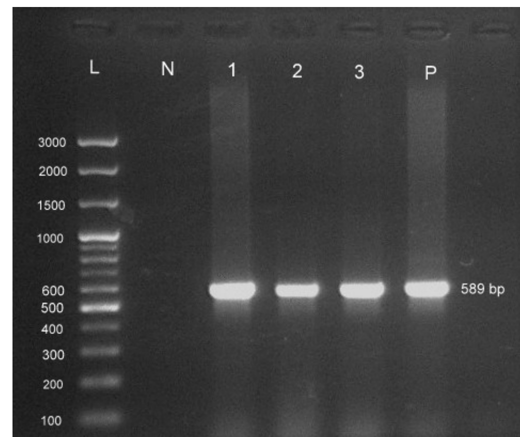

Figure 4.4-Result of PCR assay for *mapA* gene of *C. jejuni* isolates; Lane L: 1kb plus DNA ladder; Lane P: Positive control; Lane N: Negative control; Lane 1, 2 and 3: *mapA* gene-sized (589 bp) amplicon

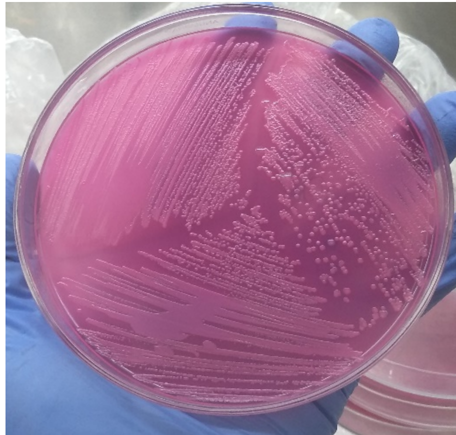

Figure 4.5-Growth of *E. coli* on a MacConkey agar plate

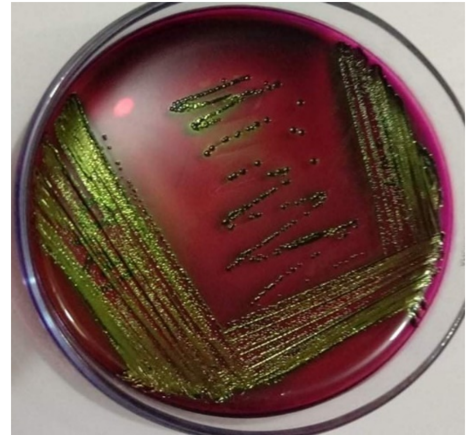

Figure 4.6-Growth of *E. coli* on an EMB agar plate

Figure 4.7-Pure culture of *E. coli* on Blood agar

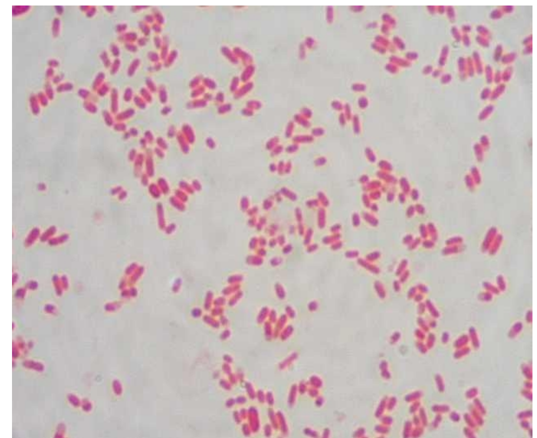

Figure 4.8-Gram's staining results of *E. coli*

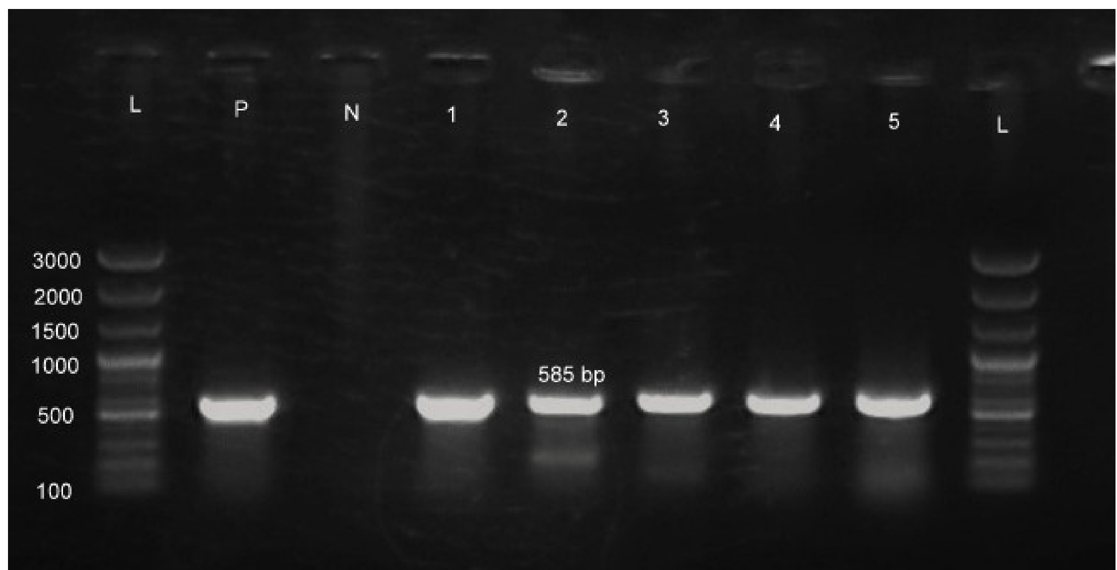

Figure 4.9-Result of PCR assay for the *16S rRNA* gene of AFEC isolates tested; Lane L: 1kb plus DNA ladder; Lane P: Positive control; Lane N: Negative control; Lane 1-5: *16S rRNA* gene-sized (585 bp) amplicon

Table 4.1- Proportionate prevalence of *E. coli* and *Campylobacter spp* colonization in Dhaka and Chattogram in commercial broiler farm (N=216)

Organism	Positive (n)	Proportionate prevalence; %	95% CI
Avian fecal *E. coli* (AFEC)	177	81.94	76.2-86.9
VAG AFEC	120	55.6	48.7-62.3
Campylobacter spp.	98	45.37	38.7-52.3
Campylobacter jejuni	27	12.5	8.5-17.7

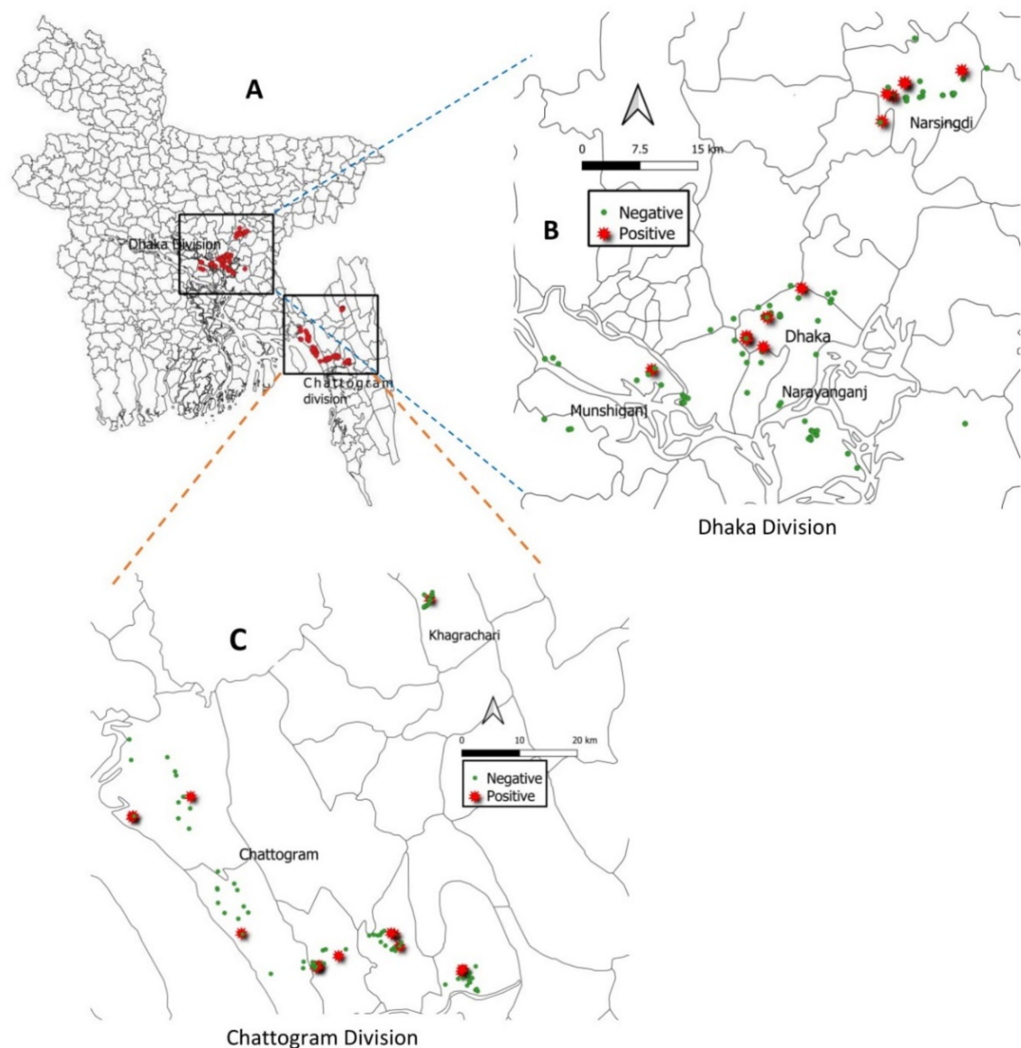

Figure 4.10-Map of Bangladesh showing: A) Geographical location of farms included into the study; B) Locations of *C. jejuni* positive and negative farms in Dhaka Division; C) Locations of *C. jejuni* positive and negative farms in Chattogram Division

4.1.1.2: Prevalence of virulent gene of AFEC isolated from broiler fecal samples at farm level

All AFEC isolates were examined for genes *astA, iss, irp2, papC, iucD, tsh, vat*, and *cva/cvi* using both simplex and multiplex PCR **(Figure 4.11 and 4.12)**. Prevalence of various virulence genes among isolates were used for the detection and characterization of VAG AFEC. Based on the genetic criteria for the pathogenecity, isolates containing at least one virulent gene was considered as the VAG AFEC strain. Among the 177 AFEC isolates, we found 120 VAG AFEC based on different virulent genes identified in multiplex PCR. Proportionate prevalence of virulent genes encode an enteroaggregative heat-stable toxin (*astA:* 100, 46.3%, 95% CI 39.6%-53.2%); a protein for increased serum survival (*iss*: 38, 17.6% 95% CI 12.8%-23.4%); iron-acquisition systems like yersiniabactin (*irp2*: 28, 12.9%, 95% CI 8.7%-18.2%); aerobactin (*iucD*: 55, 25.5%, 95% CI 19.8%-31.9%) and a colicin V plasmid (*cva/cvi:* 6, 2.8%, 95% CI 1.1%-5.9%). We identified no adhesion-related factors such as P-fimbriae (*papC*); a temperature-sensitive hemagglutinin (*tsh*) and vacuolating autotransporter toxin (*vat)* in this study **(Table 4.2).**

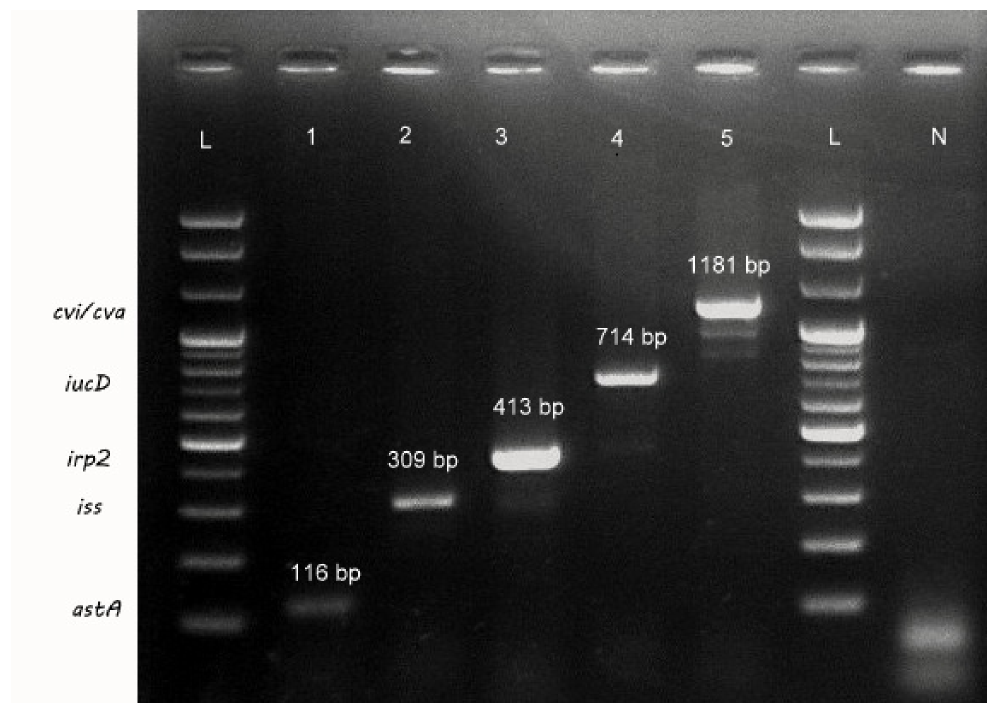

Figure 4.11-Agarose gel electrophoresis of the simplex PCR products with representative VAGs AFEC isolate carrying various combinations of virulence genes

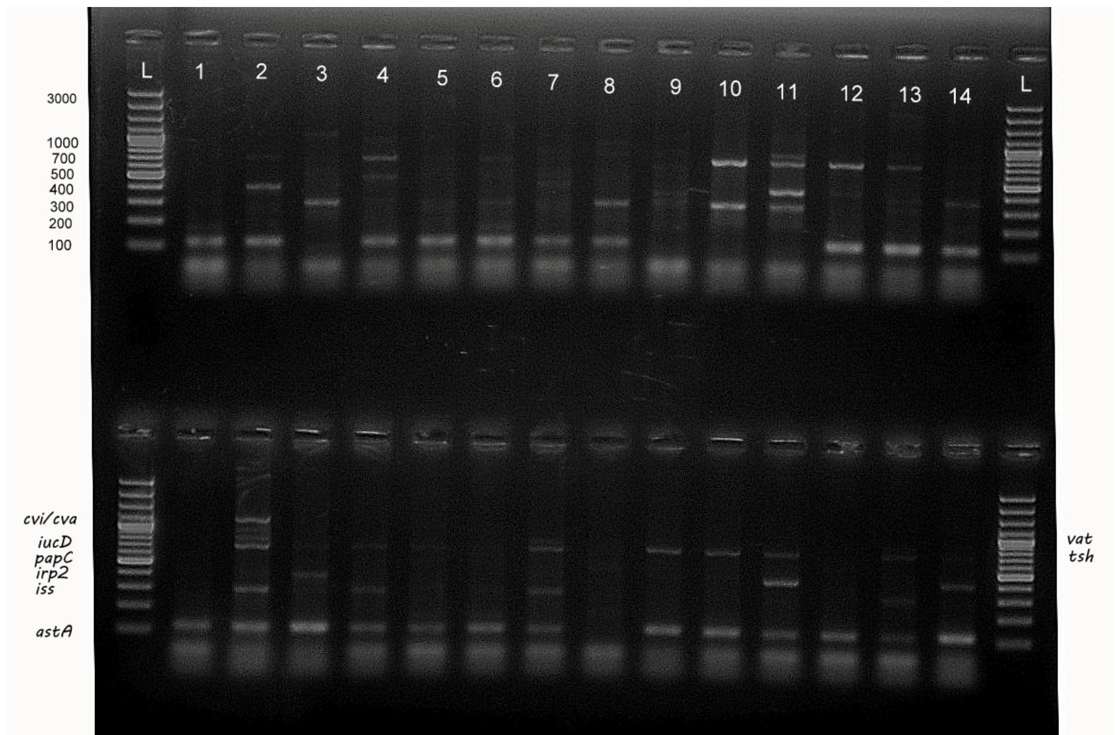

Figure 4.12-Agarose gel electrophoresis of the multiplex PCR products with representative VAG AFEC isolates carrying various combinations of virulence genes.

Table 4.2- Prevalence of virulent gene of AFEC in broiler farm (N=216)

Target genes	Positive (n)	Proportionate prevalence, %	95% CI
astA	100	46.3	39.6-53.2
iss	38	17.6	12.8-23.4
irp2	28	12.9	8.7-18.2
papC	0	0	0
iucD	55	25.5	19.8-31.9
tsh	0	0	0
vat	0	0	0
cva A/B cvi cvaC	6	2.8	1.1-5.9

4.1.2 Risk factor analysis

4.1.2.1 Univariable association of risk factors with the occurrence of *C. jejuni* in broilers at farm level

The prevalence of *C. jejuni* associated with different farm level variables in broilers are shown in **Table 4.3**. The univariable analysis covered a total of 22 variables related to housing, flock management, biosecurity and hygiene. Ten potential risk factors ($p \leq 0.1$) associated with the presence of *C. jejuni* were identified in the univariable analysis. The factors having significant effect on the prevalence of *C. jejuni* identified using univariable logistic regression were: number of chicken production over 1000, presence of more than one poultry houses, house establishment before 2010, more than one person entering the house, more than 8 flocks per shed per year, no separate household footwear, selling age more than 35 days, avoiding all out systems and less than 14 days of downtime from the previous batch.

Table 4.3- Univariable analysis to evaluate potential factors associated with *Campylobacter spp* and *C. jejuni* (N=216) status of broiler farm.

Factor	Categories	Total number (N)	*Campylobacter spp*				*C. jejuni*			
			No. positive	%	OR (95% CI)	*P value*	No. positive	%	OR (95% CI)	*P value*
Geographical area	Dhaka	9	2	22.2	Ref	0.3	0	0	N/A	0.2
	Munshiganj	23	9	39.1	2.2 (0.3-13.3)		1	4.3	Ref	
	Khagrachari	20	7	35.0	1.9 (0.3-11.7)		1	5.0	1.1 (0.1-19.8)	
	Narsingdi	27	14	51.9	3.8 (0.7-21.6)		6	22.2	6.2 (0.7-56.8)	
	Narayanganj	46	19	41.3	2.5 (0.4-13.1)		5	10.9	2.7 (0.2-24.4)	
	Chattogram	91	47	45.3	3.8 (0.8-18.9)		14	15.3	4 (0.4-32.1)	
No of chicken in production	>2000	39	19	48.3	Ref	0.6	2	5.1	Ref	0.1
	<1000	46	23	50.0	1.1 (0.4-2.4)		4	8.8	1.8 (0.3-10.1)	
	1001-2000	131	56	42.8	0.8 (0.3-1.7)		21	16.1	3.6 (0.7-15.8)	
Number of poultry shed	>1	66	28	43.5	Ref	0.5	5	7.6	Ref	0.1
	1	150	70	46.7	1.2 (0.7-2.1)		22	14.7	2.1 (0.8-5.9)	
Water Supply	Deep supply	16	6	37.6	Ref	0.5	3	18.8	Ref	0.4
	Tube well	200	92	46.0	1.5 (0.5-4.1)		24	12.0	0.6 (0.2-2.3)	
Establishment year of the house	Before 2010	72	52	72.3	Ref	0.001	16	22.3	Ref	0.002
	After 2010	144	46	31.9	0.3 (0.1-0.7)		11	7.7	0.9 (0.4-1.9)	
Person enter into shed	>1	44	30	68.2	Ref	0.001	13	29.6	Ref	0.001
	1	172	68	39.6	0.3 (0.1-0.7)		14	8.2	0.2 (0.09-0.4)	
Flock/shed (Year)	5 to 8	44	18	40.9	Ref	0.5	2	4.6	Ref	0.07

	9 to 10	172	80	46.6	1.3 (0.7-2.5)		25	14.6	3.6 (0.9-15.7)	
Litter in amount (kg)	600-800	34	19	55.9	Ref	0.3	5	14.8	Ref	0.9
	>800	81	36	44.5	0.7 (0.2-1.4)		10	12.4	0.9 (0.2-2.6)	
	200-600	101	43	42.6	0.6 (0.2-1.2)		12	11.9	0.8 (0.2-2.4)	
Separate footwear	No	88	54	61.4	Ref	0.001	18	20.5	Ref	0.003
	Yes	128	44	34.4	0.3 (0.1-0.6)		9	7.1	0.2 (0.1-0.7)	
Foot bath facilities	Yes	10	4	40	Ref	0.7	1	10	Ref	0.8
	No	206	94	45.6	1.2 (0.3-4.9)		26	12.7	1.3 (0.1-10)	
Litter types	Rice husk	7	3	42.9	Ref	0.9	1	14.2	Ref	0.9
	Saw dust	88	41	46.6	1.1 (0.2-5.6)		11	12.6	0.9 (0.1-7.9)	
	Both	112	49	43.8	1.03 (0.2-4.9)		14	12.6	0.9 (0.1-7.7)	
Flock size	≥1500	82	41	50	Ref	0.2	11	13.5	Ref	0.7
	<1500	134	57	42.6	0.8 (0.4-1.2)		16	11.9	0.9 (0.3-1.9)	
Flock age (days)	≥21	106	51	48.2	Ref	0.4	11	10.3	Ref	0.3
	<21	110	47	42.8	0.9 (0.4-1.3)		16	14.6	1.4 (0.7-3.3)	
Selling age (days)	>35	31	23	74.2	Ref	0.001	10	32.3	Ref	0.001
	<35	185	75	40.6	0.2 (0.1-0.6)		17	9.2	0.2 (0.09-0.6)	
Mortality rate	>50	81	42	51.9	Ref	0.1	13	16.1	Ref	0.2
	<50	135	56	41.5	0.7 (0.3-1.1)	-	14	10.4	0.7 (0.2-1.3)	
Season	Summer	12	5	41.7	Ref	0.8	1	8.3	Ref	0.4
	Rainy	58	25	43.1	1.07 (0.3-3.8)		5	8.7	1.03 (0.1-9.8)	
	Winter	146	68	46.6	1.2 (0.3-4.02)		21	14.3	1.9 (0.2-15.08)	
Infected neighbor broiler farms	Don't know (DK)	19	9	47.4	Ref	0.4	0	0	0	0
	Present	27	9	33.4	0.6 (0.1-1.9)		3	12.0	Ref	0.7
	Absent	170	80	47.1	1.0 (0.3-2.6)		24	14.7	1.2 (0.3-4.6)	

All in-all out system	No	50	30	60	Ref	0.01	9	18	Ref	0.1
	Yes	166	68	40.9	0.5 (0.2-0.9)		18	10.9	0.6 (0.2-1.3)	
Length of down time	<14(≤2wk)	51	37	72.6	Ref	0.001	15	29.5	Ref	0.001
	>14 (≥2wk)	165	61	36.8	0.2 (0.1-0.4)		12	7.3	0.1 (0.09-0.4)	
Vaccination	No	15	8	53.4	Ref	0.5	2	13.4	Ref	0.9
	Yes	201	90	44.8	0.8 (0.2-2.1)		25	12.5	1 (0.1-4.3)	
Previous antibiotic use history	No	31	7	22.6	Ref	0.001	1	3.3	Ref	0.09
	Yes	185	91	49.2	3.3 (1.3-8.1)		26	14.1	4.9 (0.7-37.5)	
Use of last antibiotic (days)	<4	88	39	44.4	Ref	0.7	11	12.6	Ref	1.0
	>4	128	59	46.1	1.1 (0.7-1.9)		16	12.6	1 (0.4-2.2)	

4.1.2.2 Multivariable logistic regression analysis to determine the potential risk factors associated with *C. jejuni* positive status in broiler at farm level

Ten variables with p≤0.1 in the univariable analysis were considered for inclusion in the multivariable logistic regression model to estimate the independence of effects. Entry of one person in the shed compared to more than one person (OR=0.2, p=0.001), use of separate foot ware in the shed (OR=0.29, p=0.001) and maintaining more than 14 days down time between flocks (OR=0.30, p=0.01) acted as a protective factor. On the other hand, when 9-10 flocks were raised in a shed per year it increased the risk of *C. jejuni* colonization 4.9 times compared to 5-8 flocks per shed per year **(Table 4.4)**.

Table 4.4- Risk factors for *C. jejuni* in broiler farms in selected districts of Dhaka and Chattogram during June 2019 to February 2020 identified from the final multivariable logistic regression model

Variables	Categories	OR	*P value*	95% CI
Person enter into shed	>1	Ref.		
	1	0.2	0.001	0.08-0.53
Flock/shed (Year)	5 to 8	Ref		
	9 to 10	4.9	0.05	0.97-24.52
Separate footwear	No	Ref.		
	Yes	0.29	0.01	0.11-0.76
Length of down time	<14 (≤2wk)	Ref.		
	>14 (≥2wk)	0.30	0.01	0.12-0.76

4.1.2.3 Univariable association of risk factors with the occurrence of VAG AFEC in broilers at farm level

The prevalence of VAG AFEC in broiler farm associated with different farm level factors are shown in **Table 4.5**. A total of 22 variables related to housing, flock management, biosecurity and hygiene were included in the univariable analysis. The univariable analysis identified six potential risk factors (p≤0.1) associated with the presence of VAG AFEC. According to geographical location, isolation of VAG AFEC was highest in Narsingdi compared to other locations (p=0.01). In univariable

logistic regression it was observed that farms at Narsingdi had 8 times higher risk of having VAG AFEC compared to Dhaka. Winter (OR=4.6) and rainy (OR=3) season had an increased infection than summer. Finally, presence of infected neighboring farms, water supply from tube well, flock age less than 21 days, mortality rate less than 50 during rearing were identified as significant risk factors.

Table 4.5- Univariable analysis to identify potential risk factors for the occurrence of AFEC and VAG AFEC (N=216) in broilers at farm level

Factor	Categories	No. of observation (N)	AFEC				VAG AFEC			
			No. positive	%	OR (95%CI)	p value	No. positive	%	OR (95% CI)	p value
Geographical area	Dhaka	9	7	77.8	Ref	0.09	3	33.3	Ref	0.01
	Munshiganj	23	21	91.3	3 (0.4-25.5)		10	43.4	1.6 (0.3-7.8)	
	Khagrachari	20	17	85	1.7 (0.2-11.9)		14	70	4.7 (0.9-25.1)	
	Narsingdi	27	24	88.9	2.2 (0.3-16.5)		22	81.4	8.8 (1.7-47.8)	
	Narayanganj	46	31	67.3	0.5 (0.1-3.1)		21	45.7	1.7 (0.3-7.6)	
	Chattogram	91	77	84.7	1.6 (0.2-8.3)		50	54.9	2.4 (0.5-10.3)	
No of chicken in production	>2000	39	31	79.4	Ref	0.08	20	51.3	Ref	0.3
	<1000	46	33	71.8	0.7 (0.2-1.8)		22	47.9	0.9 (0.3-2.04)	
	1001-2000	131	113	86.2	1.7 (0.7-4.1)		78	59.6	1.4 (0.7-2.9)	
Number of poultry shed	>1	66	53	80.4	Ref	0.6	34	51.6	Ref	0.4
	1	150	124	82.7	1.2 (0.6-2.5)		86	57.4	1.2 (0.8-2.2)	
Water Supply	Deep supply	16	13	81.3	Ref	0.9	6	37.6	Ref	0.1
	Tube well	200	164	82	1.06 (0.2-3.9)		114	57	2.3 (0.8-6.3)	
Establishment year of the house	Before 2010	72	60	83.4	Ref	0.7	44	61.2	Ref	0.2
	After 2010	144	117	81.3	0.9 (0.4-1.9)	-	76	52.8	0.8 (0.3-1.2)	-
Person enter into shed	>1	44	34	77.3	Ref	0.3	26	59.1	Ref	0.5
	1	172	143	83.2	1.4 (0.7-3.2)		94	54.7	0.9 (0.4-1.7)	
Flock/shed (Year)	5 to 8	44	35	79.6	Ref	0.6	21	47.8	Ref	0.2
	9 to 10	172	142	82.6	1.2 (0.6-2.8)		99	57.6	1.5 (0.8-2.9)	
Litter in amount	600-800	34	30	88.3	Ref	0.3	21	61.8	Ref	0.6

(kg)	>800	81	68	83.9	0.7 (0.2-2.1)		43	53.1	0.8 (0.3-1.6)	
	200-600	101	79	78.3	0.5 (0.1-1.6)		56	55.5	0.8 (0.3-1.8)	
Separate footwear	No	88	70	79.6	Ref	0.4	50	56.9	Ref	0.7
	Yes	128	107	83.6	1.3 (0.7-2.7)		70	54.6	1 (0.6-1.6)	
Foot bath facilities	Yes	10	7	70	Ref	0.3	5	50	Ref	0.7
	No	206	170	82.6	2.03 (0.4-8.2)		115	55.9	1,2 (0.3-4.5)	
Litter types	Rice husk	7	7	100		0.3	4	57.1	Ref	0.6
	Saw dust	88	73	82.9	Ref		52	59.1	1.09 (0.2-5.1)	
	Both	112	88	78.6	0.9 (0.4-1.9)		59	52.7	0.9 (0.1-3.9)	
Flock size	≥1500	82	73	89.1	Ref	0.03	50	60.9	Ref	0.2
	<1500	134	104	77.7	0.5 (0.1-0.9)		70	52.2	0.8 (0.4-1.2)	
Flock age (days)	≥21	106	88	83.1	Ref	0.6	54	50.9	Ref	0.1
	<21	110	89	80.9	0.9 (0.4-1.8)	-	66	60	1.5 (0.9-2.4)	
Selling age (days)	>35	31	23	74.2	Ref	0.2	15	48.4	Ref	0.3
	<35	185	154	83.3	1.8 (0.8-4.2)	-	105	56.8	1.4 (0.7-2.9)	
Mortality rate	>50	81	70	86.5	Ref	0.1	40	49.4	Ref	0.1
	<50	135	107	79.3	0.7 (0.2-1.2)	-	80	59.3	1.4 (0.9-2.6)	
Season	Summer	12	7	58.3	Ref	0.05	3	25	Ref	0.03
	Rainy	58	51	87.9	5.2 (1.2-20.9)	-	29	50	3.0 (0.8-12.2)	
	winter	146	119	81.6	3.1 (0.9-10.7)		88	60.2	4.6 (1.1-17.6)	
Infected neighbor broiler farms	Don’t know (DK)	19	16	84.2	Ref	0.8	14	73.7	Ref	0.08
	Present	27	23	85.2	1.08 (0.2-5.4)		11	40.2	0.2 (0.06-0.9)	
	Absent	170	138	81.2	0.9 (0.2-2.9)		95	55.9	0.5 (0.1-1.3)	
All in all out system	No	50	43	86	Ref	0.3	29	58	Ref	0.6
	Yes	166	134	80.8	0.7 (0.2-1.7)		91	54.9	0.9 (0.5-1.7)	
Length of down	<14 (≤2wk)	51	42	82.4	Ref	0.9	29	56.9	Ref	0.8

time	>14 (≥2wk)	165	135	81.9	1 (0.4-2.1)		91	55.2	1 (0.4-1.8)	
Vaccination	No	15	14	93.4	Ref	0.2	10	66.7	Ref	0.3
	Yes	201	163	81.1	0.3 (0.03-2.4)		110	54.8	0.7 (0.1-1.8)	
Previous antibiotic use history	No	31	23	74.2	Ref	0.2	19	61.3	Ref	0.4
	Yes	185	154	83.3	1.8 (0.8-4.2)		101	54.6	0.8 (0.3-1.7)	
Use of last antibiotic (days)	<4	88	70	79.6	Ref	0.4	49	55.7	Ref	0.9
	>4	128	107	83.6	1.3 (0.7-2.7)		71	55.5	1 (0.5-1.8)	

4.1.2.4 Multivariable logistic regression to determine the potential risk factors associated with VAG AFEC positive status in broilers at farm level

The risk factors for VAG AFEC in broiler farm identified in the final model are presented in **Table 4.6.** Two risk factors were identified in the final model namely geographical area of the farm and age of the flock. Farms located in Khagrachhari and Narshingdi showed significantly higher risk of having VAG AFEC compared to Dhaka. Flocks of less than 21 days of age had a 3.8 times higher risk of having VAG AFEC compared to flocks of ≥21 days of age.

Table 4.6- Risk factors for VAG AFEC (N=216) in broiler farms in selected districts of Dhaka and Chattogram during June 2019 to February 2020 from the final multivariable logistic regression model

Variables	Category	OR	p value	95% CI
Geographical area	Dhaka	Ref	-	-
	Munshiganj	1.5	0.6	0.3-7.7
	Khagrachari	16.9	0.04	2.4-117.5
	Narsingdi	8.8	0.012	1.6-47.7
	Narayanganj	1.6	0.499	0.3-7.5
	Chattogram	3.5	0.094	0.8-15.3
Flock age (days)	≥21	Ref	-	-
	<21	3.8	0.007	1.4-10.0

4.2 Broiler meat sample

4.2.1 Descriptive analysis

Overall prevalence of *Campylobacter spp* was 54.17% (95% CI: 39.17 – 68.63; N=48) and *C. jejuni* 27.08% (95% CI: 15.28 – 41.84; N=48) at live bird market and super shops in Chattogram.

4.2.2 Risk factor analysis

4.2.2.1 Univariable association of binary response of *C. jejuni* in different LBM of Chattogram with different factors using χ^2 test

The prevalence of *C. jejuni* estimated in terms of market location, season, hygiene score, shop-kept birds, water source are shown in **Table 4.7**. Rural and metro LBM had 30% (95% CI: 11.89-54.28; N= 20) prevalence each followed by 12.5% (95% CI: 0.31-52.65; N= 8) in super shops (p=0.022). No statistically significant association (p> 0.05) was observed using χ^2 test between different factors like season, hygiene score, birds kept in the shop before slaughter and water source with the presence of *C. jejuni* in broiler meat **(Table: 4.7).**

Table 4.7- Univariable association between different selected factors with *C. jejuni* status of the broiler meat samples in Chattogram (chi square test)

Variable	Category (n)	*Campylobacter spp* %, (95% CI)	p value (χ^2 test)	*C. jejuni* %, (95% CI)	p value (χ^2 test)
Market location	Super shop (8)	12.5 (0.31 – 52.65)	0.022	12.5 (0.31 – 52.65)	0.596
	Rural (20)	55 (31.53 – 76.94)		30 (11.89 -54.28)	
	Metro (20)	70 (45.72 – 88.11)		30 (11.89 – 54.28)	
Season	Spring (28)	42.86 (40.99 – 86.66)	0.176	25 (10.69 – 44.87)	0.416
	Winter (17)	70.59 (44.04 – 89.69)		35.29 (14.21 – 61.67)	
	Autumn (3)	66.67 (9.43 – 99.16)		0, (0 – 70.76)	
Hygiene score	Very clean (8)	12.5 (0.32 – 52.65)	0.029	12.5 (0.31 – 52.65)	0.595
	Moderate (27)	59.26 (38.8 – 77.61)		29.63 (13.75 – 50.18)	
	Dirty (13)	69.23 (38.57 – 90.91)		30.77 (9.1 – 61.42)	
Birds stay at shop	1 day (20)	50 (27.19 – 72.80)	0.761	25 (8.66 – 49.1)	0.956
	2 days (17)	52.94 (27.81 – 77.02)		29.41 (10.31 – 55.96)	
	3 days (11)	63.64 (30.79 – 89.07)		27.27 (6.02 – 60.97)	
Water source	Tube well (14)	50 (23.04 – 76.96)	0.787	35.71 (12.76 – 64.86)	0.632
	Pond (6)	66.67 (22.28 – 95.67)		16.67 (0.42 – 64.12)	
	WASA (28)	53.57 (33.87 – 72.49)		25 (10.69 – 44.87)	

4.2.2.2 Univariable logistic regression model to identify risk factors of occurring *C. jejuni* in different LBM of Chattogram

As a unit of comparison, the *C. jejuni* status of each market location was used. Altogether, pooled samples from 48 LBMs were used in the analysis. The odds of detecting *C. jejuni* in broiler meat sample was 3 times higher in both rural and metro LBMs than the odds of the super shops (p=0.350). Winter season had 1.6 times greater risk than spring and autumn in univariate analysis (p=0.462). Moreover, dirty hygienic condition of the LBMs (OR= 3.11) had an increased infection than a mild and very clean condition. Other factors tested were birds stay at the shop before slaughtering or water source. However, none of them showed a statistically significant difference **(Table: 4.8).**

Table 4.8- Association between different variables with *Campylobacter spp* and *C. jejuni* status of broiler meat collected from super shops and different live bird markets in Chattogram tested with univariable logistic regression models

Variable	Category	*Campylobacter spp.* OR, (95% CI)	p value	*C. jejuni* OR, (95% CI)	p value
Market location	Super shop	Ref		Ref	
	Rural	8.56 (0.88 – 83.06)	0.064	3 (0.3 – 30.02)	0.350
	Metro	16.33 (1.63 – 163.44)	0.017	3 (0.3 – 30.02)	0.350
Season	Spring	Ref		Ref	
	Winter	3.2 (0.89 – 11.55)	0.076	1.64 (0.44 – 6.08)	0.462
	Autumn	2.67 (0.22 – 32.96)	0.445	N/A	
Hygiene score	Very clean	Ref		Ref	
	Moderate	10.18 (1.09 – 94.83)	0.042	2.95 (0.31 – 28.03)	0.347
	Dirty	15.75 (1.42 – 174.25)	0.025	3.11 (0.28 – 34.42)	0.355
Birds stay	1 day	Ref		Ref	
	2 days	1.13 (0.31 – 4.11)	0.858	1.25 (0.29 –	0.764

				5.35)	
at shop	3 days	1.75 (0.39 – 7.91)	0.467	1.13 (0.21 – 5.97)	0.890
Water source	Tube well	Ref		Ref	
	Pond	2 (0.27 – 14.7)	0.496	0.36 (0.03 – 4.01)	0.406
	WASA	1.15 (0.32 – 4.17)	0.827	0.6 (0.15 – 2.4)	0.471

4.3 Molecular characterization of *C. jejuni:*

The *mapA* genes of randomly selected six positive samples were partially sequenced, and the sequence data were submitted to GenBank under accession numbers MT175597-99 and MT920396-98. The accession numbers of the nucleotide sequences, retrieved from Genbank, to compare the sequence data of this study, are given in the **Supplementary Table: 8.5**. The phylogenetic tree finally developed to visualize the evolutionary comparison among the strains is displayed in **(Figure. 4.13).**

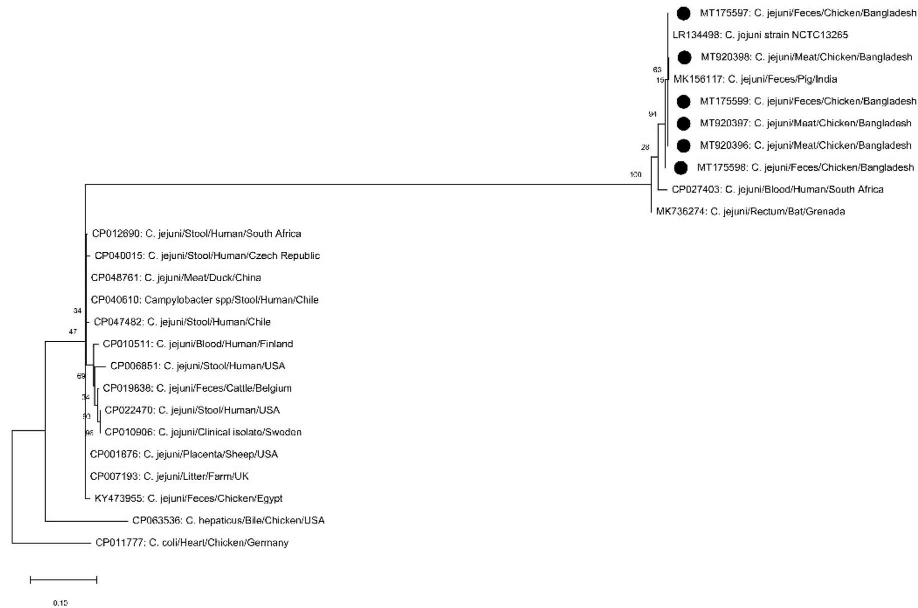

Figure 4.13-Phylogeny of *mapA* gene of selected *C. jejuni* strains from this study and other global strains.The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model.

The tree analysis revealed that three showed highest log likelehood (-2413.91). The percentage of trees in which the associated taxa clustered together is shown next to the branches. This analysis involved 25 nucleotide sequences where the outgroup of the phylogeny was *C. coli.* There were a total of 627 positions in the final dataset.Our data showed that strains of *C.jejuni* across the phylogeny are from different hosts including broiler chicken, bat, pig, duck, cattle, sheep, humans. As anticipated, strains from this study were closely clustered within same clade. At the same time, the evidence of close relatedness with the strains isolated from human, pig and bat is shown in the tree. In case of geographical point of view, the study strains were isolated from Bangladesh and there is a close relatedness with other strains from India, South Africa, Grenada.

Data availability: The *16S rRNA* gene, *mapA* gene, *astA* gene, *iss* gene, *irp2* gene, *iucD* gene, *cva/cvi* gene sequencing data (17 sequences) has been submitted NCBI database under the accession numbers-MT032361-MT032363, MT912607-MT912609, MT175597-MT175599, MT920396-MT920398, MT928164-MT928166, MT982360-MT982361.

Chapter-5: Discussion

Isolation and molecular characterization of *C. jejuni* and VAG of avian fecal *E. coli* from apparently healthy broilers is reported in this study. The infections have public health significance due to the zoonotic nature of these pathogens. The organisms' identification was based on their cultural morphology and the results of their *16S rRNA* gene sequences. In the present work, bacteriological and molecular examination showed that the farm level prevalence of *C. jejuni* was 12.5% (27/216) in fecal samples, while 27.08% (13/48) in LBM and super shop meat samples. Conversely, AFEC's farm level prevalence was 81.94% (177/216) and VAG AFEC's was 55.6% (120/177).

5.1 *C. jejuni* in broiler farms

Poultry intestines are a favorable environment for the colonization of *Campylobacter*, thus increasing the likelihood of human campylobacteriosis due to the consumption of its contaminated meat, which is of great concern to human health (Kaakoush et al., 2015). *Campylobacter* is an extremely important food-borne, zoonotic pathogen that infects millions of people each year worldwide. Human beings can get infections in different ways, but studies showed that broiler is the most important source (Mirzaie et al., 2011). Not many studies on *C. jejuni* in poultry in Bangladesh have been reported. Fresh scientific information is therefore expected to be added to the existing literature by this study. This study provides an insight into the infection/colonization burden and potential risk factors of *C. jejuni* in several poultry production and supply chain sources in Bangladesh.

We recorded the overall farm level prevalence of *Campylobacter spp* as 45.37% (98 from 216, 95% CI 38.7 % -52.3 %), which is consistent with previous findings; ranged from 40% to 43% (Hasan et al., 2020; Neogi et al., 2020). Kabir et al. (2014a), however, reported 78% prevalence in broilers at flock level. The present study analyzed each farm's pooled cloacal samples. The authors believe that the isolation rate of *Campylobacter* would have been increased if cloacal samples at individual level were screened. The overall occurrence of *C. jejuni* in this study was 12.5% (27 from 98). Previous researches both at home and abroad supports the positivity status estimated in this study. In Bangladesh, the prevalence of *C. jejuni* in broiler samples was reported to be 17.9% (Alam et al., 2020) and 6.25% in India (Malik et al., 2014).

In contrast, a higher occurrence of 65% was reported in Bangladesh at broiler farm level by Neogi et al. (2020). Due to effect of season, rearing system, farm management, biosecurity and hygiene, the prevalence might differ (Cardinale et al., 2004; Guerin et al., 2007; Näther et al., 2009; Lyngstad et al., 2008; Sommer et al., 2013). In addition, differences in laboratory techniques used in various studies can also fluctuate the results (Rahimi and Ameri, 2011; Vinueza-Burgos et al., 2017).

The most significant factor associated with *C. jejuni* infection in this study was the weak poultry shed management system, particularly when more than one individual entered the broiler house during bird rearing. Therefore, it seems likely that Campylobacters can be tracked by individuals entering the poultry house from the external environment. It can be speculated that the most likely source of this bacterial infection is environmental contamination during the rearing period (Newell et al., 2011). In addition, the present study revealed that the positive *C. jejuni* status was associated with not using separate footwear, less downtime length, and increased number of flocks per shed per year.

When more than one individual entered the broiler house, the present study showed a higher risk of *Campylobacter* colonization. The results of Chowdhury et al. (2012b) are consistent with this finding. Human trafficking is an important route (via boots, hands, cloths) for the introduction of *Campylobacter* in broiler houses (Hald et al., 2000; Cardinale et al., 2004) and molecular studies have confirmed the subsequent colonization into the broiler flocks of similar strains isolated from workers' shoes (Messens et al., 2009). Biologically, it is plausible that a greater risk of introduction could be associated with a large number of people entering the house (Chowdhury et al., 2012b). Personal hygiene could be an important factor that confounds the relationship between *Campylobacter's* introduction into the house and the number of people who regularly enter the house. In our analysis, however, we found a significant association between the use of separate footwear in the shed with *C. jejuni* status. Other studies have claimed that the disinfection of shoes before entering a shed is effective in reducing *Campylobacter* infection (Sibanda et al., 2018), which agrees with the present study's finding that the risk can be reduced by entering a shed with separate shoes.

In this research, a greater interval of more than fourteen days between two batches of broilers was identified as a protective factor. Theoretically, for a broiler shed to dry

completely, long downtime should be allowed so that the farm environment can become least commendable for *Campylobacter* infection. The outcome is consistent with the study finding of Lyngstad et al. (2008) and Hasan et al. (2020). Contrary to this, Høg et al. (2016) indicated that the longer downtime implied a greater risk of infection with *Campylobacter*. Nevertheless, Barrios et al. (2006) found no impact of downtime on this infection. Shorter downtime also contributes to an increase in the number of flocks per shed per year. As a proxy of the length of the depopulation period, we evaluated the number of rotations per year per shed; it turned out to be statistically significant. More than eight flocks per shed per year had an increased probability of infection with *C. jejuni*. This finding is consistent with previous studies that have shown that increased flock rotations are associated with a higher risk of infection (Hald et al., 2000; Høg et al., 2016). Due to its chemotactic and aerotactic properties, *Campylobacter* can survive on the floor until properly dried, even a few more times in the environment (Hald et al., 2000). Hazeleger et al. (1998) noted, however, that under favorable conditions, *Campylobacter* can survive in environmental materials for up to 4 months.

5.2 *C. jejuni* in LBM's and super shop

Isolation of 54.17% *Campylobacter* isolates was resulted in 176 pooled chicken meat from 40 LBMs and 8 super shops examined by molecular techniques. The reported market level prevalence of 54.17% of chicken meat with *Campylobacter spp* is comparable to the 49% recovery rate reported by Neogi et al (2020).

The current study found that 27.08% raw chicken meat was contaminated with *C. jejuni* isolates. The reported prevalence has differed with the prevalence of 68% of *C. jejuni* estimated by Neogi et al. (2020) in Bangladesh and 70.96% by Kabir et al (2014b). Numerous other studies have shown the prevalence of *C. jejuni* in poultry meat in different parts of the world, and the observed prevalence has allegedly varied. Ilida and Faridah (2012) reported a prevalence of *C. jejuni* of 51.06% in chicken meat and chicken-based products in Malaysia. In European countries, Skarp et al. (2016) reported 11-71% prevalence of *Campylobacter* in retail poultry meat. Another literature survey on Campylobacters in retail poultry in various countries reported between 7.1% and 100% prevalence (Suzuki and Yamamoto, 2009). Several factors including difference in the infection rates in food animals and food production system can cause these variations. In addition, the phases beginning with farm production,

transport to slaughter, the process of slaughtering and subsequent processing of chicken meat products, the retail sale of products, the handling and consumption at home all might play a role in *Campylobacter* transmission in chicken meat.

Similarly, in this study, the effects of differential anthropogenic practices and environmental variations may contribute to the variations in the occurrence of *Campylobacter* at farms and LBMs. Compared to broiler farms, the higher occurrence of *C. jejuni* in meat samples of LBMs is likely to be associated with the floor environments, facilitate widespread secondary transfer of this zoonotic pathogen (Neogi et al., 2020). Personal and environmental hygiene maintenance is rarely practiced in these LBMs in Bangladesh. Regular poultry product contamination by *C. jejuni* in retail markets and slaughter houses was found to be a major cause of foodborne illness in developed countries in Europe, even with good farming practices and health interventions (Kramer et al., 2000; Reich et al., 2018).

We observed that in live bird markets, the level of hygienic and bio-safety measures, such as regular hand washing, the use of disinfectants and the washing of floors and cages is inadequate compared to super shops. The optimal slaughtering process can effectively reduce bacterial loads in chicken carcasses by reducing cross-contamination and proper washing with chlorinated water (Pissol et al., 2013).

As in this study, a face-to-face interview was conducted with the farmers and shop owners, to some extent there could be presence of information bias. A translated questionnaire was used to minimize it, and the facts were discussed in detail with the farmers and broiler handlers in LBM's. In addition, in some cases, if there was any inconsistency with the data generated through physical observation of the settings and management of the farm and market, the participants were cross-questioned.

5.3 Molecular Characterization of *C. jejuni*

The analysis of the *mapA* sequence data from this study revealed that all the six strains sequenced belonged to the same clade which indicates these strains have less variation at the gene level. Since the *mapA* genes of the study isolates were partially sequenced, it is not enough to interpret their complete clonal similarity or dissimilarity based on the phylogenetic tree constructed here. However, from the evolutionary point of view, there are close relatedness among the study strains in spite of different sample collection site can be found from the constructed tree. This might

be due to sampling from the common species. At the same time, the relatedness of the strains with another strain from neighboring country like India indicates that these stains are well circulated irrespective of geographical location. As *C. jejuni* is a zoonotically significant organism, there is an enormous chance of human infection from poultry originated *C. jejuni.* Several previous reports of human infection by *C. jejuni* from poultry origin were anticipated using the findings of the evolutionary tree where human strains were located in the same cluster with the study strains (Friis et al., 2010). Overall, these partial gene sequencing results represent the evolutionary relatedness where whole genome sequencing will be the standard recommendation to find out more accurate relatedness.

5.4 VAG AFEC in broiler farms

The intestine of birds has previously been identified as a VAGs AFEC reservoir (Kemmettet al., 2013; Subedi et al., 2018). In the present study, VAG AFEC was isolated from apparently healthy broiler chickens in Bangladesh at a prevalence rate of 55.6% which is similar to Ibrahim et al. (2019) and Schouler et al. (2012). The analysis of the study revealed that overall prevalence of AFEC in broiler farms of the study area was 81.94% (177 out of 216 pooled fecal samples) whereas prevalence for VAG AFEC was 55.6% (N=120). The prevalence of AFEC in apparently healthy broiler farms observed in this study corroborates with the previous records of Akond et al. (2009) in Bangladesh. The prevalence of AFEC in this study was higher than the previous records of Nazir (2004) and Rahman et al. (2008). The prevalence of AFEC in farms might depend on many factors such as, biosecurity, clinical condition, flock age, geographical location etc.

Screening of multiplex PCR for eight virulent genes was performed for all AFEC isolates; the most prevalent genes were *astA* (46.3%), *iss* (17.6%), *iucD* (25.5%), *irp2* (12.9%) and *cva/cvi* (2.8%). Presence of three out of four of *iss, iucC, tsh and cvi* genes indicate that the isolate is avian pathogenic *E. coli* (APEC) (Schouler et al., 2012). Timothy et al. (2008) reported that presence of these genes are associated with avian colibacillosis and indicates presence of APEC.

In this study *astA* gene was detected with a prevalence of 46.3% which is lower than the prevalence previously reported in Jordan (71%) (Ibrahim et al., 2019). The prevalence of increased serum survival protein coded by *iss* gene (17.6%) was lower

than what was detected in USA and Germany where 80.5 and 82.7% of APEC isolated from birds with colibacillosis possess such gene (Dissanayake et al., 2014; Ewers et al., 2004). In general, VAGs are integrated within the plasmid, the pathogenicity islands (chromosomally or extra chromosomally) or the bacteriophages, the acquisition of VAGs is usually occur through horizontal gene transfer (Hacker et al., 2000; Ochman et al., 2000) which may explain the absence or the low prevalence of the remaining virulent genes. The presence of virulence markers in poultry-isolated AFECs is genetically similar to different pathogenic clones, indicating their potential for transferring virulence genes to pathogenic *E. coli* clones in humans and animals (Ahmed et al., 2020).

The eight virulent genes selected for this study do not represent an exhausted list of VAGs AFEC determinants (Johnson et al.,2008). An investigation published after this study recommended a new virulotyping protocol offering vastly improved error margins in APEC detection, might be ideal for future epidemiological studies (Schouler et al., 2012).

The predisposing epidemiologic factors such as geographic locations, farm housing types, flock age, varying sample collection, transportation and preservation methods, and management practices are likely to contribute to the differences in frequency of VAG AFEC isolation (Saud et al., 2019). This study tested the risk factors that were hypothesized to be associated with the presence of virulent genes associated AFEC in broiler farms in Bangladesh. Variations in geographical locations were observed in this study was supported by the findings of a recent study (Saha et al., 2020). Another important factor associated with the presence of VAG AFEC was farms having flock age less than 21 days. Kemmett et al. (2014) identified intestines of 1-day-old chicks as a reservoir rich in potentially pathogenic *E. coli*. Colibacillosis has often been associated with the disease of older broiler chickens (>2 weeks old), but several studies have taken an "integrated poultry production" approach and suggested broiler breeders and hatcheries may be significant reservoirs of early APEC infections either via environmental contamination or vertical transmission (Giovanardi et al., 2005; Petersen et al., 2006). Another reason might be use of antibiotics in flocks after 21 days as a preventive measure reduces occurring any diseases as selling age gets closed.

Overall, this is the first report in Bangladesh on the prevalence of common virulence associated genes in AFEC in broiler. The results of this study are in accordance with those reported in several geographical areas all over the globe, describing a high prevalence of these virulence associated genes in APEC than AFEC. Though this virulence associated genes are responsible for the degree of severity in colibacillosis caused by APEC, the acquisition of such virulence associated genes can be possible by AFEC which may trigger them to spillover within other organisms. Once the AFEC cross the intestinal barrier and get entry into the chicken's body through different routes like respiratory it might pose a potential risk of causing colibacillosis. Therefore, it is necessary to carry out further studies in large scale to correlate the characteristics with the pathogenicity levels of APEC and AFEC in chicken. The presence of these genes in AFEC isolates, however, poses a potential risk that they may subsequently cause colibacillosis. Therefore, it is necessary to carry out further studies to establish their importance in the pathogenesis of this disease.

Chapter-6: Conclusions

The study provides insights into epidemiology, i.e., the prevalence at the farm and live birds market, along with the risk factors of *C. jejuni* infection in Bangladesh's commercial broiler chicken. Clearly, the observed contamination of *C. jejuni* strains in broiler samples and various environmental sources at poultry farms and their magnified occurrences at LBMs might be linked to the practice of poor hygiene, bio-safety and health management measures, reflecting an alarming food safety situation in Bangladesh. Like many studies conducted before, our findings emphasize that biosecurity measures are most important for keeping infections outside of bird flocks. Therefore, every action that could function as a vector for bringing *Campylobacter* into broiler houses should be limited. Both in broiler farms and LBMs, as few people as possible should enter the house using separate footwear that could function as a source of *Campylobacter*. Farm management practices were related to the occurrence of *C. jejuni* infection, including flock rotation per shed per year and downtime between successive batches. Therefore, in each broiler house an optimal empty period between flocks is highly recommended to reduce the environmental load and successive infection in new flocks. Based on the potential risk factors of *Campylobacter* observed, it is recommended that not only farm owners but also, more assertively, poultry market handlers should be included in motivational training programs for behavioral change to take preventable measures, including strict maintenance of personal and environmental hygiene and regular monitoring of poultry health. Phylogenetic studies conducted on *mapA* gene sequences in poultry sources revealed the evolutionary history of *C. jejuni*. Phylogeny has shown that *C. jejuni* isolated from the various sources of poultry are closely related.

This study also revealed a high frequency of virulence genes in AFEC strains isolated from Bangladesh's apparently healthy broiler chickens. Significant risk factors associated with the presence of VAG AFEC in broiler chickens were geographic locations and flock age of less than 21 days. The results obtained in this study provide insights into the most common combination of virulence-associated genes in AFEC strains that allowed us to create a multiplex PCR that supplements PCR as a diagnostic tool for the rapid and specific detection of APEC. The established tools could also be used to investigate infection chains, which would provide valuable information on infection pathways in and between poultry flocks. Finally, the results

obtained in this study provide the basis for further epidemiological studies and forthcoming experiments on in vivo (chicken) and in vitro (cell culture) infection, providing substantial information for a better understanding of the pathogenesis of poultry colibacillosis.

Chapter-7: Limitations

We have identified some limitations in our study. Firstly, the study was conducted on a small scale because of time and resource restrictions. Secondly, in Bangladesh, the study was conducted in a certain geographical region. A more detailed and comprehensive picture of the condition of the farm would be generated by adding more farms from different regions of the country. Thirdly, this study investigated only broiler chickens, while layer and backyard chickens are also a major part of the production system of poultry in Bangladesh. Finally, although *C. jejuni* was identified in the study, other *Campylobacter spp* could not be studied.

Chapter-8: Appendix

Appendix-A

1. Buffered peptone water

Composition	Gm./Liter
Peptone	10.0
Sodium chloride	5.0
Disodium phosphate	3.5
Potassium dihydrogen phosphate	1.5

2. Culture media for isolation of *C. jejuni*

a) Base agar

Campylobacter agar base (Micro-Master)	19.75 g
Distilled water	500 ml

Suspend 19.75 grams in 500 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 Lbs pressure (121°C) for 15 min. Cool to 45-50°C and aseptically add 25 ml lysed sheep blood and reconstituted contents of 1 vial of Skirrow supplement. Mix well before pouring into sterile petri plates.

b) Composition of Campylobacter agar base

Composition	Gm. / Liter
Proteose peptone	15.0
Liver digest	2.5
Yeast extract	5.0
Sodium chloride	5.0
Agar	12.0

c) Campylobacter selective supplement (Micro-master)

Polymyxin B sulphate	1,250 IU
Vancomycin	5.00 mg
Trimethoprim	2.50 mg

Rehydrate the contents of 1 vial aseptically with 2 ml of sterile distilled water and mix well to dissolve. Avoid frothing of the solution. Aseptically add the rehydrated contents to 500 ml of sterile, molten, cooled (45-50°C) Campylobacter agar base. Mix gently and pour into sterile petri plates.

d) Blood agar medium

Blood agar medium had the same composition as Nutrient agar medium except addition of 5 per cent lysed sheep blood. Poured 12-15 ml in each petri plates, allowed to solidify and sterile blood agar medium plates were kept at 4°C till further use.

3. Culture media for isolation of *E. coli*

a) Composition of MacConkey agar (Oxoid)

Composition	Gm. / Litre
Peptone	20.0
Lactose	10.0
Bile Salts	5.0
Sodium chloride	5.0
Agar	12.0

Suspend 52g of MacConkey agar powder in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes.

b) Composition of Eosin Methylene Blue (EMB) agar (Oxoid)

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

Suspend 37.5g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 60°C and shake the medium in order to oxidize the methylene blue (i.e. restore its blue colour) and to suspend the precipitate which is an essential part of the medium**.**

4. Reagents used for molecular characterization of *C. jejuni* and VAG AFEC.

A. Ethidium bromide (10 mg/ml)

Ethidium bromide	50 mg
Distilled water	5 ml

Stored the solution in amber colored vial at 4°C

B. EDTA (0.5 M, pH 8.0)

EDTA. 2H 2 O	18.61 g
Distilled water	100 ml

Adjusted the pH to 8.0 with 5M NaOH. The solution was filtered through Whattman filter paper no.1 and stored at room temperature.

C. Tris-acetate-EDTA (TAE) stock solution (50X)

Tris base	121.0 g
Glacial acetic acid	28.5 ml
EDTA (0.5 M, pH 8.0)	50.0 ml
Distilled water (DW)	500 ml

For working solution (1X), stock solution was diluted fifty times in distilled water.

D. Loading dye (6X)

Sucrose	40% w/v in DW
Bromophenol blue	0.25% w/v in DW
Xylene cyanol	0.25% w/v in DW

The solution stored at 4°C until use.

E. TE-buffer (pH,

Tris-HCl (1.0 M)	1.0 ml
EDTA (0.5 M)	0.2 ml

Mixed with distilled water to make 100 ml, sterilized by autoclaving at 15 lb pressure (121°C) for 15 min and stored at 4°C.

Appendix-B

Questionnaire on broiler flocks rearing system

General information

Study area:

Date:

Name of the farm:

Longitude: Latitude:

Farm ID:

Sample code:

Owner's information

Name of the owner:

Contact number:

Farm information

1. Number of chicken production of the farm:
   a. 1000 b. 2000 c. more than 2000
2. Number of houses in the farm:
   a. 1 b. 2 c. more than 2
3. Water supply of the farm:
   a. Deep tube well b. tube well c. pond d. others
4. What is the disposal system of dead birds? Ans:
5. How do you store litter materials? Ans:

House information:

1. In which year house was established? Ans:
2. What is the length of house (in feet)? Ans:
3. What is the width of the house (in feet)? Ans:
4. Number of person enter into the house:
   a. 1 b. 2 c. more than 2

5. Number of Flocks per house per year:

   a. 4 b. 6 c. 8 d. 10

6. Litter amount(kg):

   a. 200-600 b. >600-800 c. >800

Observational checklist:

7. Is there any kind of fly net? a. Present b. absent
8. Use of any distinct cloth to enter the house:

   a. Yes b. no

9. Use of separate foot wear to enter the house:

   a. Yes b. no

10. Foot bath facility in the house:

    a. Yes b. no

11. Type of floor:

    a. Mud b. Bamboo c. Wood d. Tin e. Brick f. Others

12. Litter type:

    a. rice husk b. saw dust c. both a & b d. others

13. Type of cooling system during summer season:

    a. Fan b. water sparkling c. others

Flock information:

- Density of broiler per square meter of the house(1 square meter= 10.764 square feet)

  (1 meter=3 feet 3.37 inches):

- Flock size:
- Flock age:
- Average slaughter age of the bird:

  a. <35 days b. >35 days

- Number of dead birds per flock:

  a. 0-50 b. 50-100 c. 100-200 d. more than 200

- Season of the sample collection:

  a. Summer b. Autumn c. Spring d. Winter

- Number of day old chicks per meter square house area:
- Presence of infected neighboring broiler farms?

(2km, 30 days before and 14 days after Sample collection)

a. presence b. absent

- Practice of ‘all in all out’ system: yes/no
- Disinfection of farm before restock: yes/no
- Broiler house empty for >14 days between flocks: yes/no
- Presence of rodents in the poultry house: yes/no
- Elimination of dead birds every day: yes/no

Questionnaire on broiler meat sample

A. General Information

1. Sample ID (bird ID):	2. Address of stall/market:
3. Sampling date (DD/MM/YY):	4. Sample collector's name:
5. *Google map location (Decimal degrees, DD format): Longitude……………………….Latitude……………………	
6. Source of sample: LBM-ctg-metro LBM-ctg-rural Supermarket-ctg-metro	

B. Packaging information (applicable for supermarket sample)

7. Name of the packaging company	
8. Date of packaging	
9. Expiration date	
10. Lot/batch number	
11. Storage temperature	

C. Hygiene and biosecurity information (applicable for LBM sample)

12. Overall hygiene and cleanliness score of the stall (a score given by the sample collector)	Grade A stall (very clean) Grade B stall (moderate clean) Grade C stall (dirty)
13. Use disinfectant spray in the stall	Yes No
14. How long the sampled bird kept at the stall before slaughtering (Days)?	

Appendix-C

Table 8.1- Descriptive statistics of demographic data of broiler farms (N=216) included into the study

Name of the variable	Coding/categories	n	%
Geographical area	Chattogram	91	42.1
	Dhaka	9	4.1
	Munshiganj	23	10.7
	Narayanganj	46	21.3
	Narsingdi	27	12.5
	Khagrachari	20	9.2
No chicken production	<1000	46	21.4
	1001-2000	129	59.8
	>2000	41	18.9
Overall number of poultry shed/house	<1	124	57.4
	≥1	53	24.6
Water Supply	Deep supply	16	7.5
	Tube well	200	92.6
Establishment year of the house	After 2010	144	66.7
	Before 2010	72	33.3
Person enter into shed	1	172	79.7
	≥ 1	44	20.4
Flock/shed (Year)	5 to 8	44	20.3
	9 to 10	172	79.7
Litter in amount (kg)	200-600	79	78.3
	600-800	30	13.9
	>800	68	31.4
Use of distinctive cloth	No	215	99.6
	Yes	1	0.5
Separate footwear	No	88	40.8
	Yes	128	59.2
Foot bath facilities	No	206	95.4
	Yes	10	4.7

Litter types	Rice husk	7	3.3
	saw dust	88	42.8
	Both	112	52.9
Flock size	<1500	134	62.1
	≥1500	82	37.9
Flock age (days)	<21	89	41.2
	≥21	88	40.8
Selling age (days)	<35	185	85.7
	>35	31	14.3
Dead bird2/Mortality rate	<50	135	62.5
	>50	81	37.5
Season	Spring	16	7.4
	Autumn	58	26.9
	Winter	142	65.7
Infected neighbor broiler farms	Absent	170	78.7
	Don’t know (DK)	19	8.8
	Present	27	12.5
All in all out system	No	50	23.2
	Yes	166	76.9
Disinfection before restock	No	51	23.7
	Yes	165	76.3
Vaccination	No	15	6.9
	Yes	201	93.1
Previous antibiotic use history	No	31	14.3
	Yes	185	85.7
Use of last antibiotic (days)	<4	88	40.8
	>4	128	59.2
Maintain withdraw period of antibiotic	No	216	100
Awareness bad effects of AMR	No	216	100

Table 8.2- Univariable associations between different selected factors with *Campylobacter spp* status of different part of broiler meat collected from super shops and live bird markets in Chattogram (chi-square test)

Variable	Category	Thigh muscle %, (95% CI)	P value	Breast muscle %, (95% CI)	P value	Liver %, (95% CI)	P value	Gizzard %, (95% CI)	P value
Market location	Super shop	12.5, (0.32 – 52.65)	0.027	0	0.007	N/A	0.058	N/A	0.077
	Rural	15, (3.21 – 37.89)		20, (5.73 – 43.66)		10, (1.23 – 31.7)		15, (3.21 – 37.89)	
	Metro	50, (27.19 – 72.8)		55, (31.53 – 76.94)		35, (15.39 – 59.22)		40, (19.12 – 63.94)	
Season	Spring	14.29, (4.03 – 32.66)	0.022	14.29, (4.03 – 32.66)	0.008	10, (1.23 – 31.7)	0.166	15, (3.21 – 37.89)	0.051
	Winter	52.94 (27.81 – 77.02)		58.82, (32.92 – 81.56)		35.29, (14.21 – 61.67)		47.06, (22.98 – 72.19)	
	Autumn	33.33, (0.84 – 90.57)		33.33, (0.84 – 90.57)		33.33, (0.84 – 90.57)		0	
Hygiene score	Very clean	12.5, (0.31 – 52.65)	0.517	0	0.112	N/A	0.952	N/A	0.748
	Moderate	33.33, (16.52 – 53.96)		37.04, (19.4 – 57.63)		22.22, (8.62 – 42.26)		25.93, (11.11 – 46.28)	

	Dirty	30.77, (9.09 – 61.43)		38.46, (13.86 – 68.42)		23.08, (5.04 – 53.81)		30.77, (9.09 – 61.43)	
Birds stay at shop	1 day	35, (15.39 – 59.22)	0.615	30, (11.89 – 54.28)	0.434	25, (5.49 – 57.18)	0.440	33.33, (9.92 – 65.11)	0.844
	2 days	29.41, (10.31 – 55.96)		41.18, (18.44 – 67.07)		29.41, (10.31 – 55.96)		23.53, (6.81 – 49.9)	
	3 days	18.18, (2.28 – 51.78)		18.18, (2.28 – 51.78)		9.09, (0.23 – 41.28)		27.27, (6.02 – 60.97)	
Water source	Tube well	14.29, (1.78 – 42.81)	0.188	28.57, (8.39 –	0.164	7.14, (0.18 – 33.87)	0.149	7.14, (0.18 – 33.87)	0.101
	Pond	16.67, (0.42 – 64.12)		0		16.67, (0.42 – 64.12)		33.33, (4.33 – 77.72)	
	WASA	39.29, (21.5 – 59.42)		39.29, (21.5 – 59.42)		35, (15.39 – 59.22)		40, (19.12 – 63.94)	

Table 8.3- Univariable associations between different selected factors with *C. jejuni* status of different part of broiler meat collected from super shops and live bird markets in Chattogram (chi square test)

Variable	Category	Thigh muscle %, (95% CI)	P value	Breast muscle %, (95% CI)	P value	Liver %, (95% CI)	P value	Gizzard %, (95% CI)	P value
Market location	Super shop	12.5, (0.32 – 52.65)	0.572	0	0.319	N/A	0.548	N/A	1
	Rural	5, (0.13 – 24.87)		10, (1.23 – 31.7)		10, (1.23 – 31.7)		5, (0.13 – 24.87)	
	Metro	15, (3.21 – 37.89)		20, (5.73 – 43.66)		5, (0.13 – 24.87)		5, (0.13 – 24.87)	
Season	Spring	7.14, (0.88 – 23.5)	0.444	7.14, (0.88 – 23.5)	0.217	10, (1.23 – 31.7)	0.784	5, (0.13 – 24.87)	0.911
	Winter	17.65, (3.8 – 43.43)		23.53, (6.81 – 49.9)		5.88, (0.15 – 28.69)		5.88, (0.15 – 28.69)	
	Autumn	0		0		0		0	
Hygiene score	Very clean	12.5, (0.32 – 52.65)	0.348	0	0.284	N/A	0.974	N/A	0.314
	Moderate	14.81, (4.19 – 33.73)		11.11, (2.35 – 29.16)		7.41, (0.91 – 24.29)		7.41, (0.91 – 24.29)	
	Dirty	0		23.08, (5.04 – 53.81)		7.69, (0.19 – 36.03)		0	

Birds stay at shop	1 day	15, (3.21 – 37.89)	0.414	15, (3.21 – 37.89)	0.887	8.33, (0.21 – 38.48)	0.944	8.33, (0.21 – 38.48)	0.458
	2 days	11.76, (1.46 – 36.44)		11.76, (1.46 – 36.44)		5.88, (0.15 – 28.69)		0	
	3 days	0		9.09, (023 – 41.28)		9.09, (0.23 – 41.28)		9.09, (0.23 – 41.28)	
Water source	Tube well	7.14, (0.18 – 33.87)	0.520	14.29, (1.78 – 42.81)	0.613	7.14, (0.18 – 33.87)	0.635	7.14, (0.18 – 33.87)	0.798
	Pond	0		0		16.67, (0.42 – 64.12)		0	
	WASA	14.29, (4.03 – 32.66)		14.29, (4.03 – 32.66)		5, (0.13 – 24.87)		5, (0.13 – 24.87)	

Table 8.4- List of the accession number given by NCBI Genebank

Sl. No.	Sample source	Organism name	Gene name	Protein name	DNA bp	Accession number	Sequence authors
1.	Broiler fecal	*Campylobacter spp*	*16S rRNA*	16S ribosomal RNA	792	MT032361	Islam et al.,2020
2.	Broiler fecal	*Campylobacter spp*	*16S rRNA*	16S ribosomal RNA	792	MT032362	Islam et al.,2020
3.	Broiler fecal	*Campylobacter spp*	*16S rRNA*	16S ribosomal RNA	823	MT032363	Islam et al.,2020
4.	Broiler meat	*Campylobacter spp*	*16S rRNA*	16S ribosomal RNA	828	MT912607	Islam et al.,2020
5.	Broiler meat	*Campylobacter spp*	*16S rRNA*	16S ribosomal RNA	817	MT912608	Islam et al.,2020
6.	Broiler meat	*Campylobacter spp*	*16S rRNA*	16S ribosomal RNA	826	MT912609	Islam et al.,2020
7.	Broiler fecal	*C. jejuni*	*mapA*	outer membrane lipoprotein	564	MT175597	Islam et al.,2020
8.	Broiler fecal	*C. jejuni*	*mapA*	outer membrane lipoprotein	561	MT175598	Islam et al.,2020
9.	Broiler fecal	*C. jejuni*	*mapA*	outer membrane lipoprotein	571	MT175599	Islam et al.,2020
10.	Broiler meat	*C. jejuni*	*mapA*	outer membrane lipoprotein	565	MT920396	Islam et al.,2020

11.	Broiler meat	*C. jejuni*	*mapA*	outer membrane lipoprotein	565	MT920397	Islam et al.,2020
12.	Broiler meat	*C. jejuni*	*mapA*	outer membrane lipoprotein	565	MT920398	Islam et al.,2020
13.	Broiler fecal	*E. coli*	*astA*	arginine N-succinyltransferase	67	MT928164	Islam et al.,2020
14.	Broiler fecal	*E. coli*	*irp2*	peptide synthetase-like protein	395	MT928165	Islam et al.,2020
15.	Broiler fecal	*E. coli*	*iucD*	IucD protein	682	MT928166	Islam et al.,2020
16.	Broiler fecal	*E. coli*	*iss*	increased serum survival lipoprotein	270	MT982360	Islam et al.,2020
17.	Broiler fecal	*E. coli*	*cvaC*	colicin V immunity protein	291	MT982361	Islam et al.,2020

Table 8.5- List of the accession number NCBI Genebank that were used for phylogenetic analysis of *C. jejuni*

SL No.	Accession ID	Gene	Species	Sample source	Host	Year	Country	Ref.
1	MT175597	*mapA*	C. jejuni	Feces	Chicken	2020	Bangladesh	Islam et al.
2	MT175598	*mapA*	*C. jejuni*	Feces	Chicken	2020	Bangladesh	Islam et al.
3	MT175599	*mapA*	*C. jejuni*	Feces	Chicken	2020	Bangladesh	Islam et al.
4	MT920396	*mapA*	*C. jejuni*	Meat	Chicken	2020	Bangladesh	Islam et al.
5	MT920397	*mapA*	*C. jejuni*	Meat	Chicken	2020	Bangladesh	Islam et al.
6	MT920398	*mapA*	*C. jejuni*	Meat	Chicken	2020	Bangladesh	Islam et al.
7	CP040610	Whole genome	*Campylobacter spp*	Stool	Human	2020	Chile	Gonzalez-Escalona et al.
8	CP048761	Whole genome	*C. jejuni*	Meat	Duck	2020	China	Tang, B.
9	MK156117	*mapA*	*C. jejuni*	Feces	Pig	2019	India	Muralikrishna and Sunil
10	LR134498	Whole genome	*C. jejuni*	N/A	N/A	2018	UK	Reference strain
11	CP007193	Whole genome	*C. jejuni*	Litter	Farm	2017	UK	Timms,A.R.
12	CP012690	Whole genome	*C. jejuni*	Stool	Human	2015	South Africa	Parker et al.
13	CP001876	Whole genome	*C. jejuni*	Placenta	Sheep	2014	USA	Luo et al.
14	CP047482	Whole genome	*C. jejuni*	Stool	Human	2020	Chile	Gonzalez-Escalona et al.
15	CP040015	Whole genome	*C. jejuni*	Stool	Human	2020	Czech Republic	Cejkova et al.
16	CP019838	Whole genome	*C. jejuni*	Feces	Cattle	2019	Belgium	Li et al.

17	CP010511	Whole genome	*C. jejuni*	Blood	Human	2016	Finland	Akinrinade et al.
18	CP022470	Whole genome	*C. jejuni*	Stool	Human	2017	USA	Parker et al.
19	CP010906	Whole genome	*C. jejuni*	Clinical isolate	Human	2016	Sweden	Ghaffar et al.
20	CP027403	Whole genome	*C. jejuni*	Blood	Human	2019	South Africa	Kerrigan et al
21	MK736274	*mapA*	*C. jejuni*	Rectum	Bat	2020	Grenada	Lee et al.
22	CP063536	Whole genome	*C. hepaticus*	Bile	Chicken	2020	USA	Wu et al.
23	CP011777	Whole genome	*C. coli*	Heart	Chicken	2015	Germany	Zautner et al.
24	KY473955	*mapA*	*C. jejuni*	Feces	Chicken	2017	Egypt	Ghoneim et al.
25	CP006851	*mapA*	*C. jejuni*	Stool	Human	2015	USA	Eucker et al.

Chapter-9: References

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Chapter-10: Biography

Md. Sirazul Islam is a veterinarian, son of Late Akbar Ali Khan and Mrs. Nasreen Akther Khanam who was born in Chattogram Upazilla at Chattogram, Bangladesh. He completed his Secondary School Certificate (SSC) Examination in 2009 from Chattogram Government High School and Higher Secondary Certificate (HSC) Examination in 2011 from Chattogram College. He completed Doctor of Veterinary Medicine (DVM) from Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh in 2018. He have been studying Masters of Science at the Department of Pathology and Parasitology of Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh. Currently he is working as a frontline fighter in CVASU COVID-19 detection laboratory, Bangladesh. He is also a research assistant in the Department of Pathology and Parasitology, CVASU under the project "Determination of Antimicrobial Resistance and residues in Livestock and Poultry Food Products and Feed in Bangladesh" funded by Livestock Division, Bangladesh Agricultural Research Council (BARC). He has published 1 article as a first author and 3 articles as co-authors. His expertise and research interests lie in the realm of infectious diseases, especially emerging and reemerging pathogens in tropical environments, clinical & molecular pathology, veterinary & medical microbiology, genomics, and bioinformatics. He has an affiliation with various bodies, including the American Society for Microbiology (ASM), Bangladesh Veterinary Council (BVC), One Health Bangladesh, Hubnet (One Health South Asian Network), Bangladesh Veterinary Association (BVA). He has received Scholarships from the United States Department of Agriculture (USDA), Bangladesh Agriculture and Research Council (BARC), and a fellowship from the National Science & Technology & University Grants Commission (UGC) Bangladesh Post Graduate Research Grant.