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The Author

December, 2019

Prevalence of Gut p^H and Antibiotic Resistant *Escherichia coli* in Dahi of Chattogram Metropolitan area, Bangladesh

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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

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Dedication

DEDICATED TO MY PARENTS

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LIST OF ABBREVIATION

Abbreviation	Elaboration
ml	Mililiter
Mm	Milimeter
PCR	Polymerase Chain Reaction
FDA	Food and Drug Administration
E. coli	*Escherichia coli*
GEN	Gentamycin
CT	12-O-tetradecanoylphorbol 13-acetate
AMP	Ampicillin
CIP	Ciprofloxacin
TEC	Tetracyline
DO	Doxycycline
AMX	Amoxicillin
CL	Cephalexin
R	Resistance
I	Intermediately resistant
S	Sensitive
MDR	Multi Drug Resistance
BCSIR	Bangladesh Council of Scientific and Industrial Research
GMP	Good Manufacturing Practice
PRTC	Poultry Research and Training Centre
CVASU	Chattogram Veterinary and Animal Sciences University
FAO	Food and Agriculture Organization
WHO	World Health Organization
APHA	American Public Health Association
ISI	Indian Standard Institution
ARB	Antibiotic Resistant Bacteria

ABSTRACT

Dahi is one of the most popular fermented dairy products in Bangladesh, which could pose public health concern due to their indiscriminate preparation and vending scheme. To address this issue, the present study was carried out to investigate the prevalence of gut pH and antibiotic resistant *Escherichia coli*, a potential public health concerned bacteria in dahi; sampled from randomly selected retail shops of Chattogram metropolitan area from May to October, 2019. Dahi samples were analyzed at the laboratory of Poultry Research and Training Centre in Chattogram Veterinary and Animal Sciences University, Bangladesh. All the samples were inoculated on different bacteriological media for the confirmation of the isolates. The results revealed that *Escherichia coli* were not found in all dahi samples. Therefore, gut pH and antibiotic resistance could not be observed. The absence of *E. coli* in dahi samples in this study could possibly due to the boiled milk used for the preparation of dahi and it could also be successively developed acidity of dahi formation. The absence of *E. coli* is also a indication of Good Manufacturing Practice (GMP) employed by the producers and handlers. Present study revealed that the dahi collected from selected areas were of acceptable quality in term of gut microorganisms.

Key words: Dahi, *Escherichia coli*, pH resistance, Antibiotic resistance, Public health

CHAPTER-I: INTRODUCTION

Dahi, a fermented dairy product, is the most popular and delicious milk product in Bangladesh that is consumed by our people either as a part of diet or as a refreshing beverage. It is believed that dahi has valuable therapeutic properties and helps curing gastrointestinal disorders like constipation, diarrhea, dysentery, etc (Athar, 1986; Gandhi and Nambudripad, 1975). About 4% of the total milk produced in Bangladesh is used for the preparation of dahi (Mustafa et al., 1997).

Food safety and quality is a topic of public concern throughout the world. Well published and widespread food borne disease outbreaks have created an awareness of potential threats to human health from food products. Milk is supposed to constitute a complex ecosystem for various microorganism including bacteria. Milk products like dahi is widely consumed in many parts of the world for many generations. There is an increased demand by the consumer for high quality natural foods, free from artificial preservatives, and contaminating microorganisms. Contamination of milk products with pathogenic bacteria is largely due to processing, handling and unhygienic conditions. Under such conditions many microorganisms can find access to dahi. A frequently contaminating organism is *Escherichia coli* and also a reliable indicator of fecal contamination generally in insanitary conditions of dairy products (Diliello, 1982). Milk products that carry *E. coli* indicate the presence of enteropathogenic microorganisms. Although most *Escherichia coli* are harmless, but some are known to be pathogenic bacteria (Kaper et al., 2004). Acid resistance is a defense offering attribute of various microorganisms and enables the enteric pathogens to thrive in the low pH environment of stomach. The phenomenon has been extensively studied in *E. coli*. However, the acid resistant phenotype of *E. coli* to the extreme acidic status has been investigated (Gorden & Small, 1993; Waterman & Small, 1996).

Food like dahi contaminated with antibiotic resistant bacteria (ARB) can be a major threat to public health. The antibiotic resistance determinants can be transferred to other pathogenic bacteria potentially comprising the treatment of severe bacterial infections. Nowadays, AR has become a major threat for public health and has been reported worldwide and increasing rates of resistance among *E. coli* is a rising disquiet in both developed and developing countries (Bell et al., 2002). Occurrence and susceptibility profiles of *E. coli* show tangible geographic variations with significant differences in various populations and environments (Erb et al., 2007).

Dahi production must be free from all types of undesirable microorganisms. The knowledge about the *E. coli* contamination in dahi is of great significance for further development of its hygienic processing into high quality consumer products. And to best of my knowledge, data on such aspects is quite meager and scattered. Research is needed to create awareness and protect the consumer's health and rights. Considering the above facts the present study was designed to investigate the following objectives:

i. To investigate the prevalence of gut pH resistant *Escherichia coli* in dahi sold under market conditions at Chattogram metropolitan area.
ii. To investigate antibiotic resistant *Escherichia coli* in dahi.

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CHAPTER-II: REVIEW OF LITERATURE

2.1 Dahi

Dahi, a fermented milk product, is the most popular and delicious milk product in Bangladesh that is consumed by our people either as a part of diet or as a refreshing beverage. From the time immemorial it is being used for its nutritive and therapeutic values. The starter culture used in dahi is not definite and that's why the quality of dahi varies according to the culture uses for the preparation of dahi. Dahi is mostly prepared by using mixed culture of *Streptococcus lactis, Lactobacillus bulgaricus, Streptococcus thermophilus, Streptococcus citrophilus, Lactobacillus plantarum* etc (Islam et al., 2017). But the lactic acid bacteria are the main microbial agent to produce this fermented milk product locally (Dewan and Tamang, 2007).

2.1.1 Difference between yoghurt and dahi

Although yoghurt and dahi both are cultured or ripened dairy products still there are little differences between those. Yoghurt is prepared by using starter organisms *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in a proportion of 1:1, whereas dahi is prepared by using mixed culture of *Streptococcus lactis, Lactobacillus bulgaricus, Streptococcus thermophilus, Streptococcus citrophilus, Ixictobacillus plantarum* etc. Higher temperature (45°-50 ° C) and shorter (3-4h) incubation period is required for yoghurt making. On the other hand, lower temperature (37°-42°C) and long incubation period (8-15h) is required for dahi preparation. Comparatively yoghurt is softer and dahi curd is reverse of that. (Varnam and Sutherland, 2001).

2.1.2 Types of dahi/yoghurt

1. **Sweet dahi:** Sweet dahi is generally prepared from mixed culture of *Streptococcus lactis, Streptococcus thermophilus* and *Streptococcus citrophilus*, while highly aromatic sweet dahi results using *Lactobacillus planetarium* in place of *Streptococcus citroilus.* Sugar was added at the rate o f 8 to 10% during preparation.

2. **Sour/Plane dahi:** Sour dahi is made by seeding milk with a combination of *Streptococcus thermoilus* and *Lactobacillus bulgaricus.* (Singl, J. and S.Jasjit, 1979).

2.1.3 Proportionate production of Dahi or Yoghurt in all over the world

Mortensen (1990) reported in Turkey in 1989, milk production (cow, ewe, goat and buffalo) was approx. 7.7 million ton. Average annual milk production cow is very low (approx. 581 litters). Approx. 66% o f the milk produced is utilized directly by the farmers and 33% is utilized by dairy factories. He also reported in Turkey in 1989, approx. 5, 07000 ton of yoghurt was produced.

Abdel Muttaleb (1990) reported that annual milk production in Egypt increased from 1.361 million ton 1960 to 2.2 million ton in 1986. Cow, goat and ewe milk respectively account for 61.4, 37.3 and 1.3% o f total production. 50% of milk is utilized in the rural areas and 50% is available for manufacturing by public or private sector plants. Egypt imports dried milk for recombining as part of the World Food Programmed scheme. It is then utilized for manufacture of pasteurized and UHI milk, yoghurt, processed cheese.

Fourcaud (1991) studied on a husband-and-wife team of farmers in Montalembert, France, who decided to specialize in the production milk. The farm has producing an average of 70,000 liters of milk per year. Two-thirds of the milk produced is delivered to a local dairy and one-third is manufactured into yoghurt in the farm dairy. The yoghurt produced is flavored either with fruits or with honey.

The majority of milk is consumed locally as liquid milk or processed into dried milk. There is some yoghurt, and milk product consumption in china is low (5kg/person/year) compared with the world average of 100kg/person/year.

The Felles Dairy Association (1998) informed that, in 1997, sales of fresh and cultured milk products decreased by 3.7 and 0.4% respectively, but there was increase of 17.5% for yoghurt. During 1997, yoghurt production was 19, 5,000 ton and per capita consumption was 3-4kg.

Anonymuus (1998) observed that consumption of milk and milk products in Belgium increased slightly from 1980 to date, to 120kg/inhabitant per year (+4%). The greatest increase in consumption was for yoghurt (+136%).

Seljeskog (1999) gave the information on the Tine Sor Dairy Association. He informed that the Association has 4405 producers, receives and processes 341.5 and 178 million liters respectively of cow and goat milk annually. Sor supplies local and national markets and exports frozen goat milk Curd. Market milk sales continued to decrease in 1998. The 4-5% decrease for standard products was offset by increase of 3.3% for yoghurt.

Henze and Kosmatschov (1999) reported that in 1997, in the region of St. Petersburg, Russia, only 85% of milk was transported to dairy factories, while the remainder was used for own consumption or as feed. He also reported that recent investments were mainly in packaging and product development (yoghurt and UHT milk).

2.1.4 Standards and Standardization of Yoghurt or dahi

APHA (1976) set the standards for both coli forms and yeasts in yoghurt at less than 10 colonies per ml. and in only, one sample out of four.

Eschmann (1968) reported referring the Swiss standards that fermented milk products in Switzerland should be negative PR (Phosphates Reaction), lactic acid bacteria should present in at least 16c' dilution and frozen bacteria not more than 50000/ml on retailing.

ISI (1973) prescribed the types, requirements, method of sampling and test for fermented milk products (plain and flavored) i.e. (i) sweet, dahi produced using a culture of *streptococcus lactis, S. diacetilactis*, *V. diacetilactis* either alone or in combination with or without *Leuconostoc spp*(ii) sour dahi, using cultures as above but with *Lactobacillus bulgaricus* and /or *S. thermoilus*, and (iii) yoghurt, produced using cultures of *S. thermoilus* and /or *L. bulgaricus.* Requirents are: maximum acidity (as lactic acid), 0.7, 1.0 and 0.8% by mass for (i), (ii) and (iii) respectively, maximum yeast and mould count 100/g, maximum coliform count, 10/g and osatase test negative.

Spanish Standard (1976) specified requirements applicable to the following types o f yoghurt for the Spanish market: ordinary yoghurt (> 2% milk fat, > 8.5% S.N.F.), skim-milk yoghurt (< 0.5% milk fat, > 8.5% S.N.F.), yoghurt with fruit, juices and /or other natural products (must contain 70% yoghurt by weight). In all the fermentation organisms *Streptococcus thermoilus* and *L. bulgaricus* must be viable and abundant in the final product.

2.1.5 Nutritive value of dahi or yoghurt:

Table 1: According to Laxminarayana et al., (1983), composition of plain dahi prepared from whole milk and skimmed milk is represented below:

Product	Water %	Fat %	Protein %	Lactose %	Lactic acid %	Ash %	Ca %	P %
Dahi(whole milk)	85 to 88	5 to 8	3.2 to 3.4	4.6 to 5.2	0.5 to1.1	0.70 to o.75	0.12 to 0.14	0.09 to 0.11
Dahi(skimmed milk)	90 to 91	0.05 to 0.10	3.2 to 3.5	4.7 to 5.3	0.5 to 1	0.70 to o.75	0.12 to 0.14	0.09 to 0.11

Table 2: According to Balasubramanian and Basu (1995), average Mineral and Vitamin Contents of Milk and Dahi is represented below:

Constituent	Amount (in 100 gm of sample)	
	Milk	Dahi
Mineral matter (g)	0.8	2.1
Calcium (mg)	149	149
Phosphorus (mg)	96	93
Vitamin A (i.u)	118	102
Vitamin Bi (fig)	55	49
Riboflavin (jig)	167	157
Nicotinic acid (jig)	96	86
Biotin (ng)	29	23
Pantothenic acid (jig)	202	183
Folic acid (jig)	161	178
Vitamin B 12 (jig)	0.15	–
Ascorbic acid (jig)	1.4	1.3

Yogurt is more nutritious as compared to milk. It has good amount of calcium, phosphorus, vitamin B2, B6, B12,in addition to protein, zinc, potassium and

molybdenum. One hundred grams of yogurt provide 72calories, 3.6 g protein, 3.4 g fat, 4.9 g carbohydrates, and 145 mg of calcium, 114 mg phosphorus, 47 mg sodium and 186 mg potassium (Deeth and Tamime, 1981).

Dairy products provide 23% of thiamine, 40% of riboflavin and 14% of nicotinic acid in an average diet. Although traditionally, dairy products have played an important role in the diet, their reputation has suffered recently on the accounts of doubts expressed about the quantity and nature of fats in the diet. The animal fat and cholesterol in dairy products have both been linked with modern diseases of affluence which include; type2diabetes, asthma, coronary heart disease, cerebrovascular disease, obesity, hypertension cancer and many others (Fox and Cameron, 1989).

El-Salragy and Samragy (1988) used milk to manufacture the Egyptain cultured milk 'zabady' (or zabadi), which is similar to yoghurt. They reported that fresh zabady contained 14.16% TS, 4.3% fat, 3.55% lactose, 3.80% protein and 0.78% ash. They also reported that after 15 day storage there were no major differences in chemical composition, microbiological or organoleptic characteristics of zabady manufactured form milk.

Yaygin and Mehanna (1988) studied on Yoghurt prepared form three different milk which were analyzed by gas chromatogram (lead-space technique) for volatile flavor components. They reported that yoghurt made form three different group of milk, respectively. (15 samples each mean values in parentheses), acetaldehyde content ranged from 4 to 26 (12.5), form 5 to 19 (9.7) and form 6 to 2$ (13.4) ppm; acetone content correspondingly ranged from 3 to 25 (11.4), form 3 to 40(14.6) and form 5 to 30 (15.9) ppm; and ethanol content ranged from 19 to 365 (92.8), form 25 to 355 (12S.S) and form 5 to 195 (71.9) ppm.

Aneja (1990) reported that buffalo milk fat has less cholesterol and more tocoerol, which is a natural antioxidant. Buffalo milk is richer in Ca and P and lower in Na and K than cow milk. The per-oxidase activity in buffalo milk is much higher than in cow milk, which accounts for the natural preserving ability o f buffalo milk. He also reported that buffalo milk is a better tea/coffee whitener, and makes richer, firmer curd and yoghurt.

Agnihotri and Prasad (1993) reported that sample market milk either singly or and mixed with cow or buffalo milk, can be utilized for conversion into value added products such as ghee, khoa, chana, dahi (curd), yoghurt, cheese and dried milk products.

Park (1994) evaluated eight varieties of commercial milk yoghurt plain and flavored produced in the USA. Mean percentages of TS, protein, fat, carbohydrate and ash for plain yoghurts form 3 companies were: 11.5 ±0.56, 3.99 ± 0.12, 2.25 ± 0.13, 4.49 ± 0.56, and 0.818 ± 0.019, respectively.

Fomtini (1996) observed the nutritional value of milk, yoghurt, butter and cream. He suggested that milk product like yoghurt is very healthy of its served in the breakfast table. He also suggested distributing milk and milk- products at a reduced price to school students.

Buttriss (1997) suggested that yoghurt improved lactose intolerance, protect against gastrointestinal infections, relief from constipation, improved immunity, reduced cholesterol level and protects against cancers and so many other diseases. Patel and Renz (1997) concluded that the nutritive attributes of yoghurt, e.g. its richness in Ca, P, K, easily-absorbed carbohydrate, vitamins and free amino acids. Other therapeutic benefits of yoghurts are lactose intolerance, reduction of blood ammonia levels, patients with hepatic encealopathy, cholesterol reduction and relief from constipation.

Gajewska et al., (1997) studied on the nutrient and mineral composition of goat milk products. They reported that the nutritional composition of milk products is comparable to that of yoghurt. They are also suitable for use as substitutes for cow milk.

2.2 Causes of bacterial contamination of dahi through milk

2.2.1 Udder health and milking hygiene:

The bacteria that cause udder infections in a herd mainly come from infected quarters or cows and the environment in which the animals are kept (Radostits and Blood, 2000). The rate of new infections is however, greatly reduced if proper milking hygiene practices are followed at milking times (Robert, 1996).

2.2.2 Personal hygiene:

All people involved in dairying should maintain cleanliness and must be in sound health. It is important for milkmen to be in good health so that they not become a source of infectious diseases such as tuberculosis (Kurwijila, 1998).

2.2.3 Bacteriological Quality of Milk:

Total bacteria counts in milk mainly reflect its storage temperature and time elapsed since milking. Coliforms counts indicate the level of hygiene, since coli forms are microorganism's of faecal origin. The standard plate count per milliliter (or gram) for raw reconstituted (prepared) milk or pasteurized milk (at the plant in the final container) shall not exceed 30,000 (EAS, 2007).

2.3 Enterobacteriaceae

The large family Enterobacteriaceae includes Gram-negative bacteria along with many harmless symbionts, many o f the more familiar pathogens, such as *Salmonella*, *Escherichia coli*, *Yersinia pestis*, *Klebsiella* and *Shigella.* Other disease-causing bacteria in this, family include Proteus, *Enterobacter, Serratia*, and *Citrohacter.* Members of the Enterobacteriaceae are rod-shaped, and are typically I -5 nm in length. Like other proteobacteria, enterobacteria have Gram-negative stains, and they arc facultative anaerobes, fermenting sugars to produce lactic acid and various other end products.

2.3.1 *Escherichia coli*

Escherichia coli (commonly abbreviated *E. coli*) is a Gram-negative, rod shaped bacterium that is commonly found in the lower intestine of warm blooded organisms. Most E. coli strains are harmless, but some serotypes can cause serious food poisoning in humans, and are occasionally responsible for product recalls due to food contamination (Vogt and Dippold, 2005). The species E. coli comprises oxidase-negative straight cylindrical rods measuring 1.1-1.5 x 2.0-6.0 film. They are aerobic and facultative anaerobic, rendered motile by peritrichous flagella, or non-motile (Schcutz and Strockbine, 2005).

2.3.2 *E. coli* taxonomy and phylogeny

Escherichia and Shigella have been historically separated into different genera within the enterobacteriaceae family. DNA sequence analysis of their genomes reveals a high degree of sequence similarity and suggests that they should be considered a single species. Currently, the two organisms continue to be regarded as two different genera anchored in the historical perception of their disease potential and ecology (Sousa. 2006).

Scientific classification of *E. coli* is summarized below:

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: Escherichia

Species: *Escherichia coli* (VetBakt, 2007)

Table 3: General characteristics of *E. coli*

Test	**Properties**
Gram stain	Gram-negative
Cell morphology	Cells are typically rod-shaped, and are about 2.0 micrometers (μm) long and 0.25–1.0 μm in diameter, with a cell volume of 0.6–0.7 μm3 (Yu *et al*., 2014, Kubitschek, 1990).
Growth condition	Facultative anaerobic that makes ATP by aerobic respiration if oxygen is present, but is capable of switching to fermentation or anaerobic respiration if oxygen is absent
Sporulation	Nonsporulating
Motility	Strains that possess flagella are motile. The flagella have a peritrichous arrangement (Darnton*et al.*, 2007*)*
Metabolism	Produce lactate, succinate, ethanol, acetate, and carbondioxide

2.3.3 Acid resistant *Escherichia coli*

Enteric organisms, such as *Escherichia coli*, colonize and cause disease in the human intestinal tract. Intestinal pathogens arrive at their human hosts usually via

contaminated food or water, travel through the host's upper digestive tract, which includes the stomach, before reaching the bowel where pathogenesis occurs. The normal human stomach averages a pH of 2 with an emptying time of approximately 2hr (Texter et al., 1968). Consequently, *E. coli* must survive an acid challenge of less than 3 and as low as 2 for a considerable period of time in order to gain entrance into the less acidic environment of the intestinal tract (Texter et al., 1968; Gorden and Small, 1993). An organism's capacity to survive this challenge may be directly correlated with infectious dose (ID50). For example, *Vibrio cholerae*, an acid sensitive organism, has an infectious dose in the range of 106–1011 organisms, whereas *E. coli* and *Shigella*, which are more acid-resistant organisms, have very low infectious doses as low as 10 organisms (Boyd, 1995). This correlation underscores the importance of acid resistance in the pathogenesis of enteric organisms. Although an effective system to provide resistance to pH 2 would seem a requirement for such a low ID50, *E. coli* grown to exponential phase in the laboratory is quite acid sensitive. However, *E. coli* becomes acid resistant upon entry into stationary phase. Research from several laboratories has shown that stationary phase triggers at least three genetically and physiologically distinct acid resistance systems (Shi and Bennett, 1994; Lin et al., 1995, 1996; Stim-Herndon et al., 1996; Castanie-Cornet et al., 1999; Tramonti et al., 2002). The one characteristic common to all three systems is that each protects stationary phase cells from acid stress, albeit under different conditions.

Acid resistance system 1 (AR1) is a stationary phase, acid-induced, glucose-repressed system that requires the alternative sigma factor RpoS to be expressed. The structural components of AR1 as well as the mechanism(s) by which it protects are still unknown. Acid resistance system 2 (AR2) is a stationary phase–induced and glutamated ependentsystem. This system requires glutamate decarboxylase and a putative glutamate: aminobutyric acid (GABA) antiporter, as well as exogenous glutamate to function at pH 2.0. Acid resistance system 3(AR3) is acid induced under anaerobic conditions. It is argininedependent, and requires the presence of an acid-inducible argininedecarboxylase (AdiA) to function. Analogous to AR2, AR3 will protect cells from extreme acid only if arginine is present extracellularly. It is postulated that both AR2 and AR3 confer acid resistance by consuming intracellular protons. Proton consumption may produce a less acidic internal pH that allows cells to survive in extremely acidic environments. All of these acid resistance systems provide different levels of protection. The glutamate-dependent system (AR2) provides the

highest level of protection, functioning at pH 2.0 or less. The arginine-dependent (AR3) and the oxidative systems (AR1) provide a lower level of protection, functioning at pH 2.5 or higher (Audia et al., 2001). These three stationary phase–induced systems are the main constituents of *E. coli* acid resistance and the primary focus of this review.

In addition to these acid resistance systems, other less effective acid tolerance/habituation systems have been reported for *E. coli* (Goodson and Rowbury, 1989a). Acid tolerance in exponential phase has been described for various enteric organisms, and the exponential phase induction of acid tolerance has been reported in *E. coli* (Foster and Hall, 1991; Lin et al., 1995), although the mechanisms are not well characterized. The net result of these different stationary and exponential phase systems is an overlapping protection against pH stresses ranging from pH 4 to 1.5.

2.3.4 Cultural growth

E. coli grows at 37°C (98.6°F) at optimum level, but there is some exception. Some laboratory strains can reproduce at temperatures up to 49°C (Fotadar et al., 2005). *E. coli* grows well in variety of laboratory media, such as lysogeny broth, or any medium that contains glucose, ammonium phosphate, monobasic, sodium chloride, magnesium sulfate, potassium phosphate, dibasic, and water. Growth can occur on aerobic or anaerobic respiration, using a large variety of redox pairs, including the oxidation of pyruvic acid, formic acid, hydrogen, and amino acids, and the reduction of substrates such as oxygen, nitrate, fumarate, dimethyl sulfoxide, and trimethylamine N-oxide (Ingledew, 1984). *E. coli* is classified as a facultative anaerobe. It uses oxygen when it is available. It can, however, keep on growing in the absence of oxygen by using fermentation or anaerobic respiration. The ability to continue growing in the absence of oxygen is a benefit to bacteria because their endurance is amplified in environments where water predominates (Tortora, 2010).

2.3.5 Diversity

E. coli encompasses a vast population of bacteria that display a very high degree of both genetic and phenotypic diversity. Genome sequencing of a large number of isolates of *E. coli* and associated bacteria shows that a taxonomic reclassification would be advantageous. However, this has not been done, largely due to its medical importance (Krieg, 1984) and *E. coli* remains one of the most sundry bacterial species: only 20% of the genes in a typical *E. coli* genome are shared among all

strains (Lukjancenko et al., 2010). In fact, from the evolutionary point of view, the members of genus Shigella (*S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*) should be classified as *E. coli* strains, a phenomenon termed taxa in disguise (Lan, 2002). Likewise, other strains of *E. coli* (e.g. the K-12 strain usually used in recombinant DNA work) are adequately different that they would merit reclassification.

2.3.6 Strains

A strain is a subgroup within the species that has sole characteristics that differentiate it from other strains. These differences are often obvious only at the molecular level; however, they may result in changes to the makeup or lifecycle of the bacterium. A familiar subdivision system of *E. coli*, but not based on evolutionary relatedness, is by serotype, which is based on major surface antigens (O antigen: part of lipopolysaccharide layer; H: flagellin; K antigen: capsule), e.g. O157:H7 (Orskov et al., 1977). It is, however, common to cite only the serogroup, i.e. the O-antigen. At present, about 190 serogroups are known (Stenutz et al., 2006). The common laboratory strain has a mutation that prevents the formation of an O-antigen and is thus not typeable. Different strains of *E. coli* are often host-specific, making it probable to determine the source of fecal contamination in environmental samples (Feng et al., 2002).

2.4 Cultural identification of *E. coli*

Various solid media has been used to detect *E. coli* by observing their cultural properties.

2.4.1 MacConkey agar

MacConkey Agar with Sorbitol can successfully differentiates the most common serotype of *Escherichia coli* associated with hemorrhagic colitis (O157:H7) from most other nonpathogenic *E. coli.* On MacConkey Agar this strain of *E. coli* is indistinguishable from other lactose-fermenting E. coli. However, when plated onto MacConkey Agar with Sorbitol, the O157:H7 strain fails to ferment sorbitol, thus producing colorless colonies, while other *E. coli* yield sorbitol-positive pink to red colonies. (Peter et al., 2011)

2.4.2 Eosin methylene blue (EMB) agar

EMB agar medium contains lactose and the dyes eosin and methylene blue that permit differentiation between enteric lactose fermenters and non-fermenters as well as identification of the gram-negative bacillus *E. coli.* The *E. coli* colonies are black colonies with a metallic green sheen caused by the large quantities of acid that is produced and that precipitates out the dyes onto the growth's surface.

2.4.3 Blood agar

Escherichia coli are cultivated on blood agar in an aerobic atmosphere and incubated at 37°C for 24 hours. *Escherichia coli* are usually non-hemolytic, but many strains isolated from infections are beta-hemolytic.

2.5 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a technique used in molecular biology to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. PCR has become a very rapid and sensible tool for the molecular biology-based diagnosis of a variety of infectious disease (Fredricks and Relman, 1999). DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group; it needs a primer to which it can add the first nucleotide. This requirement makes it possible to depict a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies. Potential PCR inhibitors found in stool specimens include heme, bilirubins, bile salts, and complex carbohydrates (Demeke and Adams, 1992; Lantz et al., 1997; Monteiro et al., 1997). PCR-based methods have been developed to identify STEC E. coli O157 in clinical (Takeshi et al., 1997), food and water (Oberst et al., 1998) and environmental samples (Fortin et al., 2001; Ibekwe et al., 2002). Real-time PCR methods make detection of the synthesized DNA fragments possible during the PCR reaction using fluorescent techniques in combination with an on-line fluorescent detection system. Direct real-time PCR detection of STEC O157 on DNA isolated from concentrated water samples has the advantage of being a quick and quantitative method. Serogroup specific PCR amplifies identical regions in the rfb gene cluster which are preserved for each serogroup. There are a number of serogroup specific PCR for EHEC/STEC and E. coli that causes blood stream infections (Clermont et al., 2007; Lin et al., 2011). Serogroup specific PCR specifically O-antigen specific PCR

assays are very simple, specific and cost effective. The use of real time PCR assays makes the detection and identification of serogroups faster than conventional PCR (Lin et al., 2011). However, this method is highly precious and is only useful for known serogroups for which specific primers are included in the assay. On the other hand Serogroup specific PCR is limited to detecting a maximum of 12 to 15 serogroups at a time (Clermont et al., 2007), which makes this method ineffective in identifying new serogroups which could cause infection and also in identifying previously uncharacterized serogroups.

2.5.1 Pulsed-field gel electrophoresis

Pulsed field gel electrophoresis is a technique used for the separation of large deoxyribonucleic acid (DNA) molecules by applying to a gel matrix an electric field that periodically changes direction. It is currently considered as a gold standard method for typing of *E. coli* for many epidemiological investigations, due to the stability of this technique (Riley, 1983; Goering, 2010). The procedure for this technique is relatively similar to performing a standard gel electrophoresis except that instead of constantly running the voltage in one direction, the voltage is periodically switched among three directions; one that runs through the central axis of the gel and two that run at an angle of 60 degrees either side. The pulse times are equal for each direction resulting in a net forward migration of the DNA. For extremely large molecules (up to around 2 Mb), switching-interval ramps can be used that increases the pulse time for each direction over the course of a number of hours take, for instance, increasing the pulse linearly from 10 seconds at 0 hours to 60 seconds at 18 hours. PFGE is highly reproducible and easily interpreted and compared within and across different laboratories (Gordon, 2010).

2.5.2 Restriction fragment length polymorphism

RFLP is a technique that exploits variations in homologous DNA sequences. It refers to a difference between samples of homologous DNA molecules from different locations of restriction enzyme sites, and to a related laboratory technique by which these segments can be illustrated. RFLP-based methods are routinely used in molecular epidemiology research. The general working principle of these techniques is detection of restriction enzyme sites on genomic DNA. RFLP-based methods are based on pattern recognition of DNA fragments which can be visualized by gel electrophoresis. Several studies were conducted to assess a simplified method for inter

strain differentiation of *Escherichia coli* O157:H7 and other Shiga-like toxin-producing *E. coli* (SLTEC) strains.

2.5.3 Multilocus sequence typing (MLST)

Multilocus sequence typing is a procedure in molecular biology for the typing of multiple loci. The system characterizes isolates of microbial species using the DNA sequences of internal fragments of several housekeeping genes (Gordon, 2010). Approximately 450-500 bp internal fragments of each gene are used, as these can be accurately sequenced on both strands using an automated DNA sequence (Cooper and Feil, 2004). These sequences are identified as an allele and each unique combination of alleles corresponds to a specific sequence type (ST) (Cooper and Feil, 2004). These specific sequence profiles can be used to determine the origin and relatedness of each strain (Gordon. 2010). MLST can be biased based on the core genes selected and cannot be applied to all E. coli groups using the same set of 7 genes (Gordon. 2010). However, MLST has a lesser discriminatory power than PFGE for epidemiological studies (Tartof et al., 2005).

2.5.4 Random amplified polymorphic DNA (RAPD)

Random amplified polymorphic DNA (RAPD) is a type of PCR reaction, but the segments of DNA that are amplified are random. A number of methods had been used for typing and differentiating E. coli isolates but RAPD is more sensitive and more cost effective than other molecular methods (Osek, 2000; Wang, 1993). The random sequences bind to random priming sites and amplify DNA segments of variable lengths (Hadrys et al., 1992). This results in a pattern of small and large DNA fragments (Williams et al., 1990). These patterns would be similar for colonel bacterial populations (Gordon, 2010). The power of RAPD is in the use of multiple primer sequences. However in some instances, RAPD has shown to have higher discriminatory power than multi locus enzyme electrophoresis (MLEE) (Desjardins et al., 1995).

2.5.5Multilocus variable-number tandem repeat analysis (MLVA)

MLVA is a method engaged for the genetic analysis of particular microorganisms, such as pathogenic bacteria, that takes benefit of the polymorphism of tandemly repeated DNA sequences. It is a fast, portable method that analyzes multiple VNTR loci, which are areas of the bacterial genome that evolve quickly. Different

electrophoresis tools can be used depending on the requisite size estimate accuracy, and the local laboratory set-up, from basic agarose gel electrophoresis up to the more complicated and high-throughput capillary electrophoresis devices (Vergnaud, 2009). From this size estimate, the number of repeat units at each locus can be deducing. The resulting information is a code which can be simply compared to reference databases once the assay has been harmonized and standardized (Grissa et al., 2008). MLVA has become a main typing tool in a number of pathogens. In addition to being a simple and validated method for E. coli O157:H7 outbreak detection, MLVA appears to have a sensitivity equal to that of PFGE and a specificity superior to that of PFGE (Van Belkum et al., 1998). The MLVA protocol is amenable to the handling of large sample sets and can easily be standardized for comparisons of results among different laboratories.

2.6 Antimicrobial resistance (AMR)

One of the most important problems facing global public health today is antimicrobial resistance. The problem is most horrible in developing countries, where the bacterial infections causing human disease are also those in which emerging antibiotic resistance is most evident (Shears 2000; Fathpour et al., 2003; Kalantar et al., 2008). Antibiotics are often used for therapy of infected humans and animals as well as for prophylaxis and growth promotion of food producing animals. Many findings suggest that inadequate selection and abuse of antimicrobials may lead to resistance in various bacteria and make the treatment of bacterial infections more difficult (Kolár et al., 2001). AR in microorganisms is either genetically inherent or the result of the microorganism being exposed to antibiotic. Most of the antibiotic resistance has emerged as a result of mutation or through transfer of genetic material between microorganisms. A broad variety of biochemical and physiological mechanisms are responsible for the development of resistance. Recent studies of almost 400 different bacteria have demonstrated about 20,000 possible resistance genes (r genes) (Davies et al., 2010). In many developing countries the use of antimicrobial drugs for treating people and animals is unregulated; antibiotics can be purchased in pharmacies, general stores, and even market stalls. In the Rajbari district of Bangladesh, a survey of rural medical practitioners (barefoot doctors) with an average of 11 years' experience showed that they each saw on average 380 patients per month and

prescribed antibiotics to 60% of these patients on the basis of symptoms alone (Mamun, 1993).

2.6.1 Antibiotic susceptibility testing

An important task of the clinical microbiology laboratory is the performance of antimicrobial susceptibility testing of significant bacterial isolates. The goals of testing are to detect possible drug resistance in common pathogens and to assure susceptibility to drugs of choice for particular infections.

2.6.2 Disk diffusion test (Kirby Bauer method)

The disk diffusion susceptibility method (Clinical and Laboratory Standards Institute, 2009) is simple and practical and has been well-standardized. The test is performed by applying a bacterial inoculum of approximately 1–2×108 CFU/ml to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Twelve antibiotic disks are placed on the inoculated agar surface. Plates are incubated for 16–24 h at 35°C prior to determination of results. The zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug are interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards or NCCLS, 2009) or those included in the US Food and Drug Administration (FDA, 2015)-approved product inserts for the disks. The diameter of zone is related to the susceptibility of the isolates and to the diffusion rate of the drug through the agar medium.

2.7 Mechanisms of resistance

The four main mechanisms by which microorganisms exhibit resistance to antimicrobials are:

Drug inactivation or modification: For example, enzymatic deactivation of Penicillin G in some penicillin-resistant bacteria through the production of β-lactamases. The emergence of carbapenem-resistant Gram-negative pathogens poses a serious threat to public health worldwide. Klebsiella pneumonia carbapenemases (KPCs) and carbapenemases of the oxacillinase-48 (OXA-48) type have been reported worldwide. New Delhi metallo-β-lactamase (NDM) carbapenemases were originally identified in Sweden in 2008 and have spread worldwide rapidly. Most commonly,

the protective enzymes produced by the bacterial cell will add an acetyl or phosphate group to a specific site on the antibiotic, which will reduce its ability to bind to the bacterial ribosome and disrupt protein synthesis (Broszat and Grohmann, 2014).

Alteration of target- or binding site: For example, alteration of PBP the binding target site of penicillin in MRSA and other penicillin-resistant bacteria. Another protective mechanism found among bacterial species is ribosomal protection proteins. These proteins protect the bacterial cell from antibiotics that target the cell's ribosome to inhibit protein synthesis. The mechanism involves the binding of the ribosomal protection proteins to the ribosome of the bacterial cell, which in turn changes its conformational shape. This allows the ribosome to continue synthesizing proteins essential to the cell while preventing antibiotics from binding to the ribosome to inhibit protein synthesis (Butaye et al., 2006).

Alteration of metabolic pathway: For example, some sulfonamide-resistant bacteria do not require para-aminobenzoic acid (PABA), an important precursor for the synthesis of folic acid and nucleic acids in bacteria inhibited by sulfonamides, instead, like mammalian cells, they turn to using preformed folic acid (Mathew et al., 2007).

Reduced drug accumulation: By decreasing drug permeability or increasing active efflux (pumping out) of the drugs across the cell surface. These pumps within the cellular membrane of certain bacterial species are used to pump antibiotics out of the cell before they are able to do any damage. They are often activated by a specific substrate associated with an antibiotic as in fluoroquinolone resistance.

Antibiotic resistance can be a result of horizontal gene transfer, and also of unlinked point mutations in the pathogen genome at a rate of about 1 in 108 per chromosomal replication. Mutations are rare but the fact that bacteria reproduce at such a high rate allows for the effect to be significant. A mutation may produce a change in the binding site of the antibiotic, which may allow the site to continue proper functioning in the presence of the antibiotic or prevent the binding of the antibiotic to the site altogether. Antibiotic action against a pathogen can be seen as an environmental pressure. Those bacteria with a mutation that allows them to survive will reproduce; pass the trait to their offspring, which leads to the microevolution of a fully resistant colony. Chromosomal mutations providing antibiotic resistance benefit the bacteria but also confer a cost of fitness. For example, a ribosomal mutation may protect a bacterial cell by changing the binding site of an antibiotic but will also slow protein synthesis manifesting, in slower growth rate. In Gram-negative bacteria, plasmid-

mediated resistance genes produce proteins that can bind to DNA gyrase, protecting it from the action of quinolones. Finally, mutations at key sites in DNA gyrase or topoisomerase IV can decrease their binding affinity to quinolones, decreasing the drug's effectiveness.

Antibiotic resistance can be introduced artificially into a microorganism through laboratory protocols, sometimes used as a selectable marker to examine the mechanisms of gene transfer or to identify individuals that absorbed a piece of DNA that included the resistance gene and another gene of interest.

Recent findings show no necessity of large populations of bacteria for the appearance of antibiotic resistance. Small populations of *E. coli* in an antibiotic gradient can become resistant. Any heterogeneous environment with respect to nutrient and antibiotic gradients may facilitate antibiotic resistance in small bacterial populations. Researchers hypothesize that the mechanism of resistance development is based on four SNP mutations in the genome of *E. coli* produced by the gradient of antibiotic.

2.7.1 Resistance to β –lactam antibiotics

β-lactam antibiotics are a group of antibiotics which are characterized by possession of a β-lactam ring. They include penicillins, cephalosporins, carbapenems, oxapenams, and cephamycins. The penicillins are one of the most commonly used antibiotics in developing countries because of their ready availability and relatively low cost. For the activity of these antibiotics, the β-lactam ring is important which results in the inactivation of a set of trans-peptidases that catalyze the final cross-linking reactions of peptidoglycan synthesis in bacteria. The effectiveness of these antibiotics relies on their ability to reach to the penicillin-binding protein (PBP) and to intact to bind with the PBPs. Resistance to β-lactams in many bacteria occurs usually due to the hydrolysis of the antibiotic by a β-lactamase or the modification of PBPs or cellular permeability. β-lactamases constitute a heterogeneous group of enzymes which are classified according to different ways including their hydrolytic spectrum, susceptibility to inhibitors, genetic localization (plasmidic or chromosomal), and gene or amino acid sequence in protein. The functional classification scheme of β-lactamases proposed by Bush et al. (2010) defines four groups according to their substrate and inhibitor profiles.

2.7.2 Resistance to Tetracyclines

Tetracyclines are another of the very commonly used antibiotics in both human and veterinary medicine in developing countries because of their availability and low cost as well as broad spectrum of activity and low toxicity. They are broad-spectrum agents. They exhibit activity against a wide range of gram-positive and gram-negative bacteria, atypical organisms such as chlamydia, mycoplasmas, rickettsia and protozoan parasites. Examples of these include Tetracycline, Doxycycline, Minocycline, and Oxytetracycline.

Resistance to these agents occurs mainly by three mechanisms (Roberts, 1996), namely

i. Efflux of the antibiotics,
ii. Ribosome protection, and
iii. Modification of the antibiotic

Efflux of the drug occurs through an export protein from the major facilitator super family (MFS). These export proteins are membrane-associated proteins which are coded for by test efflux genes and export tetracycline from the cell. Export of tetracycline reduces the intracellular drug concentration and thus protects the ribosomes within the cell (Cohen et al., 1988).

2.7.3 Resistance to Aminoglycosides

Resistance to aminoglycosides such as gentamicin, tobramycin, amikacin, and streptomycin is widespread, with more than 50 aminoglycoside-modifying enzymes described. Most of these genes are associated with gram-negative bacteria. Depending on their type of modification, these enzymes are classified as aminoglycoside acetyltransferases (AAC), aminoglycoside adenyltransferases (also named aminoglycoside nucleotidyltransferases; ANT), and aminoglycoside phos-photransferases (APH). Aminoglycosides are modified at amino groups by AAC enzymes or at hydroxyl groups by ANT or APH enzymes and thus lose their ribosome-binding ability resulting no longer inhibition of protein synthesis. Besides aminoglycoside-modifying enzymes, efflux systems and rRNA mutations have been described for aminoglycoside resistance (Quintiliani and Courvalin, 1996).

2.7.4 Resistance to Quinolones and Fluroquinolones

Mechanisms of bacterial resistance to quinolones fall into two principal categories:

i. Alterations in drug target enzymes and

ii. Alterations that limit the permeability of the drug to the target.

In gram-negative organisms, DNA gyrase seems to be the primary target for all quinolones. In gram-positive organisms, topoisomerase-IV or DNA gyrase is the primary target depending on the fluroquinolones considered. In almost all instances, amino acid substitutions within the quinolone resistance-determining region (QRDR) involve the replacement of a hydroxyl group with a bulky hydrophobic residue. Mutations in gyrA induce changes in the binding-site conformation that may be important for quinolone DNA gyrase interaction. Changes in the cell envelope of gram-negative bacteria, particularly in the outer membrane, have been associated with decreased uptake and increased resistance to fluroquinolones, and this has not been demonstrated in gram-positive bacteria.

2.7.5 Resistance to Sulfonamides and Trimethoprim

Resistance in sulfonamides is commonly mediated by production of drug-resistant forms of dihydropteroate synthase (DHPS). Sulfonamide resistance in gram-negative bacilli generally arises from the acquisition of either of the two genes sul1 and sul2, encoding for the production of enzyme dihydropteroate synthase that are not inhibited by the drug. The sul1 gene is normally found linked to other resistance genes in class-1 integrons, while sul2 is usually located on small non-conjugative plasmids or large transmissible multi-resistance plasmids. Trimethoprim is an analog of dihydrofolic acid, an essential component in the synthesis of amino acid and nucleotides that competitively inhibits the enzyme dihydrofolatereductase (DHFR). At least 15 DHFR enzyme types are known based on their properties and sequence homology. Trimethoprim resistance is caused by a number of mechanisms (Byarugaba, 2010)

Including:

i. Over production of the host DHFR enzyme.

ii. Mutations in the structural gene for DHFR.

iii. Acquisition of a gene (dfr) encoding a resistant DHFR enzyme which is the most resistant mechanism in clinical isolates.

2.8 Antimicrobial Resistance (AR) in *E. coli*

Escherichia coli is usually a common bacterium of humans and animals. Pathogenic variants cause intestinal and extra intestinal infections, including gastroenteritis, urinary tract infection, peritonitis, meningitis and septicemia (Chatterjee et al., 2012). Therapeutic options vary depending on the type of infection. For example, for urinary

tract infections, trimethoprim/sulfamethoxazole and fluoroquinolones are treatments of choice (Taur and Smith, 2007), whereas for Shiga toxin producing *E. coli* infections, antimicrobial drug therapy is not recommended (Igarashi et al., 1999). *E. coli* is sometimes used as a sentinel for monitoring antimicrobial drug resistance in fecal bacteria as it is found more frequently in a wide range of hosts, acquires resistance easily (Tadesse et al., 2012). Surveillance data show that resistance in *E. coli* is consistently highest for antimicrobial agents that have been in use the longest time in human and veterinary medicine. Major increases in emergence and spread of multidrug-resistant bacteria and increasing resistance to newer compounds, such as fluoroquinolones and certain cephalosporins have witnessed by the past two decades (Levy and Marshall, 2004). For example, a study of the susceptibility of E. coli isolates recovered from hospitals during a 12-year period (1971–1982) showed no major change in resistance to any of the antimicrobial drugs tested (Lorian and Atkinson, 1984). In contrast, a retrospective analysis of E. coli from urine specimens collected from patients during 1997–2007 showed an increasing resistance trend for Ciprofloxacin, Trimethoprim/Sulfamethoxazole, and Amoxicillin/Clavulanic acid (Tadesse et al., 2012). Similarly a 30-year (1979–2009) follow-up study on *E. coli* in Sweden showed an increasing resistance trend for Ampicillin, Sulfonamide, Trimethoprim, and Gentamicin (Kronvall, 2010). Although studies of farms have shown an association between multidrug-resistant E. coli and chronic antimicrobial drug exposure (Byarugaba, 2004), there are few data on temporal trends of antimicrobial drug resistance in food animal *E. coli* isolates, particularly those recovered before 1980. Recent data are available in several countries establishing resistance monitoring programs during the mid-1990s.

2.9 Development of Resistance

Antibiotic resistance is a global problem in public health and is growing around the world (WHO, 2006). Antibiotics have been used for 70 years but during the last decade some treatments have become ineffective and this may lead to spread of some infections in the future. Antimicrobial resistance (AMR) is created by use of antibiotics in a wrong way and develops when a microorganism have mutated or acquired inappropriate use of antibiotics in human and veterinary medicine leads to higher frequencies of AMR (Rahman et al., 2014). Antibiotics are often used in animals. Transfer to human's food of these antibiotics can affect the safety of the

meat, milk and eggs produced and can be the source of superbugs. The resistant bacteria in animals can transfer to humans by three pathways, consumption of meat or other food, direct contact with animals or through the environment.

2.10 Public health significance of acid resistant *Escherichia coli*

Food borne diseases area growing public health problem in both developed and developing countries today (Elmi, 2004). Food borne diseases (FBD) can be defined as those associated with the ingestion of contaminated food and these diseases encompass a wide spectrum of illnesses (WHO, 2007). The World Health Organization (WHO) estimated that in developed countries, up to 30% of the population suffer from food borne diseases each year, whereas in developing countries up to 2 million deaths are estimated per year (WHO, 2007a; WHO, 2007b). To give assurance about the microbial quality to the consumer and to ensure that the product is free from pathogenic microorganisms is of utmost importance to promote the consumption of products in domestic and export markets (Dhanze, 2011; Dhanze *et al*., 2013).

Acid resistance is a defense offering attribute of various microorganisms and enables the enteric pathogens to thrive in the low pH environment of stomach. The phenomenon has been extensively studied in *E. coli*. However the acid resistant phenotype of *E. coli* to the extreme acidic status has been investigated (Gorden & Small, 1993; Waterman & Small, 1996). *E. coli* is usually a non-pathogenic member of the human colonic flora. However, certain strains have acquired virulence factors and may cause a variety of infections in humans and in animals. Among the pathogenic strains, diarrhoeagenic *E. coli* (DEC) represents a major public health problem in developing countries (Nataro & Kaper, 1998).

E. coli are generally regarded as part of the normal flora of the human intestinal tract and that of many animals. Most of the strains of *Escherichia coli* are non-pathogenic; however, some of them are major food borne pathogen of public health importance and responsible for watery and bloody diarrhoea, infantile diarrhoea, traveler's diarrhea ,hemorrhagic colitis, hemolytic uremic syndrome inman (Johnson *et al.*, 1996; Mead and Griffin, 1998; Shiferaw *et al*., 2000; Banerjee *et al.*, 2001; Hazarika *et al.*, 2005). Based on their pathogenic phenotypes and the diseases that they cause, diarrhoeagenic *E. coli* have been classified into 6 groups: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC),

enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and Shigatoxin producing *E. coli* (STEC)/enterohemorrhagic *E. coli* (EHEC)/ verocytotoxin producing *E. coli* (VTEC)(Paniagua *et al.,* 1997; Nataro and Kaper, 1998; Matar *et al.,* 2002; Kaper *et al.,* 2004; Bischoff *et al.,* 2005).Other diarrhoeagenic *E. coli* pathotypes have been proposed, such as cell detaching *E. coli* (CDEC);however, their significance remains uncertain (Clarke,2001; Abduch-Fabrega *et al*., 2002).

Antibiotic usage is considered the most important factor promoting the emergence, selection and dissemination of antibiotic-resistant microorganisms in both veterinary and human medicine (Levy, 1982; Witte, 1998).

CHAPTER-III: MATERIALS AND METHODS

The research work was conducted at the laboratory of Poultry Research and Training Center (PRTC), Chattogram Veterinary and Animal Sciences University (CVASU), Chattogram. The study was conducted for a period of six months from May to October 2019.

3.1 Collection of sample

A total of 50 dahi samples were collected randomly from local retail shops (Appendix figure 5) of Chattogram metropolitan area (Figure 2) considering where large numbers of dahi were sold regularly that mentioned in Figure 1.

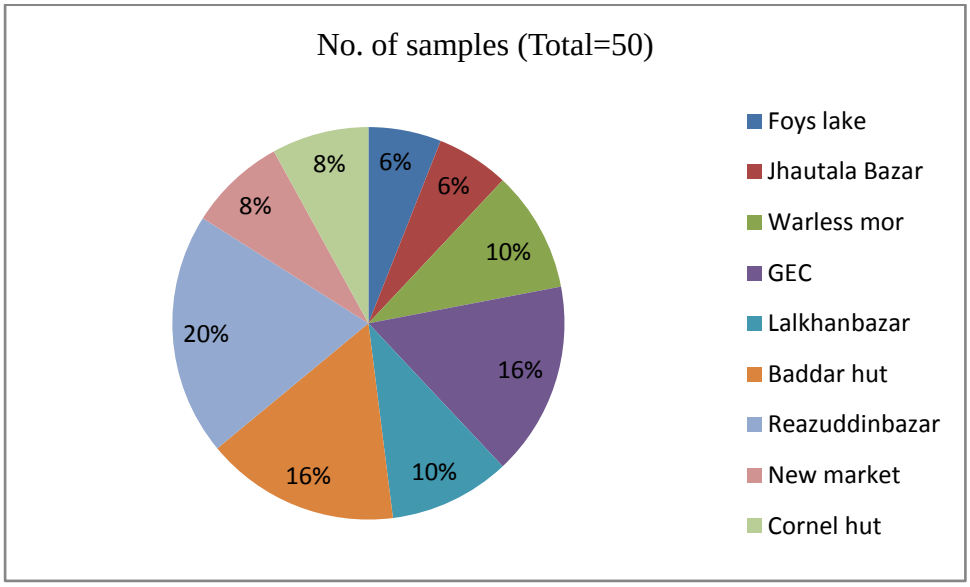

Figure 1: Name of the area and the number of collected samples

Samples were collected from the branded and non-branded shops and wrapped properly to avoid the contact with air. All possible efforts were made to minimize the time lag between collection and analysis, so that no significant change in dahi quality could occur. Samples were then transported to the laboratory as soon as possible in an insulated container with ice to maintain temperature ranging from 4°C to 6°C. Collections of samples from the shops were done five times and same procedures were followed to get optimum results of the study.

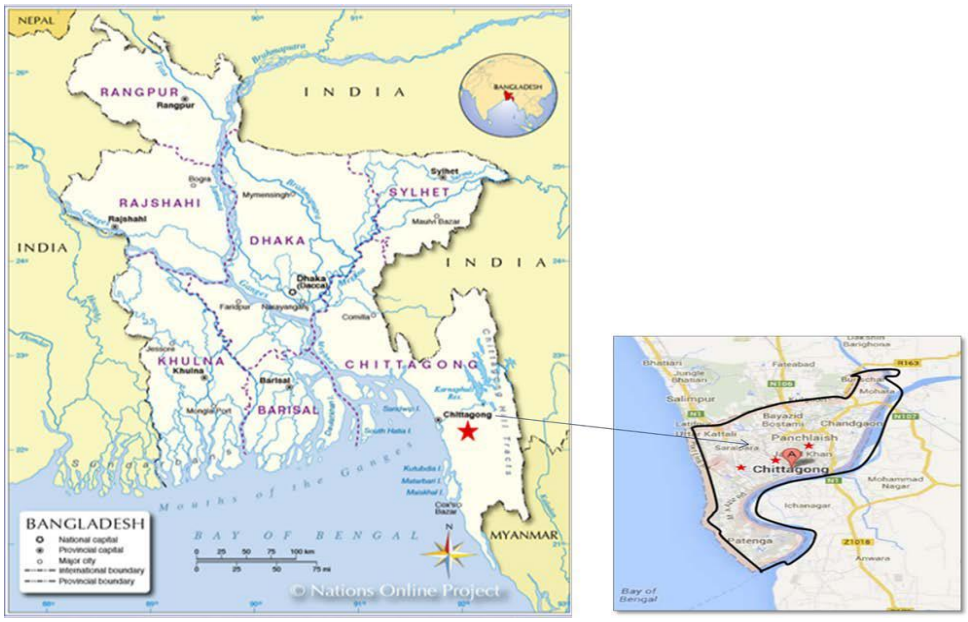

Figure 2: A map of Bangladesh and Chattogram city showing the geographical locations of the sampling areas (Star marked).

3.2 Materials required for the study

Petri dish, Conical flask, Eppendrof tube, Zipper Bag, Inoculating loop, Spirit lamp, Distilled water, 70% Ethanol, Mask, Gloves, Tissue, Permanent marker, Falcon tube/test tube rack were the required materials for the present study.

3.3 Agar media used

MacConkey (HiMedia Pvt. Ltd.) and Eosin Methylene Blue (EMB) (HiMedia Pvt. Ltd.) agar were used as selective media. All media were prepared according to the manufacturer's directions (Appendix Figure 7, 8).

3.4 Isolation and identification of *Escherichia coli*

The samples were pre-enriched in buffered peptone broth at 37°C Serial dilution was done according to APHA (1967) in which 1 ml of dahi from a homogenous sample was serially diluted into 9 ml of sterile distilled water to prepare tenfold dilution upto 10^4 (Appendix Figure 6). Then 50 µl of each diluted samples were inoculated on MacConkey agar and incubated at 37°C for 24 hours**.** After incubation the plates were observed for the *Escherichia coli.* Less amount of very light pink colored colonies appeared in all plates were considered as presumptive of *E. coli*. For the confirmation

of the presence of *Escherichia coli* in dahi sample a well isolated colony was picked from each plates and streaked on selective media Eosin Methylene Blue (EMB) agar. All the plates were incubated at 37°C for 24 hours. The colonies with green metallic sheen on EMB agar which is typical feature for the confirmation of *E. coli* (Appendix Figure 9, 10).

CHAPTER-IV: RESULTS

4.1 Isolation of *E. coli*

Escherichia coli were not detected in any of the fifty dahi samples. Thus, these dahi samples were considered as safe for consumption. The samples were pre-enriched in buffered peptone broth. Then the colonial growths observed on to MacConky agar and Eosin Methylene Blue (EMB) was displayed in Figures 3 and 4, respectively.

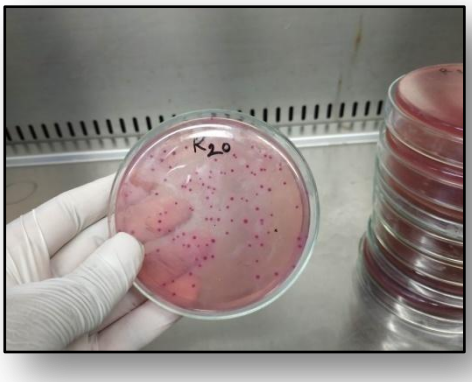

Figure 3: Suspected colony of *E. coli* on MacConkey agar

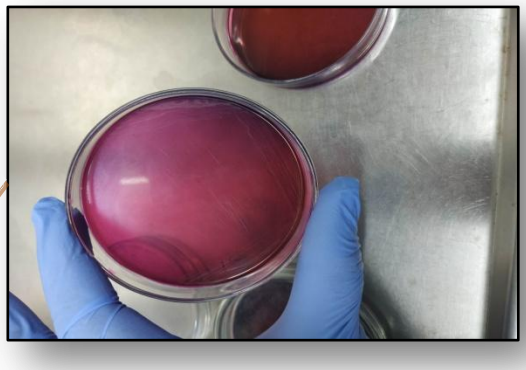

Figure 4: No growth of *E. coli* on EMB agar

4.2 Results of cultural examination

All of the studied samples had showed negative result in EMB agar with no green metallic sheen. Prevalence of *E. coli* in dahi was displayed below in table 4

Table 4: Prevalence of *E. coli* in dahi samples collected from different areas of Chattogram.

Sampling area	**No. samples**	**No. positive**	**Prevalence (%)** (95% CI)
Foys lake	3	Nil	Nil
Jhautala Bazar	3	Nil	Nil
Warless mor	5	Nil	Nil
GEC mor	8	Nil	Nil
Lalkhan bazar	5	Nil	Nil
Baddarhut	8	Nil	Nil
Reazuddin bazar	10	Nil	Nil
New market	4	Nil	Nil
Cornel hut	4	Nil	Nil
Total	**50**	**Nil**	**Nil**

Prevalence of *E. coli* in dahi samples collected from different areas of Chattogram was shown in Table-4. All the dahi samples (N=50) were found negative indicating zero prevalence of *E. coli*. This denotes the good hygienic status of available marketed dahi in those particular areas.

CHAPTER-V: DISCUSSION

The study was conducted with the aim of isolation and identification of *Escherichia coli* present in dahi which is resistant to gut pH (1.5-3.5) and to know the resistance pattern against different antibiotics. In the present study no *Escherichia coli* was not found in any of the dahi samples. Therefore, gut pH and antibiotic resistance could not be observed.

Hossain, et al., (2015) also studied the microbial quality of yoghurt in sylhet city area where the samples were negative for *E. coli* which is similar to our study findings.

A previous study was conducted by Chowdhury et al., (2011) to assess the quality of curd (Dahi), locally available in Bangladesh market ,who remarked that total coliform was nil in their microbial studies which is supported our study findings.

Similar result was found by Sarkar et al., (2012) in Mymensingh, Bangladesh. They studied the chemical and bacteriological quality of popular dahi available in some selected areas of Bangladesh. The data for coliform count has not statistically tested as because the sample of Comilla and Sylhet had Nil count and other groups raw data also possess some Nil figure. However, the count for Bogra, Khulna, Barisal and Savar were 2.01, 0.76, 1.20 and 0.76 log cfu/g, respectively.

The result of present study corresponds with the statement of Mac Graw et al., (1977) who remarked that processed milk should contain no trace of coliform. However, Tamime and Robinson (1985) recommended that yoghurt should contain less than 0.1x10 cfu/g.

Similar result was also found by Rahimi et al., (2011) in Iran. They also studied the prevalence and antimicrobial resistance of *E. coli* isolated from traditional yoghurt. They remarked that survival of *E. coli* in foods depends on the sample acidity. The bacteria disappear when the pH falls to 3.5. Furthermore, the absence of *E. coli* in dahi samples in this study could possibly be accounted for by the acidity of these products. However, it could also be due to the boiling stage performed during the processing of these products.

Similar results have been reported by Younus et al., (2002) in Pakistan. They studied quality evaluation of market yoghurt/dahi. They found most coliform bacteria were absent in most of the yoghurt samples which is very close to our study findings.

Lopez et al.,(1997) also found low number of coliforms in yoghurt sample.

On the other hand, F. Saleem et al., (2014) in Pakistan studied the prevalence of acid and antibiotic resistant coliform bacteria in yoghurt. In their study they found dahi sample contained coliform contents of 6446 C.F.U./ml, of which 38390 were *E.coli.* 83% of the coliform isolates of that study appeared acid resistant. While, 46% were resistant to various antibiotics used in that study which is opposite to our present study.

Dey et al., (2011) studied the quality of dahi available in Sylhet metropolitan city. They found Average highest Coliform count was found in Modhuban Sweetmeat Shop made Dahi and lowest Coliform count was found in Fulkoli Sweetmeat Shop made dahi which do not supported our present study.

The findings of our present study are dissimilar with the findings of Saad et al., (1987). They assessed the microbiological quality of yogurt produced in Assiut city, Egypt. Forty random samples of yogurt were collected from Assiut city markets and the microbial flora analysed. Mean values of counts of coliform in samples were 5.28x103. Indian Standard Institution (1973) and American Public Health Association (1967) set the standards for both coliforms and yeasts in yogurt at less than 10 colonies per ml. and in only one sample out of four.

Soomroet al., (2014) studied isolation of *Escherichia coli* from raw milk and milk products in relation to public health. Amoung 160 studied samples 20 were dahi. They found 11 (55%) of dahi sample *E. coli* positive which do not support our present study.

Another study was done by Kumar et al., (2010) at pantnagar, India. They studied on detection of *E. coli* and *Staphylococcus* in milk and milk products. Among 15 dahi sample they found *E. coli* in 2 samples only, which is nearly close to our present study, suggesting that it could be due to contaminated environment and unhygienic handling or preparation.

CHAPTER-VI: CONCLUSION

In Bangladesh the production of dahi takes place in a much disorganized way. Another cause, to be unfortunate, that there is no legal standard in respect of hygienic production, distribution and nutritional status of the product. The aim of the present study was to determine the prevalence of gut pH and antibiotic resistant *Escherichia coli* in dahi sampled from Chattogram metropolitan area. Although an overall analysis of the results of this study revealed that the dahi collected from selected areas were of acceptable quality. The absence of *E. coli* is an indication of Good Manufacturing Practice (GMP) employed by the producers and handlers. The existence of *E. coli* is the indication of contamination in dahi samples with acid resistant strains. This might be result of the poor hygienic condition of the production, processing, handling and storage period. The possible sources of contamination of product are poor personnel hygiene, contaminated utensils and poor production facilities. Incidence of coliform organisms in dahi can be checked by employing proper sanitary measures in every steps of production and processing.

CHAPTER-VII: RECOMMENDATIONS

The current study revealed that there was no prevalence of *Escherichia coli* in dahi sold in Chattogram metropolitan area. On the basis of the present study following recommendations are given as under:

- Further detailed study will be needed with large number of samples for a longer period from different areas of Chattogram.
- Various government institutes like BSTI should be performed this type of test periodically.
- Food manufacturer sand specialists should design comprehensive programs as good manufacturing practices (GMP) and implementation of HACCP system to ensure the freedom of such foods from these pathogens.
- A comprehensive research work will be required to set a standard for commercial production of dahi in Bangladesh to have uniformity and superiority in its organoleptic and microbiological quality.

CHAPTER-VIII: REFERENCES

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APPENDIX
PERFORMING LABORATORY PROCEDURES


Figure 5: Collection of dahi sample


Figure 6: Preparation of working sample


Figure 7: Preparation of agar

PERFORMING LABORATORY PROCEDURES

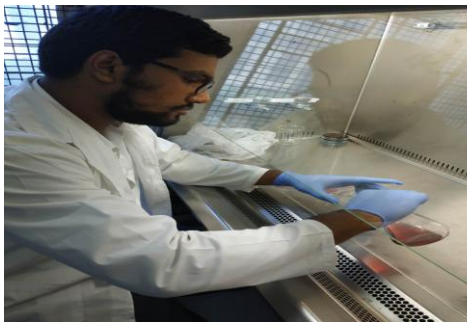

Figure 8: Preparation of agar plate

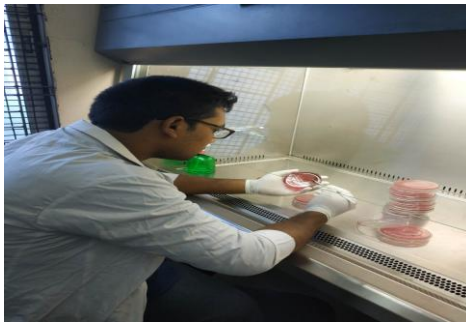

Figure 9: Inoculation of dahi sample at agar plate

Figure 10: Incubation of agar plateand Observing agar plate

BIOGRAPHY

DR. S M Ahosanul Alam Rajon was born in Saghatta, Gaibandha, Rangpur, Bangladesh in 1993. He is the youngest son of MD. Abdul Bari Sardar and Most. Anowara Begum. He passed the Secondary School Certificate Examination from Gaibandha Govt. Boys High School in 2009 with GPA 5.00 followed by Higher Secondary Certificate Examination from Gaibandha Govt. College in 2011 with GPA 4.80. He completed his graduation degree on Doctor of Veterinary Medicine (DVM) from Chattogram Veterinary and Animal Sciences University (CVASU), Bangladesh in 2016 with CGPA 3.34. During his graduation, he received clinical training from Madras Veterinary College and Veterinary College & Research Institute, Namakkal, Tamilnadu, India. Now, he is a Candidate for the degree of MS in Dairy Science, Dept. of Dairy and Poultry Science, Faculty of Veterinary Medicine, CVASU.