PREVALENCE AND RISK FACTORS OF *CAMPYLOBACTER* SPP. COLONIZATION IN BROILER FARMS OF MUNSHIGONJ, NARAYANGANJ AND NARSINGDI DISTRICTS IN BANGLADESH

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PREVALENCE AND RISK FACTORS OF *CAMPYLOBACTER* SPP. COLONIZATION IN BROILER FARMS OF MUNSHIGONJ, NARAYANGANJ AND NARSINGDI DISTRICTS IN BANGLADESH

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This is to certify that the thesis entitled **"PREVALENCE AND RISK FACTORS OF *CAMPYLOBACTER* SPP. COLONIZATION IN BROILER FARMS OF MUNSHIGONJ, NARAYANGANJ AND NARSINGDI DISTRICTS IN BANGLADESH"** submitted to the Department of Microbiology & Parasitology, Faculty of Animal Science & Veterinary Medicine, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTERS OF SCIENCE (M.S.)** in **MEDICINE**, embodies the result of a piece of bonafide research work carried out by **MUHAMMAD AL-MARUF**, Registration No. **19-10259** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

I further certify that any help or source of information, received during the course of this investigation has been duly acknowledged.

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DEDICATED TO
MY
BELOVED PARENTS AND
TEACHERS

DECLARATION

I declare that the thesis hereby submitted by me for the MS degree at the Sher-e-Bangla Agricultural University is my own independent work and has not previously been submitted by me at another university/faculty for any degree.

Date: 01.12.2021   **MUHAMMAD AL-MARUF**

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LIST OF ABBREVIATIONS AND ACRONYMS

ABBREVIATION FULL MEANING

PFGE	:	Pulse field gel electrophoresis
RAPD	:	Random amplification of polymorphic DNA
GBS	:	Guillian–Barré Syndrome
BHI	:	Brain heart infusion
TSI	:	Triple sugar iron
EFSA	:	European food safety authority
bp	:	Base pair
rpm	:	Revolution per minute
ELISA	:	Enzyme linked immuno sorbent assay
PCR	:	Polymerase Chain Reaction
IFT	:	Immunofluorescence test
lpx	:	Lipopolysaccharide
TAE	:	Tris Acetate EDTA
DNA	:	Deoxyribonucleic Acid
g	:	Gram(s)
mCCDA	:	Modified charcoal cefoperazone deoxycholate agar
TAE	:	Tris-acetate-EDTA
μl	:	Microliter
mg	:	Milligram(s)
ml	:	Milliliter(s)
CFU	:	Colony forming unit
sec	:	Second(s)
^{0}C	:	Degree celcius
%	:	Percent
Fig.	:	Figure

ABSTRACT

Poultry origin zoonotic *Campylobacter* spp. is considered as one of the leading causal agents of human foodborne illness. A cross-sectional study (October 2020 to January 2021) was conducted to estimate the prevalence of *Campylobacter* spp. colonization and its associated risk factors in the broiler farms of Munshigonj, Narayanganj and Narsingdi Districts in Bangladesh. Cloacal swab samples were collected and pooled from the broiler farms Standard bacteriological and molecular techniques were followed to isolate and identify *Campylobacter* spp. Data on management, biosecurity, mortality and hygiene practices were collected using a structured questionnaire. The majors risk factors were analyzed at the farm level. Among 100 pooled (five samples from each broiler farm) cloacal swab samples from 100 broiler farms, the prevalence of *Campylobacter* spp. was estimated to be 24% (95% CI 16.02–33.57). The tended to be higher prevalence *Campylobacter* spp. colonization was found in Narsindi district (32.35%) followed by Narayanganj (27.78%) and Munshiganj (10%) (p=0.09). In risk factor analysis, the factors more significantly associated with *Campylobacter* colonization were human traffic (more than one person enter into the shed, p=0.006), source of water (tube well, p=<0.001), not using of dedicated cloth or footwear into the farms (p=0.032), no use of disinfection of farms before restock (p=0.014*)* and less gap (minimum 14 days) between two batches during broiler rearing (p=0.008) associated with tended risk factors included absence of footbath facilities, presence of rodents in the farms, litter store outside of farms ($0.05<p<0.1$). The study gathered evidence of the presence of *Campylobacter* spp. colonization in the broiler farms and identified the factors that could help set effective interventions in the controlling of *Campylobacter* infection in chickens to reduce *Campylobacter* infection in humans through broilers.

Keywords: *Campylobacter* spp., cloacal swab, prevalence, risk factors, Biosecurity.

CHAPTER I

INTRODUCTION

Foodborne diseases are a growing public health problem in developed and developing countries (Elmi, 2004). Especially bacteria are a common cause of foodborne illness responsible for high levels of morbidity and mortality in the general population, but particularly for at-risk groups, such as infants and young, children, the elderly, and the immunocompromised. There has been a rise in the global incidence of campylobacteriosis in the past decade. *Campylobacter* spp. is one of the most important human pathogens causing diarrhea and other diseases like septicemia, meningitis, and complications reactive arthritis and Guillain-Barré syndrome (Richard AC Hughes, David R Cornblath, 2005; Leirisalo-Repo, 2005; C. Uzoigwe, 2005; RKI, 2006). Most human campylobacter severe cases are foodborne. Handling or consuming raw or undercooked poultry meat is a risk factor for human infection (Loewenherz-Lüning *et al.,* 1996; RKI, 2006; Adak *et al.,* 2005).

Thermophilic *Campylobacter* species have become the most frequent cause of bacterial gastroenteritis worldwide (Man, 2011). Campylobacteriosis exceeds the total number of cases caused by *Salmonella, Shigella, and Escherichia coli O157:H7* in humans (EFSA, 2011). There has been a rise in the global incidence of campylobacteriosis in the past decade. The number of cases of campylobacteriosis has increased in North America, Europe, and Australia. Data from Asia, Africa, and the Middle East indicate that *Campylobacter* is endemic in these areas (Kaakoush *et al.*, 2015). Campylobacteriosis is a common worldwide cause (5– 14%) of diarrhea. An estimated 2.4 million human campylobacteriosis cases in the United States occur yearly (Bae *et al.,* 2005). *Campylobacter* is responsible for more than 2,80,000 cases of food poisoning each year in the UK. Indian studies have reported *Campylobacter* infections in 4.5-15.6% of the diarrhea cases (Rajendran *et al.*,2012, Ghosh *et al.*, 2016). In 2012, the annual incidence of the disease was reported as 4.4-9.3per 1000 people in high-income countries (WHO, 2012).

The genus *Campylobacter* family Campylobacteraceae comprises 34 speciesand 14 subspecies (http://www.bacterio.net/campylobacter.html, as accessed on

04.11.2016). *Campylobacters* are Gram-negative, spiral, catalase, oxidase, and indoxyl-acetate positive bacteria with cork-screw motility. Of all the species, *C. jejuni* and *C. coli* are the most important from the food safety point of view and cause gastroenteritis in domestic animals and human beings (Nachamkin *et al.*, 2008).

Campylobacteriosis is an acute gastrointestinal infection with severe abdominal pain, fever, nausea, headache, muscle pain, and diarrhea. The incubation lasts 3–5 days, with symptoms lasting 5–7 days. Infections are typically self-limiting. Other conditions associated with the gastrointestinal tract include inflammatory bowel disease, esophageal disease, periodontitis, celiac disease, cholecystitis, and colon cancer. Complications of *Campylobacter* are Guillain-Barre syndrome, reactive arthritis, hemolytic uremic syndrome, and meningitis (Kopyta and Wardak, 2008). Poultry has been recognized as the primary reservoir and source of transmission of campylobacteriosis to humans. Other animals like swine, cattle, and wild birds are the potential reservoir for the bacteria.

In recent years, researchers have developed many PCR-based assays for the detection and molecular characterization of *Campylobacters* in food and reported PCR tests as more sensitive and specific than conventional methods (Klena *et al.,* 2004, Maridor *et al*., 2011, Singh *et al.,* 2011 and Fontanot *et al.,* 2014). Most of these PCR assays were developed to target the chromosomally located virulence-associated gene *cadf* (Awadallah *et al.,* 2014) and the hippiuricase gene (*hipO*) characteristic of *C. jejuni*, a sequence partly covering anaspartokinase gene (*asp*) characteristic of *C. coli*, and a universal 16S rDNA gene sequence (Persson and Olsen, 2005).

Considering the public health significance of *Campylobacter* spp. as a foodborne pathogen with poultry being the primary reservoir and reports of increased resistance of *Campylobacter* isolates from poultry and human beings, the present study is proposed with the following objectives.

OBJECTIVES OF THE INVESTIGATION:

1. To estimate the prevalence of *Campylobacter* spp. colonization in broiler farms.

2. To identify the risk factors associated with *Campylobacter* spp. colonization in broiler farms.

CHAPTER II

REVIEW OF LITERATURE

Campylobacters were initially classified in the genus *Vibrio*. In 1913, two veterinarians, McFadyean and Stockman, isolated "*Vibrio fetus*" from the stomach contents of an aborted lamb (McFadyean and Stockman, 1913). In 1931, a new "*Vibrio*" was reported that caused dysentery in calves during the winter, and its name was proposed as *C. jejuni* (Jones *et al.,* 1931). In 1944, Doyle isolated a similar *Vibrio* from pigs suffering from swine dysentery, and he named it *Vibrio coli* (Doyle, 1944).

The first documentation of *Campylobacter* was in 1886 when the odor Escherich noted the presence of aspiral bacterium in stool from children deceased of what he called" cholera infantum" The first well-documented "Vibrio-related" case of human infection occurred during a diarrhoeal outbreak caused by contaminated milk (Levy, 1946) where organisms resembling "*Vibrio jejuni*" were isolated.

King (1957) was the first to culture microaerophilic isolates of *V. fetus* at 42°C successfully. However, this temperature was higher than the optimal growth temperature of traditional vibrios, and isolates were 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* (Sebald and Veron, 1963 and; Veron and Chatelain, 1973). *Campylobacter* was finally recognized as a human pathogen after successful isolation from human feces in 1972 (Dekeyser *et al.,* 1972). 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) enabled *Campylobacter* isolation from stool, a crucial step in the re-evaluation of the *Campylobacter* epidemiology.

2.1 Genus- *Campylobacter* characteristics –The genus *Campylobacter* belongs to the family *Campylobacteriaceae* in the class *Epsilonproteobacteria* of phylum Proteobacteria. *Campylobacters* are Gram-negative, curved or spiral rods 0.2–0.4 µm wide, 0.5–5µm long, and non-spore-forming. All *Campylobacters* are oxidase-positive, catalase-positive, urease- negative and motile using a single polar unsheathed flagellum at one or both ends of the cell (Ursing *et al.,* 1994),

except *Campylobacter gracilis* (oxidase-negative and aflagellate). *Campylobacters* are 'microaerophilic' and generally require a 3–5% concentration of carbon dioxide, 3–15% concentration of oxygen, and a temperature of 42°C for optimum growth. As *Campylobacter* cells age, they become coccoid in shape (Moran and Upton, 1987). Currently, the genus *Campylobacter* consists of 34 species and 14 subspecies (http://www.bacterio.net/campylobacter.html, accessed on 04.11.2016).

2.2 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 primary sources of infection (Adak *et al.,* 2005 and Fhogartaigh & Edgeworth, 2009). Infectious 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).

2.2.1 Symptoms - The incubation period of *Campylobacter* is usually between one and three days but can be as long as ten days (Koenraad *et al.,* 1997). The symptoms of human campylobacteriosis include an initial period of fever, headaches, and malaise that lasts up to 24 hours. This is then followed by diarrhea and severe abdominal pain. The fever persists, but nausea and vomiting are less familiar features of the infection, occurring in approximately 40% of cases (Gillespie *et al.,* 2002 and Koenraad *et al.,* 1997). The patient may excrete *Campylobacter* organisms for up to three weeks post-infection, with the *Campylobacter* count in feces from infected humans in the range of 10^6 to 10^8 bacteria per gram (Taylor *et al.,* 1993).

Most cases of campylobacteriosis are self-limiting; however, infections arising from the direct spread of *Campylobacter* from the gastrointestinal tract can include cholecystitis, pancreatitis, peritonitis, and massive gastrointestinal hemorrhages (Allos, 2001). Although rare, the following illnesses can occur extra-intestinal manifestations of meningitis, endocarditis, septic arthritis, osteomyelitis, and neonatal sepsis. Bacteraemia is detected in less than 1% of cases of campylobacteriosis and occurs predominantly in the very young or old or the immunocompromised (Allos, 2001). Two well-recognized complications arising from infection by *C. jejuni* are Guillian–Barré Syndrome (Endtz *et al.,* 2000) and

the related Miller-Fisher Syndrome (Salloway *et al.,* 1996). These syndromes are neuro-paralytic, autoimmune disordersthat affect the peripheral nervous system and have led to fatal respiratory paralysis in 5% of Guillian–Barré Syndrome (GBS) cases. Typical symptoms reportedly include progressive weakness beginning in the legs and moving upwards to the arms and cranial nerves (Hadden and Gregson, 2001). Patients may experience numbness, pain, and difficulty swallowing. GBS follows in 0.1% of campylobacteriosis cases (Nachamkin *et al.,* 1998).

2.2.2 Epidemiology - *Campylobacter* is one of the most frequently reported causes of acute bacterial gastroenteritis in developed countries. Significant variations in incidence rates have been observed between different countries (Kaakoush *et al.,* 2015). Several factors, including differences in infection rates in food animals, food production systems, or different food consumption patterns, can be responsible for these variations. These incidence rates can also occur because of differences in diagnosis, reporting systems, or case definitions used in each country's surveillance systems (Brieseman, 1990). In the United States, the annual number of campylobacteriosis cases from 1998 to 2008 was estimated to be 8 45,024, resulting in 8,463 hospitalization and 76 deaths (Batz *et al.,* 2012).

Based on the Community Zoonosis Reports of the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), campylobacteriosis has been the most commonly reported zoonosis in the EU followed by salmonellosis and yersiniosis (EFSA, 2007, 2010c). In 2008, campylobacteriosis was humans' principal cause of zoonotic disease, with 1,90,566 confirmed cases (EFSA, 2010c). Although *Campylobacter* species are recognized as among the most common causes of diarrhea worldwide, the epidemiology of *Campylobacter* infections in the developing world differs markedly from that in the developed world. Cases of human campylobacteriosis have been reported from China, Japan, the Middle East, Australia, and Africa (Kaakoush *et al.,* 2015).

2.3 Human Outbreaks/infection – Several outbreaks caused by *Campylobacter* species have been reported from different parts of the world, including the UK, USA, Germany, Norway, Poland, Mexico, China, Japan, etc. (Kaakoush *et al.,* 2015).

The first well-documented incident of the human *Campylobacter* outbreak due to contaminated milk with *C. jejuni* took place in Illinois in May 1938 that affected 355 inmates of two adjacent state institutions (Levy, 1946).

Unicomb *et al.*, 2009 reviewed reports of outbreaks of campylobacteriosis in Australia from 2001 to 2006, with 33 outbreaks affecting 457 persons. The foodborne transmission was reported or suspected in 27 (82%) outbreaks. A vehicle or suspected vehicle was determined for 16 (59%) outbreaks; poultry was associated with 11 (41%) of these, and unpasteurized milk and salad were associated with two outbreaks each.

Karagiannis *et al.*, 2010 investigated a waterborne *Campylobacter jejuni* outbreak in Crete, Greece, where most of the cases originated from rural areas and found solid epidemiological evidence that tap water was the vehicle of the outbreak.

Stuart *et al.*, 2010 investigated one of the most significant reported campylobacteriosis outbreaks in Canada in June 2007 in British Columbia, affecting 225 bike racers, and contaminated mud was found to be the source of *Campylobacter* infection.

Taylor *et al.*, 2013 reviewed reports of campylobacteriosis in the US from 1997 to 2008 where 262 outbreaks with 9,135 illnesses, 159 hospitalizations, and three deaths. The foodborne transmission was reported in 225 (86%), water in 24 (9%), and animal contact in seven (3%) outbreaks.

A multistate outbreak involving 148 *Campylobacter* infections associated with unpasteurized milk was reported in the US (Longenberger *et al.*, 2013). Edwards *et al.,* 2014 investigated a foodborne outbreak associated with chicken liver pate affecting 49 persons (22 culture positive for *Campylobacter* spp.) following a wedding party in the East of England.

2.4. Prevalence in animals – *Campylobacter* has been detected in many domestic and wild animals, poultry (commercial chickens, ducks, turkey, and quails), and wild birds. Poultry is a significant food vehicle of *Campylobacters*. Commercially raised poultry is nearly always colonized by *C. jejuni* and *C. coli,* and slaughterhouse procedures amplify the contamination. The transportation of poultry, overcrowding of birds, and aerosol contaminate healthy birds or carcasses. Several reports incriminating chicken as a primary source of

Campylobacter species to humans have been reported in the literature (Suzuki and Yamamoto, 2009, Silva *et al*., 2011, Kaakoush *et al*., 2015 and Skarp *et al*., 2016)

Cattle act as a second primary source of *Campylobacter* spp. to humans. *C. jejuni* is a commensal organism of the intestinal tract of cattle. Recent studies from Denmark showed that cattle were the attributed source for 16 to 17% of the total cases of campylobacteriosis (Boysen *et al.,* 2014). Similarly, in Switzerland, cattle have been estimated to be responsible for 19.3% of *Campylobacter* infections, which is substantially higher than the contribution from pigs (1.2%) (Kittl *et al*., 2013).

Campylobacter spp. is also prevalent in pigs and piglets. However, compared to cattle, pigs are more readily colonized by *C. coli* (Alter *et al*., 2005). A carriage rate of 32.8 to85.0% has been observed, depending on the age of the pig (Weijtens *et al*., 1993; Alter *et al.,* 2005).

The prevalence of *Campylobacter* spp. in sheep and goats in different countries varies. 6.8% of intestinal samples from sheep were positive for *Campylobacter* in Nigeria (Raji *et al.,* 2000). However, a higher prevalence rate of 17.5% was reported for caecal samples from sheep from Swiss abattoirs (Zweifel *et al.,* 2004). In both studies, *C. jejuni* was the major *Campylobacter* species detected. *Campylobacter* spp. is also prevalent in dogs and cats. In a study, 58% of healthy dogs and 97% of diarrheic dogs have been found to be positive for *Campylobacter* spp. (Chaban *et al.,* 2010). However, *C. upsaliensis* has been the significant species detected in dogs and cats (Baker *et al.,* 1999).

Wild birds are most likely to carry Campylobacters among all the host species studied. The prevalence of *Campylobacter* spp. varies greatly depending on the type of wild birds and their geographic regions. A study in Colorado examined caecal contentsfrom 445 wild ducks and found that 35% were positive for *C. jejuni* (Luechtefeld *et al*., 1980). A similar study in Norway examined 540 wild birds of 40 different species and found that 28.4% were positive for *C. jejuni*.

2.5. Prevalence in foods – *Campylobacter* species are ubiquitous and isolated from awide variety of foods, including chicken meat, milk, beef, chevon, and water, but poultry meat remains the primary source of *Campylobacter* spp. to humans. It has been estimated that 71% of human campylobacteriosis cases in

Switzerland between 2001 and 2012 were attributed to chickens (Kittl *et al.*, 2013; Wei *et al.*, 2014). The UK Food Standards Agency reported that 72.9% of fresh whole retail chickens surveyed from 2014 to 2015 were infected with *Campylobacter* (Food Standards Agency, 2014). Suzuki and Yamamoto (2009) surveyed the literature on contamination of retail poultry meats and by-products worldwide. They reported that in most of the countries (both developed and developing countries), despite their sanitary conditions, most retail poultry meats and by-products were contaminated with *Campylobacter* spp. The observed prevalence of *Campylobacter* in poultry in different countries varies. Australia (100%), Argentina (92.9%), Czech (100%), New Zealand (89.1%), and Oceanica (90.4%) have a much higher prevalence. In comparison, Belgium (17%), Estonia (8.1%), Former Soviet Union and Eastern Europe (19.1%), Switzerland (25.1%), and Vietnam (30%) have a lower prevalence of *Campylobacter* in poultry (Suzuki and Yamamoto, 2009).

Malik et al. (2014) investigated the prevalence of *Campylobacter* among broilers in the Bareilly region and found 32% of samples positive for *Campylobacter* species.

Pallavi and Kumar (2014) studied the prevalence of *Campylobacter* species in foods of animal origin. The prevalence of *Campylobacter* spp. in chicken meat, chevon, and milk samples was observed at 17.33%, 6%, and 0%, respectively.

Raw milk has also been identified as a vehicle of human gastroenteritis caused by *Campylobacter* spp. (Blaser *et al*., 1979, Robinson *et al*., 1979, Porter and Reid, 1980, Potter *et al*., 1983). *C. jejuni* may be present in milk due to fecal cross-contamination during milking or as a result of udder infection (Doyle and Roman, 1982, Orr *et al*., 1995).

Water is an effective vehicle for transmitting *Campylobacter* species to humans and animals, and contaminated water has been responsible for several outbreaks in different countries (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 drinking water samples (5/500 samples) in Northern Greece. Popowski and colleagues detected *C. jejuni, C. coli,* or *C. lari* in 70% of water samples from rivers or lakes in the Warsaw region of Poland (Popowski *et al*., 1997).

2.6. Detection methods - As food safety has become an increasing concern for consumers, there is a growing need for fast and sensitive methods for specific detection and identification of zoonotic microorganisms. Laboratory diagnosis of *Campylobacter* infection requires the use of culture-dependent and/or culture-independent methodologies. Recently several new approaches have been used to detect different bacteria from foods. These methods include ELISA, IFT, nucleic acid probes, PCR, etc. The PCR technique has several advantages over classical bacteriology concerning detection limit, speed, and the potential for automation and has successfully been 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).

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

Skirrow (1977) developed a selective medium for isolating *Campylobacter* from stool samples. This medium enabled the successful recovery of *Campylobacter* and therefore provided evidence linking disease to the contamination of food, particularly chicken. Wang *et al.* (1980) developed an enriched brucella medium for storing and transporting cultures of *Campylobacter fetus* sub sp. *jejuni.*

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

Bolton *et al*. (1983) studied the comparison of selective media to isolate *Campylobacter jejuni/coli.* They compared five different selective media, Skirrow's, Butzler's, Blaser's, Campy-BAP and Preston's medium, to isolate *Campylobacter*. It was reported that Preston medium preceded by enrichment on

modified Preston Enrichment Broth was found to be the most selective medium for *Campylobacter*, while Butzler was the least effective.

Zanetti *et al*. (1996) tested several selective agars for their efficacy in isolating campylobacters. Preston, charcoal cefoperazone deoxycholate (CCDA), and Butzler agars are equally effective. The use of CCDA and incubation at 42˚C rather than 37˚C is usually the methodology of choice since it allows for isolating more *Campylobacter* strains.

Baylis *et al*. (2000) compared the performance of pre-enrichment media to recover *campylobacters* from food using artificially and naturally contaminated samples. All pre-enrichments included an initial period of resuscitation, 4 hr at 37°C after pre-enrichment; all were subcultured to mCCDA agar. The pre-enrichment broths were: Bolton broth, *Campylobacter* enrichment broth (CEB), and Preston broth. Preston broth supported the growth of the most significant number of *Campylobacter* strains but failed to inhibit some competitor organisms. In contrast, CEB inhibited all competitors but failed to support all *Campylobacter* strains.

The standard method (ISO, 2006a) for detection and isolation and a direct plating method for enumeration of *Campylobacters* (ISO, 2006b) use mCCDA as the selective agar. In these methods, Bolton broth is used for the enrichment step with incubation at 37˚C in a microaerophilic atmosphere for 4–6 hr and with further incubation at 41.5˚C for 40–48 hr and plating on mCCDA and another agar medium of the operator's own choice.

2.6.2 Molecular methods

Isolation of *Campylobacter* from clinical, food, and environmental samples is laborious and takes up to 4-5 days for incubation. Moreover, culture-dependent methods can misidentify species. Thus, various methods for detecting *Campylobacter* spp. from environmental, clinical, and food samples have been investigated. Numbers of techniques like random amplification of polymorphic DNA (RAPD), pulsed field gel electrophoresis (PFGE), DNA hybridization, Latex agglutination, Polymerase Chain Reaction are in use and being the most sensitive, specific, and reliable test, PCR is usually accepted for detection of *Campylobacter* in food.

Researchers have recently developed several PCR-based assays for detecting *Campylobacter* in food. Most of these PCR assay developed targets the 16S rRNA for rapid detection and identification of *Campylobacter* (Kulkarni *et al*., 2002, Maher *et al*., 2003), 23S rRNA gene (contain strain-specific intervening sequences) and the internal transcribed spacer (ITS) region which contains sequence composition depending on the species (Eyers *et al*., 1993, Fermer and Engvall, 1999 and Man *et al*., 2010). When all three regions (16S rRNA, ITS-region and 23S rRNA) were combined to create a phylogenetic tree, the resultant tree had the highest resolution in differentiating between members of the *Campylobacter* genus (Man *et al*., 2010). More recently, real-time PCR methods have been developed that show the potential of detecting as few as 1cfu in chicken samples and less than 2 hr. (Debretsion *et al*., 2007).

Linton *et al*. (1997) developed a method for PCR detection, identification to species level, and fingerprinting of *C. jejuni* and *C. coli* direct from diarrheic samples. Three primers were designed for PCR detection and differentiation of *C. jejuni* and *C. coli*. The first PCR assay was designed to coidentify *C. jejuni* and *C. coli* based on their 16S rRNA gene sequences. Based on the hippuricase gene sequence, the second PCR assay identified all tested reference strains of C. jejuni and strains of that species that lack detectable hippuricase activity. The third PCR assay, based on the sequence of a cloned (putative) aspartokinase gene and the downstream open reading frame, identified all tested reference strains of *C. coli*.

Wage *et al*. (1999) developed a rapid and sensitive assay to detect small numbers of *C. jejuni* and *C. coli* cells in environmental water, sewage, and food samples. A semi-nested PCR based on specific amplification of the intergenic sequence between the two *Campylobacter* flagellin genes*, flaA* and *flaB,* was performed the PCR products were visualized by agarose gel electrophoresis. The assay detected 3 to 15 CFU of *C. jejuni* per 100 ml in water samples containing a background flora consisting of up to 8,700 heterotrophic organisms per ml and 10,000 CFU of coliform bacteria per 100 ml. The assay was also conducted with food samples analyzed with or without overnight enrichment. As few as <3 CFU per g of food could be detected with samples subjected to overnight enrichment, while variable results were obtained for samples analyzed without prior enrichment.

Klena *et al*. (2004) developed a multiplex PCR assay to identify and discriminate between isolates of *C. coli, C. jejuni, C. lari,* and *C. upsaliensis*. The multiplex PCR assay was validated with 105 genetically defined isolates of *C. coli, C. jejuni, C. lari,* and *C. upsaliensis*, 34 strains representing 12 additional *Campylobacter* species, and 24 strainsrepresenting 19 non-*Campylobacter* species. Applying the multiplex PCR method to whole-cell lysates obtained from 108 clinical and environmental thermotolerant *Campylobacter* isolates resulted in 100% correlation with biochemical typing methods.

Persson and Olsen (2005) developed a multiplex-PCR method specifically designed for application in routine diagnostic laboratories to detect *C. coli* and *C. jejuni*. Primers were directed towards the following loci: the hippuricase gene (*hipO*) characteristicof *C. jejuni*, a sequence partly covering an aspartokinase gene characteristic of *C. coli*, and a universal 16S rDNA gene sequence serving as an internal positive control for the PCR. The method was tested on 47 *C. coli* strains and 88 *C. jejuni* strains and found to be almost 100% in concordance with biochemical analyses (all except for one *C. coli* strain), regardless of whether the DNA was prepared from colonies by a simple boiling procedure or by DNA easy Tissue Kit. Pure cultures of *C. coli* and *C. jejuni* were identified at 10–100 cells per PCR.

Asakura *et al.,* (2008) developed a cytolethal distending toxin *(cdt)* gene-based species-specific multiplex PCR assay for the detection of *cdtA, cdtB or cdtC* gene of *C. jejuni, C. coli* and *C. fetus,* respectively, was developed and evaluated with 76 Campylobacter strains belonging to seven different species and 131 other bacterial strains of eight different genera. The specific primer set for the *cdtA, cdtB* or *cdtC* gene of a particular species could amplify the desired gene from a mixture of DNA templates of any of two or all three species. The detection limit of *C. jejuni*, *C. coli* or *C. fetus* was 10–100CFU/tube by the multiplex PCR assay on the basis of the presence of the *cdtA, cdtB* or *cdtC* gene.

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 October 2020 to January 2021 in three districts of Dhaka (Narsingdi, Narayanganj, Munshigonj) (Fig:1). The study area's 100 commercial broiler farms (20% farm population from each district) were selected using simple random sampling and from each farm five birds were randomly sampled (pooled) for this study.
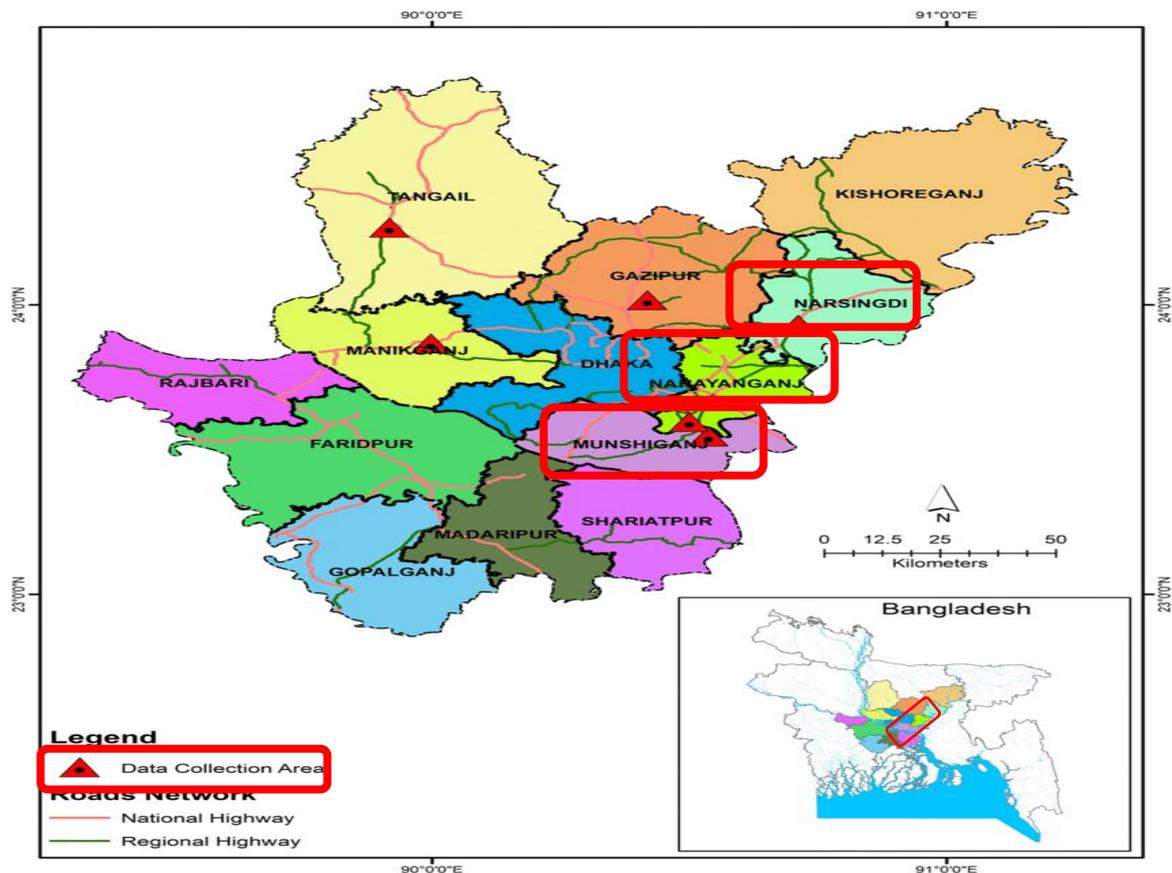
Figure 1: Study Location Munshigonj, Narayanganj & Narsingdi districts of Bangladesh

3.2 Data collection

Necessary verbal permission was taken from the individual farmer before sampling the birds, and epidemiological data was recorded on the questionnaire. The respondents were informed about the study's purpose and sample collection procedure. A farm was included in the study only upon the affirmative response, otherwise excluded. A pre-

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designed structured questionnaire was used to record epidemiological data at the farm through face-to-face interviews and physical observation. Focal points in the questionnaire were –

Sl	Major Risk Factors	Parameters
1	**Farm management**	Number of houses
		Establishment year
		Space, Shed length and width
		Type of floor
		Water supply
		Litter materials
2	**Biosecurity and hygiene**	Use of footwear and dedicated cloth
		Footbath facility
		All in the all-out system
		Disinfection of farm before restocks
		House empty for >14 days before restock
		Number of Flocks per house per year
		Dead bird's disposal system
3	**Flock attributes**	Flock size
		Age of birds
		Number of dead birds per flock
4	**Use of vaccine**	Vaccine types and age of vaccination

3.3 Sample collection procedure

3.3.1 Samples from the broiler farms

Five birds were randomly sampled from each farm, and cloacal swabs were collected using sterile cotton swabs by inserting them into the bird's cloaca (Fig: 2). Later the cloacal swabs were pooled by placed 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)

Figure 2: Collecting of cloacal samples from the live Broiler birds in the farms

3.4 Sample evaluation

Samples were analyzed to identify *Campylobacter* spp. following the methods described by Lund *et al.* (2003) and Lund *et al.* (2004).

3.4.1 Isolation and identification of *Campylobacter* from the collected samples

Standard bacteriological approaches followed by molecular techniques were applied for isolation and identification of *Campylobacter* from the cloacal swabs 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, 1992). The plates were incubated in an anaerobic jar (Oxoid™ AnaeroJar™ 2.5L) under microaerophilic conditions with a CO_2 sachet (Thermo Scientific™ Oxoid Anaero Gen 2.5L sachet) (10% CO_2, 95% humidity) in 42° C for three days (Fig: 3)

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 at 37°C for three days under microaerophilic conditions. The presumptive *Campylobacter* isolates were subjected to microscopic examination to observe the seagull appearance of *Campylobacter spp.* with Gram staining (Vandamme et al., 2008; Boyer et al., 2021). 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.
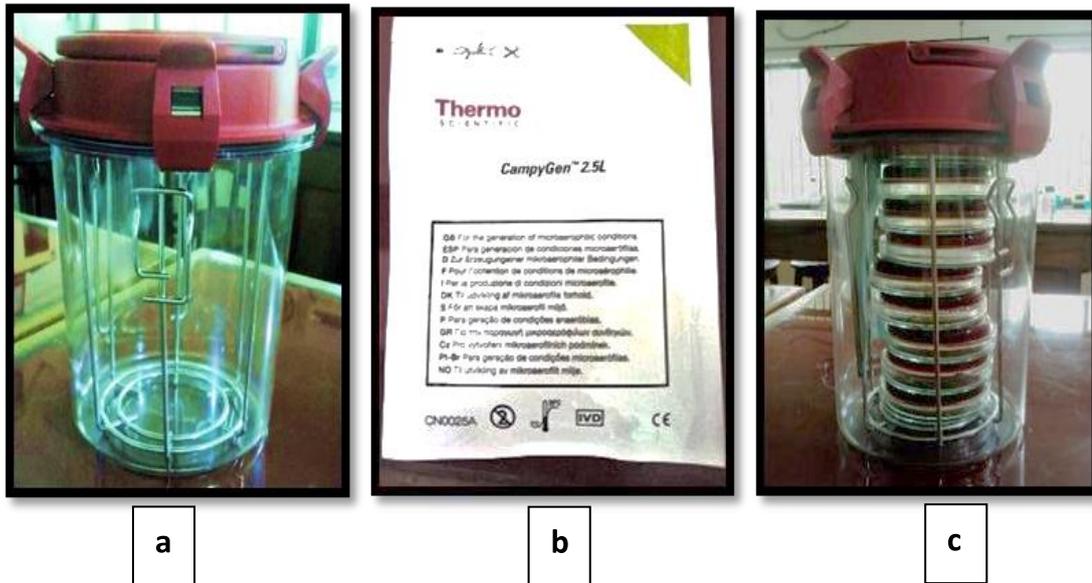
Figure 3: (a) Anaerobic jar (b) CO2 sachet (c) Anaerobic jar containing plate and sachet

3.4.2 Preservation of the isolates

Campylobacter isolates were cultured in brain heart infusion (BHI) broth and 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.

3.4.3 DNA extraction from the isolates

The boiling method was used to extract DNA from the recovered isolates (Englen and Kelley, 2000). Briefly, the procedure was as below:

(i) A loop 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 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.

3.4.4 Molecular identification of *Campylobacter*

3.4.4.1 Polymerase chain reaction (PCR) to test for the presence of *Campylobacter* spp.

A polymerase chain reaction (PCR) assay was conducted for the final confirmation of the suspected isolates by multiplex PCR using *lpx* gene primers listed in **Table-1**. The amplification of the *lpx* gene was carried out for the detection of *Campylobacter* spp. The detailed procedure followed is given below.

Table 1: Primer and oligonucleotide sequence used for the identification of *Campylobacter spp*

Gene	Primer Sequence	Amplification (bp)	Reference
lpx	lpxAF9625 (5'-TGCGTCCTGGAGATAGGC-3') lpxAC.coli (5'-AGACAAATAAGAGAGAATCAG-3') lpxAC.jejuni(5'-ACAACT TGGTGACGATGTTGTA-3') (Forward primers) lpxARKK2m (5'CAATCATGDGCDATATGASAATAHGCCAT-3') (Reverse primer)	331 (*Campylobacter jejuni*) and 391 (*Campylobacter coli*) (Biotech concern, Korea)	(Klena *et al.*, 2004)

3.4.4.2 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-2**

Table 2: Reagents used for PCR amplification of the *Campylobacter* spp.

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 20 µl using 20 picomoles of each primer concentration. Proportions of different PCR reagents are given in **Table-3**. *Campylobacter jejuni* subsp. *jejuni* ATCC 33560 strain and Nuclease-free water were used as a positive and negative control, respectively.

Table 3: Contents of each reaction mixture of PCR assay

Serial no	Name of the contents	Amount
1	Thermo Scientific PCR Master Mix (2x)	10 µl
2	Forward primer (each)	0.5µl
3	Reverse primer	0.5µl
4	DNA template	2µl
5	Nuclease free water	6µl
	Total	**20µl**

PCR was run on a thermocycler (Applied Biosystem, 2720 thermal cycler, Singapore) following the cycling conditions mentioned in **Table-4.**

Table 4: Cycling conditions used during PCR to detect the *lpx* gene of *Campylobacter* spp.

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	52°C for 1 minute
4	Initial extension	72°C for 1 minute
5	Final extension	72°C for 5 minutes
6	Final holding	4°C

3.4.4.3 Visualization of PCR products by Agar Gel Electrophoresis

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

1. 0.75 gm of agarose powder and 50 ml of 1X TAE buffer were 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 gel chamber's ends with tape and placing 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, the combs were removed, and the gel was shifted into an electrophoresis tank filled with 1X TAE buffer and kept until the gel was drowned completely.

5. Five μl of PCR product for a gene was loaded into a gel hole.

6. Three 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).

3.5 Data analysis

3.5.1 Statistical analysis

The study unit of the analysis was a farm in case of cloacal samples. A farm was considered positive if a pooled farm sample was tested positive in PCR. Therefore, the dependent variable in our study was dichotomous outcome as positive and negative. Several continuous variables (e.g., No of chicken production, Human traffic, litter amount, flock size, flock age, etc.) have been transformed into categorical variables to perform the analysis. All the data from the broiler farms (three different districts) were recorded in the 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. To evaluate the association between independent variables (risk factors/determinants) with the dependent variable (sample positive/negative), univariable analysis was performed using the $\chi 2$ test and univariable logistic regression models in STATA-IC 13. A *p*-value ≤ 0.05 was considered significant in the univariable 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 IV

RESULTS & DISCUSSION

4.1 Isolation and identification of *Campylobacter* spp.

The cultural examination of 100 broiler farm (cloacal) samples resulted in the isolation of 24 (24%) *Campylobacter* isolates **(Fig: 4).** Here, 3 (10%) of Munshigonj area broiler farm samples were found positive, 10 (27.78%) of Narayanganj area broiler farm samples got positive, and 11 (32.35%) of Narsingdi area broiler farm samples got positive (*p*=0.09). **(Table-5)**
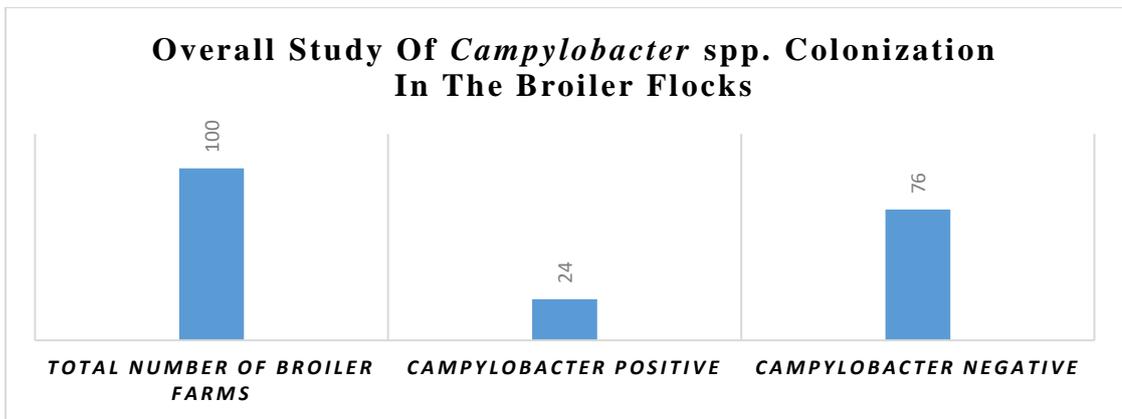
Figure 4: Graphical representation of the overall study.

Table 5: Munshigonj, Narayanganj, and Narsingdi broiler farms were positive for *Campylobacter* spp.

Variable	Category	Positive	Prevalence	*p*-value
	Munshigonj (30)	3	10	
	Narayanganj (36)	10	27.78	
Location	Narsingdi (34)	11	32.35	0.09
	Total (100)	24	24%	
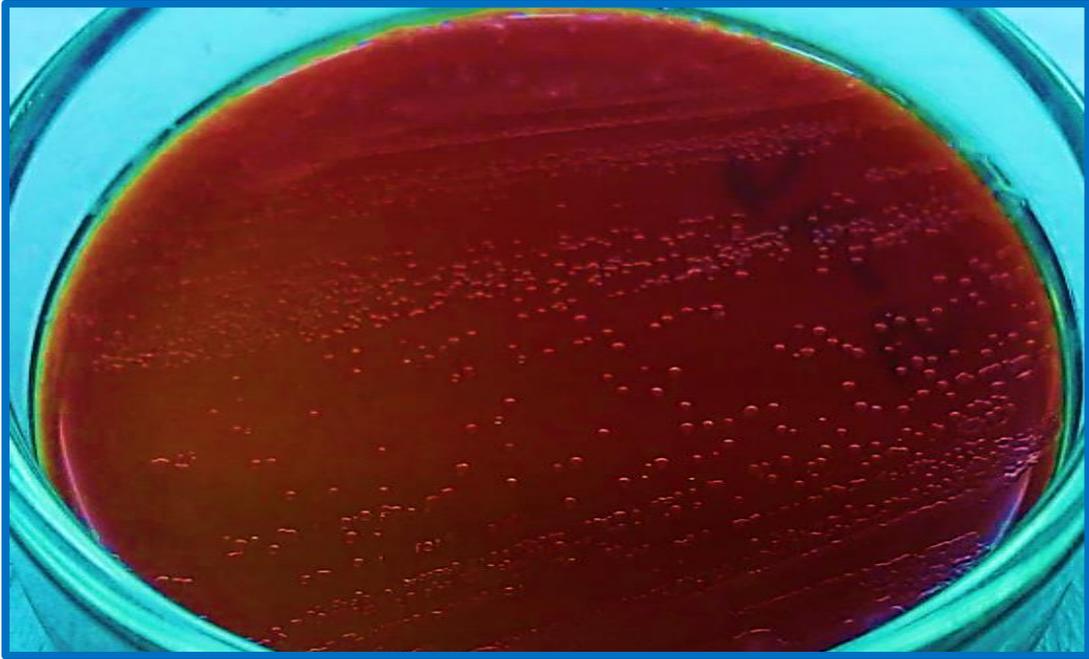
Figure 5: Cultural Response: Good-luxuriant growth of *Campylobacter* spp.
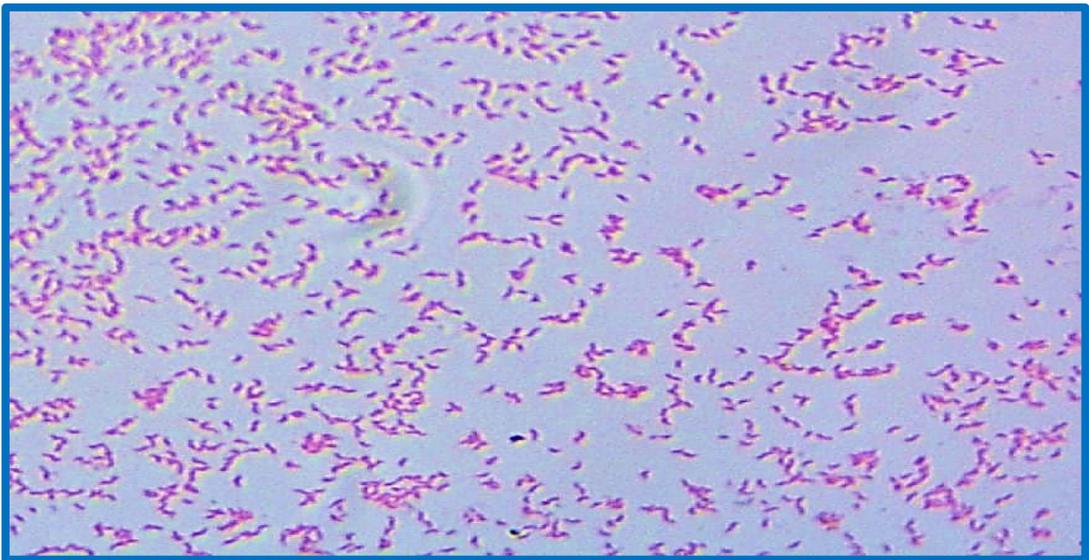
Figure 6: Gram's staining of *Campylobacter* spp. isolate showing characteristic spiral, S-shaped bacteria

4.2 Molecular characterization of *Campylobacter* spp. isolate

Molecular characterization of *Campylobacter* isolates was done using the mPCR technique. A set of published primers, one for *C. jejuni* and one for *C. coli,* were employed to amplify the *lpx* gene (Klena *et al.,* 2004).

Twenty-four (24) DNA samples, when amplified using species-specific primer revealed to be positive. Here 12 samples of amplicon product size were recorded 331 bp for a specific primer for *C. jejuni* and 12 samples were recorded 391 bp for *C. coli* specific

primer out of all the *Campylobacter* isolates when PCR products were run along with a positive reference strain and 100 bp DNA ladder in 1.5% agarose gel electrophoresis and visualized under UV gel documentation system. The following figures (**Fig: 7**) shows positive control bp of *Campylobacter* spp colonization.

(a)   **(b)**
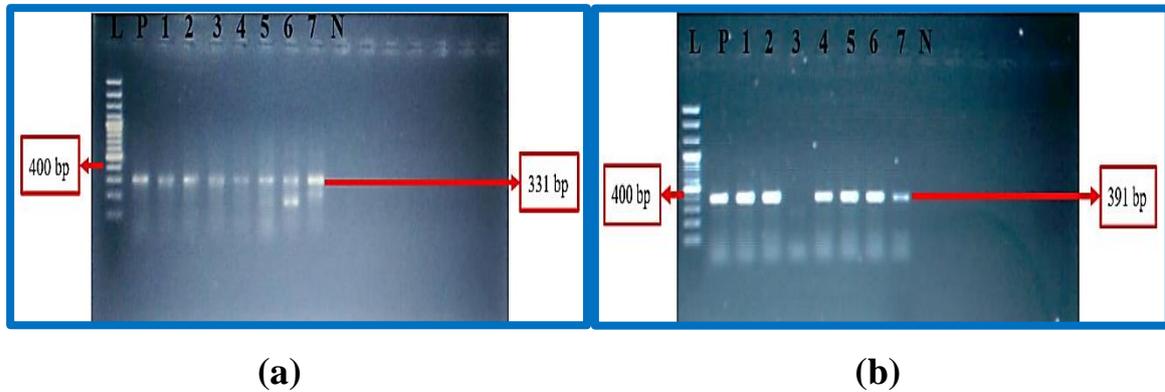
Fig 7: UV visualization of multiplex PCR of lipid A gene (*lpx*)
(a) *C. jejuni* showing 331bp (b) *C. coli* showing 391bp

4.3 Descriptive statistics of the sampled farm

Among 100 farms, 73% were large farms having more than 1000 broilers farms. Most farms (82%) comprised single houses; the rest were double or more. In 72% of farms, one person entered the bird's houses daily, and the remaining 28% were two or more people entering the house daily ($p=0.006$). The water supply in the farms was 76% Deep tube well and 24% tube well ($p=0.000$). 90% of farms store litter outside the sheds and 10% kept inside litter ($p=0.061$). 77% of farms raise more than nine flocks per year per house and 73% follow the 'all-in all-out' system. About 70% of farms did not use separate footwear to enter the shed, and 74% did not have footbath facilities ($p=0.084$). About 56% of farms were brick, and 20% had mud-type floors; the rest were bamboo. About 56% of farms used sawdust, and 44% used mixed with rice husk and sawdust ($p=0.462$). Here, 58% of flocks age under three weeks and 42% more than three weeks, where the number of dead birds per flock is 50 birds around 83% ($p=0.487$), and 58% of farms follow the elimination of dead birds every day ($p=0.608$). In 56% of farms presence of rodents ($p=0.093$), 84% of farms did not undergo disinfection before restocking ($p=0.014$), and 65% of farms did not keep their house empty for 14 days before the introduction of a new flock ($p=0.008$). **(Table-6)**

Table 6: Frequency distribution (descriptive statistics) of different variables regarding farm and farmer demography and management practices variable category frequency percentage (N=100).

Variable	Category (Number of tested samples)	Positive Number (Number of positive samples)	Prevalence (%)	Chi-square *p*-value
Number of Chicken	Minimum – 1000 (27)	5	18.52	
	1001-1500 (41)	12	29.27	0.563
	1501-maxmum (32)	7	21.88	
Number of Shed	1 (82)	21	25.61	
	2 – 4 (18)	3	16.67	0.421
Water Supply	Deep Tube well (76)	7	9.21	
	Tube well (24)	17	70.83	<0.001
Store of Litter	Inside (10)	0	0	
	Outside (90)	24	26.67	0.061
Establishment of House	2017 and after (60)	11	18.33	
	Before 2017 (40)	13	32.5	0.104
Person enters to shed	1 (72)	12	16.67	
	More than 1 (28)	12	42.86	0.006
Flocks per Shed	9 (23)	4	17.39	
	more than 9 (77)	20	25.97	0.398
Litter amount	0 – 500 (57)	14	24.56	
	501 – max (43)	10	23.26	0.88
Use of dedicated cloth to enter the shed	Yes (94)	23	24.47	
	No (6)	1	16.67	0.664

Use of separate footwear to enter the shed	Yes (30)	3	10	0.032
	No (70)	21	30	
Footbath facility	Yes (26)	3	11.54	0.084
	No (74)	21	28.38	
Floor Type	Bamboo (24)	3	12.5	0.302
	Mud (20)	5	25	
	Brick (56)	16	28.57	
Litter Type	Mixed (44)	9	20.45	0.462
	Saw dust (56)	15	26.79	
Flock Size	0-1000 (27)	5	18.52	0.563
	1001-1500 (41)	12	29.27	
	1501-max (32)	7	21.88	
Flock Age	21 (58)	14	24.14	0.970
	After 21 (42)	10	23.81	
Number of dead birds per flock	0 – 25 (50)	11	22	0.487
	26 – 50 (33)	7	21.21	
	more than 50 (17)	6	35.29	
All in all, out system	Yes (73)	17	23.29	0.784
	No (27)	7	25.93	
Disinfection before restock	Yes (16)	0	N/A	0.014
	No (84)	24	28.57	
Broiler house empty for >14 days between flocks	Yes (35)	3	8.57	0.008
	No (65)	21	32.31	

Presence of rodents in the poultry house	Yes (56)	17	30.36	0.093
	No (44)	7	15.91	
Elimination of dead birds every day	Yes (58)	15	25.86	0.608
	No (42)	9	21.43	

4.4 : Prevalence of *Campylobacter* spp. colonization:

A total of 24 pooled samples were found with *campylobacter* spp. positive out of 100 pooled samples, making the farm-level prevalence of *campylobacter* spp. colonization 24% where maximum *Campylobacter* spp. colonization area was recorded in Narsindi area (32.35%) followed by Narayanganj area were (27.78%) and Munshiganj area were (10%) (*p*=0.09). In the risk factors analysis, the prevalence's were more significant in the farms of bigger flock sizes than in smaller flocks (*p*=0.563). This finding is supported with several previous studies from industrialized countries, which have shown broiler flocks to be a significant reservoir of *Campylobacters* (Kapperud *et al.,*1993; Humphrey, 1994; Jacobs-Reitsma *et al.,* 1994; *Stern et al.,* 1995; Berndtson *et al.,* 1996; Nielsen *et al.,* 1997). The present study showed that the management-related factors might be essential drivers and increase the risk of *Campylobacter* spp. colonization. It was revealed that in older birds, more than one person entering the house (*p*=0.008) and using tube well water instead of a Deep tube well was prevalent more where (*p*=0.000). The present study showed a higher risk of *Campylobacter* spp. colonization when more than one person entered the broiler house (95% CI: 1.42-9.91) (*p*=0.008). Human traffic is an important route (via boots, hands, cloths) for introducing *Campylobacter* from the external environment (Hald *et al.,* 2000; Cardinale *et al.,* 2004) mainly if proper biosecurity is not in place.

The most positive case in frequent breeding broiler flocks means more than nine flocks per shed per year (95% CI 0.51-5.49). Most colonized shed did not have footbath facilities (95% CI: 0.82-11.2) (*p*=0.095). The litter materials of sawdust are another factor (95% CI: 0.55-3.65) (*p*=0.463). Old aged birds got more colonization the present study shows after three weeks age of broiler flocks found (95% CI: 0.39-2.490 (*p*=0.97). The present study shows the farms did not follow the all-in all-out

system (95% CI: 0.42-3.19). The present study reveal that the majority farms did not kept 14 days gap between two batches broiler breeding time which is also another source of colonization of *Campylobacter* spp. (95% CI: 1.4-18.54) (*p*=0.014). **(Table-7)**

Table 7: **Univariable logistic regression analysis to evaluate potential factors associated with *Campylobacter* spp. (N=100) status of broiler farm.**

Variable	Category	Odds Ratio	95% CI	*p*-value
Number of Chicken	Min – 1000	Ref		
	1001-1500	1.82	0.56 – 5.93	0.32
	1501-max	1.23	0.34 – 4.44	0.75
Number of Shed	2 – 4	Ref		
	1	1.72	0.45 – 6.54	0.425
Water Supply	Deep Tube well	Ref		
	Tube well	23.94	7.4 – 77.47	<0.001
Establishment of House	2017 and after	Ref		
	Before 2017	2.14	0.85 – 5.44	0.108
Person enters to shed	1	Ref		
	More than 1	3.75	1.42 – 9.91	0.008
Flocks per Shed	9	Ref		
	more than 9	1.67	0.51 – 5.49	0.401
Litter amount	0 – 500	Ref		
	501 – max	0.93	0.37 – 2.36	0.88
Use of dedicated cloth to enter the shed	Yes	Ref		
	No	0.62	0.07 – 5.56	0.667
Use of separate footwear to enter the shed	Yes	Ref		
	No	3.86	1.05 – 14.12	0.041
Footbath facility	Yes	Ref		
	No	3.04	0.82 – 11.2	0.095

Floor Type	Bamboo	Ref		
	Mud	2.33	0.48 – 11.3	0.292
	Brick	2.8	0.73 – 10.71	0.132
Litter Type	Mixed	Ref		
	Saw dust	1.42	0.55 – 3.65	0.463
Flock Size	0-1000	Ref		
	1001-1500	1.82	0.56 – 5.93	0.32
	1501-max	1.23	0.34 – 4.44	0.75
Flock Age	21	Ref		
	After 21	0.98	0.39 – 2.49	0.97
Number of dead birds per flock	0 – 25	Ref		
	26 – 50	0.95	0.33 – 2.78	0.932
	more than 50	1.93	0.58 – 6.41	0.281
All in all, out system	Yes	Ref		
	No	1.15	0.42 – 3.19	0.784
Broiler house empty for >14 days between flocks	Yes	Ref		
	No	5.09	1.4 – 18.54	0.014
Presence of rodents in the poultry house	No	Ref		
	Yes	2.3	0.86 – 6.19	0.098
Elimination of dead birds every day	Yes	Ref		
	No	0.78	0.30 – 2.01	0.609

4.5 Discussion:

Globally, Food safety is a major concern of public health irrespective of age, gender, socioeconomic status and occupation. *Campylobacter* is one of the widely recognized and significant food borne pathogen in both developed and developing countries. Thermophilic *Campylobacter* spp. have become the most frequent cause of bacterial gastroenteritis in human worldwide (Man, 2011). So far, 34 species and 14 subspecies of *Campylobacter* have been isolated, but *C. jejuni* and *C. coli* are most important

from food safety point of view and causes gastroenteritis in domestic animal and human being (Nachamkin *et al.,* 2008).

There has been a rise in global incidence of campylobacteriosis in past decade. The numbers of cases of campylobacteriosis have increased in North America, Europe and Australia. Reports from Asia, Africa and Middle East indicate that *Campylobacter* is endemic in these areas (Kaakoush *et al.,* 2015). The observed prevalence of *Campylobacter* in poultry in different country varies. Australia (100%), Argentina (92.9%), Czech (100%), New Zealand (89.1%) and Oceanica (90.4%) have a much higher prevalence, while Belgium (17%), Estonia (8.1%), Former Soviet Union and Eastern Europe (19.1%), Switzerland (25.1%) and Vietnam (30%) have a lower prevalence of *Campylobacter* in poultry (Suzuki and Yamamoto, 2009).

Keeping in view the public health significance of *Campylobacter* spp. as food borne pathogen with poultry being the primary reservoir and reports of increased resistance of *Campylobacter* isolates from poultry as well as human beings. Bangladesh is now self-reliant in meat production, of which the maximum contribution comes from broiler meat as the government has taken various measures to support the country's livestock sector (Livestock Economy at a Glance 2019).

In the present study was designed to isolate and characterize *Campylobacter* spp. from chicken cloacal sample and find out the risk factors which mostly responsible for colonization. The study was conducted in three major poultry districts of Bangladesh and these three districts provide chicken meat and eggs for the consumption of city dwellers. In this present study we estimate the prevalence and evaluated some risk factors of the occurrence in broiler farms of Munshiganj 3(10%), Narayanganj 10(27.78%) and Narsindi 11(32.35%) district. The extremely important zoonotic, food-borne pathogens *Campylobacter* spp. variety of infection vehicles has been identified, but there is general agreement that contaminated broiler meat is the most important.

The overall colonization of *Campylobacter* spp. from the Dhaka division (among all three districts) was 24% (95% CI: 16.02 – 33.57). The overall positivity status estimated under this study has been supported by other research both in home and abroad. A positive status of 32% Campylobacter in broiler flocks was found in India (Malik et al., 2014) and 29% and 21.5% in Pakistan (Nisar *et al.,* 2018; Hussai*n et*

al., 2007). However, *Campylobacter* occurrence in broiler samples was confirmed as 32% and 40.5% in Bangladesh (Hasan *et al.,* 2020; Neogi *et al.,* 2020). Conversely, relatively a higher prevalence of *Campylobacter* in broiler samples was reported in Sri Lanka as 67% as a result of higher temperature in this country comparing to the other parts of Indian subcontinent (Kottawatta *et al.,* 2017). Also, this finding is agreement with several previous studies from industrialized countries too, which have shown broiler flocks to be a significant reservoir of *Campylobacters* (Kapperud *et al.,* 1993; Humphrey, 1994; Jacobs-Reitsma *et al.,* 1994; Stern *et al.,* 1995; Berndtson *et al.,* 1996; Nielsen *et al.,* 1997).

Different studies showed that *C. jejuni* is the predominant species in poultry (Oosterom *et al.,* 1983b; Berndtson *et al.,* 1996; Wallace *et al.,* 1997; Nielsen *et al.,* 1997). However, we did not identify the isolates at species level and an extended study is in progress. The present study showed that the management related factors might be important drivers and increase the risk of *Campylobacter* spp colonization. It is revealed that older birds, more than one person entering the house and using older houses, number of shed, water supply, flock size, flocks per shed, floor type, litter type, biosecurity, use of separate foot wear to enter in to the shed, footbath facilities, all-in all-out system, disinfection before restock, presence of rodents, elimination of dead birds properly were associated with positive campylobacter status. (Table-7)

The present study showed higher risk of *Campylobacter* spp. colonization when more than one person entered the broiler house. Human traffic is an important route (via boots, hands, cloths) for introduction of *Campylobacter* from the external environment (Hald *et al.,* 2000; Cardinale *et al.,* 2004) particularly if proper biosecurity is not in place. Increased risk with increasing age of broilers has been documented previously (Bouwknegt *et al.,* 2004; Barrios *et al.,* 2006; EFSA, 2010). An extended time in the broiler house could be related with a higher risk of introduction of the organism from the environment around the house. The effect of increasing flock size on the odds of a flock being positive has been previously reported too (Berndtson *et al.,* 1996b), although other studies failed to find this association (Bouwknegt *et al.,* 2004; Cardinale *et al.,* 2004). In the present study shows the more density farms related to more colonization occurred (95%CI 0.34-4.44).

Additional time before slaughter would also allow for cecal-colony concentrations to become detectable (Stern *et al.,* 2001). Taking this association into account, a policy of slaughtering flocks at a younger age might lead to a reduction in the prevalence of *Campylobacter spp.* This effect was independent of bird density, but could be due to bigger flocks offering more chances for introduction of *Campylobacter* spp. because of increased personnel movements, or larger volume of water and air used (both potential carriers of the pathogen).

The number of flocks per house per year is directly related to the prevalence of positive flocks. In our view, when a dry-out period has appropriately maintained the prevalence of *Campylobacter* spp. gradually decrease. Because it is an unfavorable condition for *Campylobacter* to grow in dryness, which is a significant threat to the organism (Jones, 2001; Nicholson *et al.,* 2005). The present study shows more than nine flocks per house per year have a higher prevalence (95% CI: 1.4-18.54) (p=0.014). Previous epidemiological studies have identified risk factors associated with the prevalence of *Campylobacter* in chicken farms, such as the higher age of broilers at slaughter (Ansari-Lari *et al.,* 2011), drinking water distribution (Näther *et al.,* 2009), the presence of other animals in the vicinity of the farm (Hansson et al., 2010) and heavy rainfall some weeks before the slaughter (Jonsson *et al.,* 2012). The present study shows more than three weeks of age farms got more colonization (95% CI: 0.39-2.49) (p=0.97).

The *Campylobacter* can survive in hands at a log CFU loss in 45 min (Coates et al., 1987) and human moist clothing 0.5–24 h at room temperature (Griffiths *et al.,* 1990). The likelihood of enteric infections among poultry attendants is enormous as they become exposed to *Campylobacter* contamination. Therefore, personal hygienic measures to be taken immediately after working at poultry farms. However, use of protective materials like mask and gloves, aprons are needed that will minimize further exposure of zoonotic pathogens (UNICEF).

Different study has enumerated as higher occurrence of *Campylobacter* spp. in conventional farms (36.4%, 95% CI: 29.3–43.9%) in comparison to the good practice farms (16.5%, 95% CI: 11.3–22.8%) (Badrul Alam *et al.,* 2020). This finding is empirically supported by other research (Smith *et al.,* 2016) as high standards of biosecurity measures will reduce the *Campylobacter* contamination by 20–40% lower than those farms with lower standards. The study shown us in 70% farms did

not use separate foot wear to enter the shed and the *Campylobacter* colonization rate 21% (*p*=0.032), in 72% farms did not have footbath facilities where the colonization rate 21% (*p*=28.38), in 84% farms did not use proper disinfection before restock where colonization rate 2857% (*p*=0.014), in 56% farms found rodent where colonization rate 30.36% (*p*=0.093) and in 65% farms did not follow 14 days gap between two flocks where colonization rate 32.31% (*p*=0.008). Moreover, biosecurity measurement is important targeted to *Campylobacter* control when colonization happens in a poultry flocks the horizontal spread can be prompt (Battersby *et al.,* 2003). The likelihood of bacterial infection was lower in best practice farms and found to be more protective than the poor practice farms because of the implementation of key control measures related to farm biosecurity and GAP practices, i.e., provision of perimeter fencing, netting of the farm to control entrance of wild and domestic animals and birds, controlling human movement inside the farm, dedicated footwear, and footwear cleaning at the entry to the poultry shed with disinfectants, all in all out practices, along with use of safe production inputs (DOC, feed, and water) (Fraser *et al.,* 2010; Newell *et al.,* 2003; Mridha *et al.,* 2020)

Despite limitations, our study highlighted some potential risk factors for *Campylobacter* spp. colonization in broiler flocks. Restricting the caretaking of the broiler houses to a single person, putting effort into the cleaning and disinfection of houses properly and their surroundings between flocks adequately (and optimize the possibilities for doing so, i.e., more frequent renovation of houses), would possibly reduce the prevalence of *Campylobacter* positive broiler farms in Munshigonj, Narayanganj and Narsingdi Zillas of Dhaka division, Bangladesh.

Our results emphasize, like many studies conducted before, that biosecurity measures are of utmost importance to keep infections outside flocks of animals. Every action that could work as a vector for bringing *Campylobacter* into broiler house should therefore be restricted.

CHAPTER V

SUMMARY & CONCLUSION

Campylobacter spp. is a zoonotic organism that does not spread from broiler to human only via consumption of meat but also through the handling of live broilers and during the preparation of meat and meat products. The present study was designed to isolate and characterize *Campylobacter* spp. from broiler cloacal samples from 100 broiler farms, the prevalence of *Campylobacter* spp. was estimated to be 24% (95% CI 16.02 – 33.57). A tended to be higher prevalence of *Campylobacter* spp. colonization was found in Narsindi district (32.35%) followed by Narayanganj (27.78%) and Munshiganj (10%) ($p=0.09$). The study confirmed a substantial degree of *Campylobacter* contamination in a wide range of samples of a major poultry production system that signifies a huge public health concern.

In risk factor analysis, the factors more significantly associated with *Campylobacter* colonization were human traffic (more than one person enter into the shed, $p=0.006$), source of water (tube well, $p=<0.001$), not using of dedicated cloth or footwear into the farms ($p=0.032$), no use of disinfection of farms before restock ($p=0.014$) and less gap (minimum 14 days) between two batches during broiler rearing ($p=0.008$) associated with tended risk factors included absence of footbath facilities, presence of rodents in the farms, litter store outside of farms ($0.05<p<0.1$).

In this regard, raising farmers' awareness regarding good farm practices, including biosecurity measures along with personal hygiene for poultry keepers need to be ensured through participatory training under a one health approach. These measures will help for minimizing the burden of poultry origin *Campylobacter* pathogen.

However, the study gathered evidence of the presence of *Campylobacter* spp. colonization in broiler flocks and identified the factors that could help set effective interventions in controlling *Campylobacter* colonization in chickens to reduce campylobacteriosis in humans through broilers. A further extended study might provide valuable information to formulate a national control program.

CHAPTER VI
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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 *Campylobacter* spp.

2.1 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.

2.2 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

2.3 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.

3. Reagents used for molecular characterization of *Campylobacter* spp.

3.1 Ethidium bromide (10 mg/ml)

Ethidium bromide	50 mg
Distilled water	5 ml

Stored the solution in amber colored vial at 4°C

3.2 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 Whatman filter paper no.1 and stored at room temperature.

3.3 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.

3.4 Loading dye (6X)

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

The solution stored at 4°C until use.

3.5 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.

Questionnaire on broiler flocks rearing system

General information

1. Study area:

2. Date:

3. Name of the farm:

4. Longitude: Latitude:

5. Farm ID:

6. Sample code:

Owner's information

1. Name of the owner:

2. 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 persons 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. other

Flock information:

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

    (1 meter=3 feet 3.37 inches):

2. Flock size:

3. Flock age:

4. Average slaughter age of the bird:

    a. <35 days   b. >35 days

5. Number of dead birds per flock:

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

6. Season of the sample collection:

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

7. Number of day-old chicks per meter square house area:

8. Presence of infected neighboring broiler farms?

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

    a. presence   b. absent

9. Practice of 'all in all out' system: yes/no

10. Disinfection of farm before restock: yes/no

11. Broiler house empty for >14 days between flocks: yes/no

12. Presence of rodents in the poultry house: yes/no

13. Elimination of dead birds every day: yes/n