ISOLATION AND IDENTIFICATION OF BACTERIAL PATHOGENS FROM BOVINE RAW MILK SAMPLES IN AND AROUND THE DHAKA CITY

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ISOLATION AND IDENTIFICATION OF BACTERIAL PATHOGENS FROM BOVINE RAW MILK SAMPLES IN AND AROUND THE DHAKA CITY

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*This is to certify that the thesis entitled **"Isolation and Identification of bacterial pathogens from bovine raw milk samples in and around the Dhaka City"** has been submitted to the Department of Pathology, Faculty of Animal Science and Veterinary Medicine, Sher-e-Bangla Agricultural University (SAU), Dhaka in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (MS. in PATHOLOYG,** embodies the results of a piece of bona fide research work carried out by **TAHMINA SIKDER,** Registration No. **12-05033** under my supervision and guidance. No part of this thesis has been submitted for any other degree or diploma in any other institution.*

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

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DEDICATION

This thesis is dedicated to my beloved parents & siblings, respected teachers who offered me unconditional sacrifices, blessings, love and supports throughout my study periods

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ABBREVIATIONS

AMR:	Antimicrobial-resistance
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
TVBC	Total viable bacterial count
T*Ec*C	Total *E. coli* count
T*S*C	Total *Salmonella spp.* count
T*Staph*C	Total *S. aureus* count
rRNA	Ribosomal Ribonucleic Acid

Isolation and Identification of bacterial pathogens from bovine raw milk samples in and around the Dhaka City

ABSTRACT

Food-borne zoonotic hazards posed in milk and milk products are currently a serious concern of public health safety issue that plays a significant role on the emerging economies across the world especially in the low and middle income countries including Bangladesh. Raw or unpasteurized milk supports to be the excellent medium for a variety of bacterial growth, and transmits a number of bacterial pathogens cause diseases in consumers because of poor handling and also informal milk value chains. Many Previous studies reported that a number of bacterial pathogens were isolated from milk and milk products, and also from farms environments. Among these organisms, the most common pathogenic bacterial species in milk (raw) samples are *Escherichia coli, Salmonella spp*. *Staphylococcus aureus,* etc. which are the major public health concerns. The number of raw milk outlets has been continuously increasing in the local markets. These outlets retail milk to the poor people who are at high risk of the illnesses from milk-borne pathogens. So, milk could have the major public health concerns due to poses of the pathogenic bacteria for the communities who still consume raw or unpasteurized milk. Therefore, the current study conducted on a study to isolate and identify the pathogenic bacteria in bovine raw milk samples. A total of 54 bovine raw milk was collected and subjected to bacteriological analysis. In results, three bacterial species such as *E. coli, Salmonella spp.* and *S. aureus* were contaminated raw milk with a prevalence rate of 20.37%, 3.70% and 35.18 %, respectively. Besides, a high number of antibiotics were found to be resistant against all these 3 bacterial species, and the overall prevalence of MDR isolated bacteria was of 27.27%, 50% and 42.11% for *E. coli, Salmonella spp.* and *S. aureus*, respectively, which is indicated the serious public health concern. Based on the results demonstrated here, it could be concluded that proper hygienic maintenance of both milking and farm husbandry practices might reduce the chances of bacterial contamination. Besides, it is highly suggested that there is the urgency of increase adequate public awareness about the importance of hygienic milk production and also consumption of pasteurized/ processed milk to prevent milk borne infections.

CHAPTER: 1

INTRODUCTION

Milk and milk products play an important role in feeding the population due to poses of high nutritious value, which is considered the perfect single balance food. Food-borne zoonotic hazards posed in milk and milk products are currently the serious concern of public health safety issue [1-3] that plays significant role on the emerging economies across the world especially in the low and middle income countries including Bangladesh [3-5]. The food safety programs have the intended goals to prevent the contamination of food products by possible pathogenic organisms [3]. Raw or unpasteurized milk supports to be the excellent medium for a variety of bacterial growth, and transmits a number of bacterial pathogens cause diseases in consumers along with milk and milk products spoilage usually because of poor handling (milking process, personnel and/or utensils used during milking) and also informal milk value chains [5-10]. The presence of these contaminants in milk has been used as an important indicator of milk quality in the dairy farms [5] though milk contamination is entirely difficult to avoid yet with bacterial pathogens and/or other residual concentrations of contaminants for many reasons.

Many Previous studies reported that a number of bacterial pathogens were isolated from milk and milk products, and also from farms environments such as *Coliforms, Streptococci*, and *Staphylococci* with the other species of *Listeria, Brucella, Mycobacterium, Campylobacter, Leptospira, Clostridium, Pseudomonas,* and *Proteus* etc. [3-6, 11-14]. Among these organisms which cause foodborne illness, the most common pathogenic bacterial species in milk (raw) samples are *Escherichia coli, Salmonella spp. Staphylococcus aureus,* etc. are the major public health concerns [15-17]. *Escherichia coli* (*E. coli*), a Gram-negative member of the *Enterobacteriaceae* family and a major cause of foodborne infections, which is a common inhabitant of gastrointestinal tract of animals, and human. Most of E.coli. are harmless, but some are known to be highly pathogenic and cause severe intestinal and extra-intestinal diseases in human by means of their virulence factors [18]. The presence of *E. coli* in raw milk increases the risk of transmission of foodborne pathogens in terms of public health concern due to the possible presence of enteropathogenic and/or toxigenic strains impact on public health hazards. It is often used as a reliable indicator of contamination by feces, soil and contaminated water [9]. Besides,

Salmonella is another Gram-negative, facultative anaerobic bacillus belonging to the *Enterobacteriaceae* family known to cause human salmonellosis, a public health problem worldwide [19-21]. *Salmonella* are well distributed within the environment and can cause a variety of illnesses in both human and animals. Infection with *Salmonella spp.* have associated with a wide range of illness ranging from a mild self-limiting form of gastroenteritis to septicemia, localized infections and typhoid fever [5, 6, 20]. A number of studies reported that the outbreak of salmonellosis is associated with the consumption of raw milk and milk products [5, 7, 16]. Furthermore, *Staphylococcus aureus (S. aureus)*, is a pathogenic bacterium in both animals and humans, which causes a variety of diseases [13, 22, 23]. *S. aureus* is also known as one of the most prevalent and important pathogen of intra-mammary infections in ruminants that causes mastitis in dairy cattle across the world [24, 25]. Thus, *S. aureus* infections has huge economic impacts and severe public health challenges to the milk and dairy sectors [24]. Infected mammary glands are the main reservoir for infection; however, the contamination of dairy products can occur anywhere in the food chain especially during handling and processing of raw milk [23]. The consumption of contaminated milk can cause serious health hazards to the humans [24]. The *S. aureus* is associated with both nosocomial and community-acquired infections [26] in terms of the public health significance intensified in humans. Besides, the presence of proliferating pathogenic bacteria in milk and milk products they have played a major threat to the public health significance in terms of the emergence of antimicrobial-resistance (AMR) that is alarming for the rapid global spread of superbugs, which has further turned into the serious concern in current medication for clinical diseases [3, 27]. Moreover, these AMR determinants can also be transferred to the other pathogenic bacteria (especially from person to person or between people and animals, including food of animal origin) potentially compromising the treatment of severe bacterial infections [27]. Besides, the economic importance of AMR is very high usually associated with death and disability, prolonged illness results in longer periods of hospital stay, which need further more expensive medicines and financial challenges [27]. The main drivers of antimicrobial resistance include the misuse and overuse of antimicrobials; lack of access to clean water, sanitation and hygiene (WASH) for both humans and animals; poor infection and disease prevention and control in health-care facilities and farms; poor access to quality, affordable medicines, vaccines and diagnostics; lack of awareness and knowledge; and also lack of enforcement of legislation [27].

The number of raw milk outlets has been continuously increasing in the local markets usually found in the low and middle income countries. These outlets retail milk to the majority of population who have poor economic capacity to purchase high quality processed commercial pasteurized milk, and these people are at high risk of the illnesses from milk-borne pathogens [3, 5]. So, milk could have the major public health concerns due to poses of the pathogenic bacteria for the communities who still consume raw or unprocessed milk [3, 28].

Therefore, the present study aimed to carry out with the following major objectives:

i. To isolate and identify the pathogenic bacteria in bovine raw milk samples collected from the local markets/ small scale farms/ households (farmer's house) in and around the Dhaka city, Bangladesh.

ii. To evaluate the antibiotic sensitivity profile conducted on the isolated bacteria from bovine raw milk.

CHAPTER: 2

REVIEW OF LITERATURES

The following review of literatures was used for knowledge gathering to conduct this study. Isolation, identification and molecular characterization, and antibiotic sensitivity profiling of the bacteria from bovine raw milk samples was observed from the subsequent related review of literature.

2.1. Milk Composition and Nutritive value

Milk may be outlined as a yellowish-white non-transparent liquid secreted by the mammary glands of all mammals. It is the elementary source of nutrition and solely food for offspring of mammals before they are ready to eat and digest other types of food. It contains in an exceedingly balanced type of all the essential and digestible elements for growth and maintenance of the human and animal body. Milk and milk products are high-quality foods rendering both nutritional and culinary values [29]. Milk does have distinct physical, chemical and biological characteristics and its color, odor, taste, consistency, freezing point (- 0.55°C), pH (6.6) and specific gravity (1.032) and these characteristics remain significantly constant [30]. Milk is a highly nutritious, and in addition to the main milk sugar lactose, it also contains proteins (caseins, whey-proteins, and minor proteins) essential amino acids, fats, minerals, and vitamins. Its utilization may thus well have contributed to the success of human development over the centuries [31].

Cow milk has long been considered a highly nutritious and valuable human food but it is an excellent culture medium for many microorganisms, especially bacterial pathogens. There is a constant challenge in milk production to prevent or minimize the entry and subsequent growth of microorganisms in milk. Production of milk and milk products of superior quality and prolonged shelf-life with the ability to provide a safe and wholesome food for the consumers is needful [32]. Though milk has a wide range of positive nutritional values and renders a variety of essential nutrients including protein for body building, vitamins, minerals (especially calcium), fat and carbohydrate for energy [33], but act also as an excellent growth substrate for microorganisms.

2.2. Microbial Quality of Raw Milk

Microbial quality of milk refers to the cleanness of milk which may also be defined by a number of bacteria present in milk. The high bacterial count as well as the presence of pathogenic bacteria in milk is not only degrades the milk quality and shelf-life of milk or milk related products but also poses a serious health threat to the consumers [34]. Milk being a suitable medium for bacterial growth, it can serve as a source of bacterial contamination [35]. Milk is a perishable product and an ideal medium for the growth of a wide variety of bacteria [36]. Raw milk is a widely known good medium that supports the growth of several microorganisms with resultant spoilage of the product or infections/ intoxications in consumers because of poor handling (milking process, personnel and/or utensils used during milking) and also informal milk value chains [5-10]. Though milk contamination is entirely difficult to avoid yet with bacterial pathogens and other residual concentrations of milk contaminants for many reasons. However, the presence of these contaminants in milk has been used as an important indicator of milk quality in the dairy farms [5].

According to Boor et al. [37], TBC for raw milk must be less than or equal to 10^5/ml; for retailed milk, it must be less than or equal to 2×10^4/ml or gram; while for frozen desserts it must be 5×10^4/gram or less. The USA has a standard of 10^5 bacterial cells per milliliter; however, other countries take 2×10^5 colony forming units/milliliter as the acceptable bacterial limit. The microbial limit of total plate counts is used to grade milk as follows: Grade I or A ($< 2\times10^5$ bacterial cells/ml), II or B ($>2\times10^5 – < 10^6$ bacterial cells/ml) and III or C ($>10^6 – < 2\times10^6$ bacterial cells/ml) in Rwanda. A study conducted to evaluate the bacterial loads of raw milk by analyzing the four stages of the raw milk chain: dairy farmers, milk hawkers, milk collection centres (MCC) and milk kiosks in the North-western region of Rwanda. The study revealed a TBC mean values of 1.2×10^6 CFU/ml (dairy farmers), 2.6×10^7 CFU/ml (milk hawkers), 1.5×10^6 CFU/ml (MCC) and 6.9×10^6 CFU/ml (kiosks/restaurants). They mentioned that high Bacterial load was present because of using unhealthy containers for milk transport and source of water used to clean containers [38]. Another study was carried out to assess the microbial quality of raw cow milk from different dairy farms in Ogbomoso, Oyo State, Nigeria. They found that the total bacterial counts of the milk samples ranged from $0.2x10^6$ CFU/ml to $4.2x10^6$ CFU/ml. Also, They showed that the total enterobacteriaceae count ranged from $0.8x10^6$ CFU/ml to $2.6x10^6$ CFU/ml while the total salmonella-shigella count was found to range between $0.5x10^6$ CFU/ml and $1.1x10^6$ CFU/ml [32].

Similarly, a cross-sectional study was conducted in different sub-cities of Mekelle reported that overall mean viable bacterial count and standard deviation of samples from milk shop, fruit juice, and dairy milk were found to be 8.86 ± 10^7, 7.2 ± 10^7, and 8.65 ± 10^7 CFU/ml and 33.87 ± 10^6, 6.68 ± 10^6, and 22.0 ± 10^6, respectively [4]. Uddin *et al*. [39] was conducted a study in Bangladesh to assess the microbial quality of milk and reported that the highest total viable bacterial count (2.36×10^9 cfu/ml) found in samples collected from Uttara, Dhaka and the lowest total viable bacterial count (2.0×10^8 cfu/ml) which had been collected from Mohammadpur. They reported that this variation in total viable count may be due to the hygienic maintenance during milking.

Microorganisms in milk have been demonstrated to undergo rapid multiplication at high ambient temperatures [40-42]. Milking carried out in unhygienic environments increases the likelihood milk contamination by zoonotic pathogens, the level of which can subsequently increase due to the growth of pathogens when milk is stored at ambient temperatures. However, milk contains a natural inhibitory system or temporary germicidal or bacteriostatic properties which prevents a significant increase in the bacteria count during the first 2 - 3 hours [1].

2.3. Milk-borne Infections and Pathogenic Microorganisms

Previous studies reported that a number of bacterial pathogens were isolated from milk and milk products, and also farms environments from different sources, which cause milk-borne sickness such as *Coliforms, Streptococci, and Staphylococci* with the other species of *Listeria*, *Brucella*, *Mycobacterium*, *Campylobacter*, *Leptospira*, *Clostridium*, *Pseudomonas*, and *Proteus* etc. [3-6, 11-14]. Among them, the most common pathogenic bacterial species are *Escherichia coli, Staphylococcus aureus, and Salmonella spp*. All these are pathogenic bacteria that pose serious threat to human health and contribute up to 90% of all dairy related diseases [35, 43-45].

2.3.1. Escherichia coli

Escherichia coli a common inhabitant of the intestinal tract in human and animals, is a Gram-negative, facultative aerobic, rod-shaped and highly motile bacteria bacterium, a member belongs to the *Enterobacteriaceae* family Though most of the strains of *E. coli* are non-pathogenic (harmless); but a number of strains are highly pathogenic, which cause severe foodborne infection in human [46]. Farm animals, especially cattle, asymptomatically carry Shiga toxin-producing *E. coli* (STEC) and *Enterohemorrhagic E. col*i (EHEC). Due to their zoonotic nature, these pathogens

can transmit to human from farm through contaminated milk, meat, water, and direct contact with animals or their environmental equipment [47, 48]. Moreover, the pathogenic strains of *E.coli* can get access to milk and milk products as are the most frequently common contaminating organism and considered a reliable indicator of the source of contamination by manure, soil and contaminated water [49].

According to a cross sectional study conducted in the selected dairy farms in and around Mekelle, Tigray, Ethiopia, reported that the overall prevalence of *E. coli* in bovine milk (raw) was recorded about 25%, which was significantly associated with the stages of lactations and all the isolated *E. coli* showed the pattern of multidrug resistance. They reported that the highest (84.1%) rate of prevalence was found in the milk samples with the early stage of lactations than the prevalence (32.8%) found in the samples collected from the cows with four and above parity number [50]. Dadi *et al.* [51] described that raw milk was found to be contaminated with the bacterial species of *E. coli* reported in Sebeta town, Ethiopia, which also showed antimicrobial resistance having potential impact on public health. While they showed that the prevalence of *E. coli* was significantly highest (15.3%) found in the samples collected from milk collector compared to those samples of individual dairy farms bulk tank milk (4.3%). A study conducted [52] in the northeastern São Paulo State, Brazil, focused on detecting diarrheagenic *E. coli*, enteropathogenic *E. coli* (EPEC), Shiga-toxin-producing *E.coli* (STEC), enterohemorrhagic *E. coli* (EHEC or STEC:EPEC), enterotoxigenic *E. coli* (ETEC), and enteroaggregative *E. coli* (EAEC) in raw milk, water, and cattle feces collected from non-technified dairy farms. They detected that 66.67% of raw milk samples were found positive for *E. coli*. The study also described that EPEC, STEC, and EHEC strains were detected in 4.17%, 16.67% and 4.17% of raw milk samples, respectively. Lastly, they concluded that pathogenic *E. coli* contamination is mostly occurred by cowdung in non-technified dairy farms and therefore, cross-contamination is more common among feces, water, and/or raw milk. According to another cross sectional study conducted in Ethiopia, 33.9% and 2.9% of raw milk samples were contaminated with *E. coli* and *E. coli* O157:H7, respectively. The highest prevalence was recorded in samples collected from vendors (39.1%) in comparison with the samples obtained from farmers (28.1%). The contamination was found higher in the milk samples collected and transported in plastic containers (39.4%) compared with the containers made of stainless steel (23.0%) [53]. One more study [54] conducted and reported that that contaminated raw milk having multi-drug resistance bacteria may create public health hazard.

They reported that 24% were found positive for *E.coli* which was isolated from handwash of milker's, udder wash, utensil wash, raw cow milk and environment. Similarly, Lye *et al.* [55] carried out a study on raw milk samples collected from local dairy farms in the state of Selangor, Malaysia. They reported that *E. coli* O157:H7 is associated with a number of life threatening diseases such as hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). According to the study, *E. coli* O157:H7 was detected in raw cow milk (8.75%), followed by raw goat milk (7.32%) and raw buffalo milk (1.79%). The estimated quantity of E. coli O157:H7 in raw cow, goat and buffalo milk ranged from <30 MPN/g to 120 MPN/g. In raw cow and goat milk samples, E. coli O157:H7 microbial load ranged from 30 to 120 MPN/g and 30 to 36 MPN/g respectively. On the other hand, E. coli O157:H7 microbial load in buffalo milk samples was found to be the lowest, only 30 MPN/g. Therefore, the presence of coliform bacteria such as *E. coli* in milk is considered as a common indicator of fecal contamination. *E. coli* was isolated from 14 milk samples out of 22 raw milk samples. The highest coliform bacterial count was found in sample collected from Tongi ($8x10^6$ cfu/ml) and lowest total coliform count was $1.0x10^4$ cfu/ml which was collected from Ashulia [39]. Another study that was conducted by [56] in India and reported the highest level of contamination of milk samples collected from vendors (26%) followed by the dairy farm (20%) and lastly house milk (6.6%). This may be due to unhygienic handling of milk along the value chain**.**

2.3.2. Salmonella spp.

Salmonella is a genus of *Enterobacteriaceae* family, which are Gram-negative organisms. They are also oxidase negative, catalase positive, nonspore forming rods and facultative anaerobic bacteria. Almost all *Salmonella* species are motile via peritrichous flagella, with exception of the poultry pathogen *Salmonella enterica* ser. *Gallinarium* [57, 58]. *Salmonella spp*. are known to cause salmonellosis in humans. Within the genus Salmonella, S. enterica species can be further classified into six subspecies among which S. enterica subspecies enterica is responsible for 99% of the infections in humans and animals [19]. *Salmonella* are well distributed within the environment and can cause a variety of illnesses in both human and animals. Infection with *Salmonella* can cause a wide range of illnesses in human such as typhoid fever, septicemia, localized infections of various bodily tissues, and gastroenteritis [20]. Although *Salmonella* contamination in milk is lower than other bacterial contamination, milk cen get spoiled with

salmonella spp. when the post-processing contamination are present in the farm commencing from the milking system to the supply chain [59]. Rahman *et al.* [60] reported that only 1.85% milk samples were found positive for *Salmonella spp.* which are collected from Bangladesh Agricultural University (BAU) dairy farm, American dairy farm, Gazipur and different small dairy farms of municipal area. They also found that the isolates were multidrug resistant. The presence of this MDR *Salmonella spp.* in the milk and meat samples can be a consequence of unhygienic conditions of farm and marketing level. Another study [61] conducted in India stated that the overall 7.61% *Salmonella spp* were isolated from milk and milk products with higher number present in raw milk and dahi (11.9%) followed by ice-cream (9.52%) and khoa (4.76%). Interestingly, they did not find any positive sample for flavored milk. Similarly, other study was conducted in Nigeria [62] in some retail milk products and water reservoirs. They conducted a total of one hundred samples of milk products containing full cream milk and skimmed milk collected from Bulumkutu and Maiduguri Monday market. They found that the overall prevalence of *Salmonella* in milk samples was 10.00%. Yasmin et al. [63] conducted a study on milk and milk based products available within Dhaka metropolis, Bangladesh. They found that only 9 samples out of 35 samples were contaminated with *Salmonella spp* with a range of 10^4 to 10^5 cfu/ml. Another study [64] conducted on milk samples at 40 milk collection centers from four regions in Peninsular Malaysia and described that only 1.4% of the samples were contaminated by *Salmonella spp*.

2.3.3. Staphylococcus aureus

These are gram-positive, facultatively anaerobic, non-sporeforming cocci. They were described in 1897. This pathogen produces a wide range of pathogenicity and virulence factors like staphylokinase, hyaluronidases, coagulases and haemolysins [65]. *S. aureus* may gain entry to milk either by direct excretion from udders with clinical and subclinical staphylococcal mastitis or by environmental contamination during the handling and processing of raw milk [66, 67]. A cross sectional study was carried out by [68] on small scale dairy farms in Asella town, Ethiopia. They collected raw milk samples from clinically mastitic cows having significant prevalence of different Staphylococcal isolates. They found that 38.6% *Staphylococcus aureus*, 28.9% *Staphylococcus intermedius* and 7.2% *Staphylococcus hyicus* were prevalent in the tested samples. They concluded that proper hygienic maintenance of both milking and farm husbandry practices could reduce the

chances of contamination as well as mastitic condition of cows. Hoque *et al.* [69] reported the *Staphylococcus aureus* as an usual causative agent of bovine mastitis in dairy farms throughout the world. They stated that the overall prevalence of S.aureus in raw milk collected from clinical mastitic cows was found 72.7, 74.0 and 62.0% against herd, cow and quarter level respectively. Similarly, a cross-sectional study was conducted by [70] in Sebeta, Central Oromia, Ethiopia mentioning *Staphylococcus aureus* as an important cause for occurring gastroenteritis that was contaminated by foods such as milk and milk products. They reported that overall 23.4% of the samples were positive for *S. aureus*. The higher prevalence rate was found in the milk collection centres (80.0%) than at the farm levels (19.6%). On the other hand, the higher contamination rate was observed in the samples of hands of milkers'(32%) than milking buckets (11.1%). There is no positive isolates of *S. aureus* found from pasteurized milk samples. Therefore, they suggested that there was needed adequate public awareness about the importance of hygienic milk production and pasteurized milk consumption to prevent the milk borne infections. Another study [71] described that milk samples were collected from Bangladesh Agricultural University (BAU) Dairy Farm from different healthy cattle and buffaloes at one-month interval, and identify the *Staphylococcus aureus* based on cultural, staining and biochemical characteristics followed by polymerase chain reaction targeting nuc gene. They reported that the prevalence of *S. aureus* was 35.29% of total milk samples. Al- Ashmawy *et al.* [72] conducted a study in Mansoura City, Egypt reported that the toxigenic *S. aureus* isolated from milk and dairy products and its multidrug-resistant property pose a potential public health risk threat due to poor hygienic practices during milk production, retail, or storage stages. They showed that MRSA was detected in 53% among all milk and milk products with highest prevalence rate of 75% in raw milk followed by 65%, 40%, 50%, and 35% in Damietta cheese, Kareish cheese, ice cream, and yogurt samples, respectively. The overall Staphylococcal count of all samples was 3.41 log10 CFU/g. Interestingly, all isolated *S. aureus* strains were MRSA strains which were genetically verified by molecular detection of the mecA gene. Furthermore, genes encoding a-hemolysin (hla) and staphylococcal enterotoxins (sea, seb, sec) were detected in all isolates. According to a study conducted in some retail outlets of milk in North-West Province, South Africa [73], *S. aureus* isolates were confirmed on the basis of morphological (Gram staining), biochemical (DNase, catalase, haemolysis and rapid slide agglutination) tests, protein profile analysis (MALDI-TOF mass spectrometry) and molecular (nuc specific PCR) methods. They found that the prevalence of *S. aureus* isolates was 75% in raw milk

samples followed by 29% and 13% in bulk milk samples and pasteurized milk samples, respectively. Similarly, another study reported [74] in India that *S.aureus* is frequently associated with subclinical mastitis in dairy animals and therefore, contaminated milk and other dairy products causing a wide variety of infections in humans and animals. The study revealed that the overall prevalence rate of *S. aureus* in milk samples was 61% based on conventional techniques and 65.57% *S. aureus* presumptive isolates were positive by PCR which includes 73.53 % of cow's milk, 52 % of goat's milk and 50% of buffaloes milk. Similarly, Sileshi and Munees [75] conducted a cross sectional study in Bahir Dar dairy farms in North-West Ethiopia to assess the prevalence of *S. aureus* and determine their antibiotic susceptibility from lactating cow milk. They isolate and identify *S. aureus* by culturing on Mannitol Salt agar where it produces yellow colonies. They reported that overall 45% raw milk samples were found to be contaminated with *S. aureus* with average count varying between 3.3×10^2 to 7.2×10^4 CFU/ mL.

2.4. Bacterial resistance against antibiotics

Antibiotics are essential therapeutic tools for a wide variety of illnesses caused by bacterial infections. The rapid emergence of antibiotic resistant pathogens negates effective treatments and therefore is becoming a major threat to public health [76]. However, antibiotics-resistance is a significant health, social and economic problem at this time worldwide. Infections caused by antibiotic-resistant bacteria often fail to respond to standard treatments, thereby reducing the probabilities of effective treatment and increasing the risk of morbidity and mortality in serious diseases [77, 78]. In recent years, accumulating issues with bacteria that are resistant to antibiotics occur globally (Keyser *et al*., 2008). Evidence that is obtained from laboratory and epidemiological studies indicates that the persistence of resistant bacteria is related to the persistence of antibiotic use [79]. Commonly the antimicrobial agents particularly antibiotics that are used in farm level are of different groups or classes. These include the penicillins, tetracyclines, aminoglycosides, beta-lactams, sulphonamides, macrolides, and phenicols [80]. Contamination of raw milk with antibiotic resistant bacteria may pose serious threat to consumers leading to public health hazard as well. The study conducted [51] in Sebeta town, Ethiopia revealed that raw milk contaminated with the bacterial species like *E. coli* and *Salmonella* species showing resistant to certain antimicrobials. They reported that two of the isolates showed multiple drug resistance to two drugs. Though all the isolated *E. coli* were found to be 100% susceptible to gentamicin followed by

amoxicillin (92.9%), sulphamethoxazole-trimethoprim (92.9%) and tetracycline (85.7%). For dairy industry of Bangladesh, it is an alarming issue that the pathogenic *Staphylococcus aureus* causing mastitis are becoming more resistant to commercially available antibiotics and this is also a threat for both animal and public health. Antibiogram profile of *Staphylococcus aureus* revealed that 79.3% *isolates* were resistant to at least one antibiotic, 49.0% to two or more antibiotics, and clinical isolates showed more resistance to all tested antibiotics. The highest resistance rate was found to oxytetracyclin, and no resistance to ceftriaxone and azithromycin [69]. It is reported that on the basis of antimicrobial susceptibility tests, *E. coli* isolates were found to be highly susceptible to gentamicin (100%), kanamycin (92%) and sulphamethoxazole- trimethoprim (76%) whereas greatly resistant to penicillin (100%), amoxicillin (84%) and tetracycline (60%) [50]. Another study showed that *E. coli O157:H7* were resistant to tetracycline (81.8%), followed by streptomycin (81.8%), and kanamycin (63.6%) [53].

A number of milk and milk products were examined to isolate the *Salmonella* spp and to determine their antibiogram profile which is conducted by [61] in Madhya Pradesh, India. They found that Salmonella isolates showed resistant to nitrofurantoin (81.2%), tetracycline (75.0%), co-trimoxazole (68.7%), ampicillin (56.2%) and cefotaxime (50.0%).

Foodborne illness is a major public health problem in developed and developing countries now-a-days. Among the organisms causing foodborne illness, *E.coli* is considered an important one. This *E.coli* isolates were found highly resistant to ampicillin, sulfamethoxazole-trimethoprim, clindamycin, erythromycin, chloramphenicol and kanamycin. The isolates also showed high susceptibility to some antibiotics like gentamicin, norfloxacin, tracycline, polymyxin-B and ciprofloxacin [4]. Salmonella isolates originated from animal and poultry feed are found to be resistant to drugs which are commonly used in humans like azithromycin. This is a possible indication of interspecies transmission of resistance gene which is a serious threat for both human and animal. It is to be noted that *Salmonella spp.* isolated from milk samples were 100% sensitive to gentamicin, neomycin and ciprofloxacin, and 100% resistant to erythromycin, doxycycline and amoxycilin. More specifically, about 100% isolates of the Salmonella spp. originated from milk were multi-drug resistant [60]. The antibiotic resistance is associated with the availability of antibiotics in the market and their frequent use both in livestock and human. Moreover, indiscriminate and continuous use of

antibiotics as growth promoters may result antibiotic resistance. It is found that Salmonella showed 100.00% resistance to Amoxicillin, Ceftriaxone and Erythromycin, 80.00% to Gentamicin, 53.33% to Cotrimoxazole and highly sensitive to Ofloxacin (86.67%) among the isolates tested [62]. A study has been done by [71] showed that The antibiotic sensitivity test using 4 commonly used antibiotics indicating the most of the isolates (*E.coli and S.aureus*) were resistant to Gatifloxacin and one isolate showed intermediate resistance to Ofloxacin while sensitive to Ciprofloxacin and Levofloxacin. The dairy sectors of Bangladesh is progressively extended which lead to widespread use of antibiotics for the betterment of health and productivity of animals. Prolonged and indiscriminate usage of these antibiotics may leave antibiotic residues in animal originated foods that give rise to antimicrobial resistant microorganisms. The antibiotic resistance profile of the positive isolates of *E.coli* and *Staphylococcus aureu*s was determinate. They revealed that *S. aureus* showed resistance to cefoxitin (81.48%), ampicillin (64.81%), ciprofloxacin (51.85%), and gentamycin (70.37%). On the other hand, *E. coli* showed resistance to cefoxitin (69.44%), ampicillin (83.33%), ciprofloxacin (77.78%), and gentamycin (86.11%) [81]. Similarly, Yasmin et al. [63] reported that the isolated *Salmonella spp* were found to be resistant against ampicillin (10 µg), cotrimoxazole (25 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), cefixime (5 µg) and the isolates only showed sensitivity against ofloxacin (5 µg). The prolonged application of antibiotics in dairy cattle for the treatment purposes has contributed to antibiotic residues in dairy products. Penicillin is widely used to treat mastitis in dairy cattle. The excessive use of penicillin in dairy cattle left it's residues in milk which can adversely affect public health [82] [83] reported that a lot of drugs such as oxytetracycline are used abusively to treat and protect cattle against various diseases. When such drugs are administered by non-professionals correct dosages are unlikely to be ascertained as well as withdrawal period for products like milk that may lead to antimicrobial residues. The antimicrobial residues such as antibiotics and other anti-bacteria's can be found in milk as leftovers after the drugs have been administered in animal. These residues in milk are often due to farmers failing to adhere to the specified milk withdrawal periods after antibiotic use to sick lactating cows, illegal or extra label use of drugs and incorrect dosage levels and route of administration [84, 85].

CHAPTER 3

MATERIALS AND METHODS

3.1. Research conducting place and study period

This research work was conducted together at the laboratory of Pathology and Environmental Biotechnology under the department of Pathology and APMA, respectively, Sher-e-Bangla Agricultural University (SAU), Dhaka-1207; and the laboratory of Animal Biotechnology at National Institute of Biotechnology (NIB), Savar, Dhaka. This study was carried out between the periods of October'2019 to November'2020.

3.2. Materials

3.2.1. Study area, collection and transportation of samples

A total of 54 bovine milk (raw) samples were bought randomly and collected aseptically in 50 ml (sterile) conical tubes (BD FALCON™, BD Biosciences, Franklin Lakes, NJ, USA) from the outlets in the local markets (n=27) [hereafter 'outlets']; households, who was rearing 1~2 cows (n=15) [hereafter 'households'] and small scale farms, who was rearing 3~5 cows (n=12) [hereafter 'smallfarms'] in and around the Dhaka city Bangladesh. After collection, milk samples were kept on ice containing cooling box for maintaining 4˚C temperature and brought immediately to the laboratories for further analysis. On arrival at the laboratories, samples were processed to count bacterial loads and subsequently subjected to be carried out the tests for the isolation and identification of bacterial species.

3.2.2. Bacteriological media

3.2.2.1. Agar media

To conduct the bacteriological analysis, a number of different media were used such as nutrient agar, MacConkey (MC) agar, Eosin methylene blue (EMB) agar, Mannitol salt agar, Salmonella-Shigella (SS) agar, 5% blood agar and Muller Hinton (MH) agar etc.

3.2.2.2. Liquid media

A number of liquid media were used in this study such as Nutrient broth, Peptone broth, Methyl-Red and Voges-Proskauer broth (MR-VP broth) and Sugar media (dextrose, maltose, lactose, sucrose and mannitol).

3.2.3. Chemicals and reagents

The chemicals and reagents used for this study were 0.1% Peptone water, Phosphate buffered saline (PBS), reagents for Gram's staining (Crystal Violate, Gram's iodine, Safranin, Acetone alcohol), 3% Hydrogen peroxide, Phenol red, Methyl red, 10% Potassium hydroxide, Kovac's indole reagent (4-dimethylamino-benzaldehyde, concentrated HCL), Mineral oil, Normal saline and other common laboratory chemicals and reagents.

3.2.4. Glasswares and other appliances

The following glasswares and appliances were used throughout the course of the experiment. Test tubes (with or without Durham's fermentation tube and stopper), conical falcon tubes (5 ml, 15 ml), petridish, conical flask, pipette (1 ml, 2 ml, 5 ml, 10 ml), slides and cover slips, hanging drop slides, immersion oil, compound microscope, bacteriological loop, sterilized cotton, cotton plug, test tube stand, water bath, bacteriological incubator, refrigerator, sterilizing instruments (autoclave machine), thermometer, ice carrier, hand gloves, spirit lamp, gas lighter, laminar air flow, hot air oven, epi-tubes and centrifuge machine, electronic balance, pH meter, tray, forceps, thermos scientific nano drop spectrophotometer, UV transilluminator, PCR machine, Gel documentation systems etc.

3.2.5. Antimicrobial discs

Commercially available antimicrobial discs (OXOID Limited, Canada) were used to determine the drug sensitivity and resistance pattern to interpret their potency against diseases. This method allowed for the rapid detection of the efficacy of drugs against the test organisms by measuring the diameter of the zone of inhibition that resulted from diffusion of the agent into the medium surrounding the discs inhibiting the growth of the organisms. The following antimicrobial agents with their disc concentration and ranges were used to test the sensitivity and resistance pattern of

the identified bacterial pathogens (*E. coli, Salmonella spp.* and *Staphylococcus aureus)* isolated from bovine raw milk.

Table 1. List of antibiotics used in this study to assess their antibiotic sensitivity and zone of ranges of inhibition.

SL No.	Name of the Antibiotics	Disk code	Disk potency (µg)	Zone Diameter (mm)		
				Resistance (R)	Intermediate (I)	Susceptible (S)
1.	Amoxicillin	AML	10	13/19	14-17	20
2.	Ampicillin	AMP	10	13/28	14-17	17/29
3.	Azithromycin	AZM	15	13	14-17	18
4.	Erythromycin	ERY	15	13	14-22	23
5.	Nalidixic acid	NA	30	13	14-18	19
6.	Gentamycin	CN	10	≤ 12	13-14	≥ 15
7.	Tetracyclin	TE	30	14	15-18	19
8.	Levofloxacin	LEV	5	13	14-16	17
9.	Ciprofloxacin	CIP	5	≤ 15	16-20	≥ 21

Legend: µg = microgram, [86]

3.3. Methods

3.3.1. Brief description of the experimental design and methodology

The experimental design is schematically presented in Figure-1. The whole experiments were categorized into two principal steps. The first step included selection of sources, collection and transportation of samples, and isolation and identification of bacterial pathogens on the basis of their colony characteristics (cultural), morphology, motility, biochemical properties and molecular characterization for the isolated *Escherichia coli* and *Staphylococcus aureus*. Then, in the second step, the current status of drug sensitivity and resistance pattern of the isolated bacteria were evaluated.

Flowchart of the study design

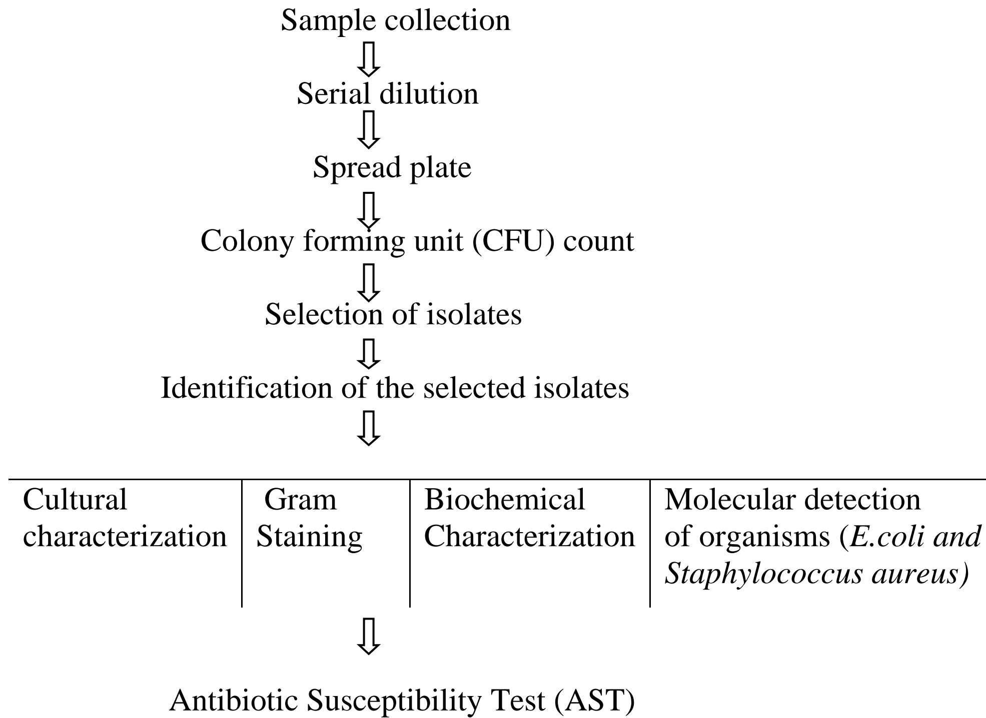


Figure-1: Schematically presentation of the experiment.

3.3.2. Preparation of various types of bacteriological culture media

3.3.2.1. Nutrient broth (NB)

Nutrient broth was prepared based on manufacturer's instruction, dissolving of 13 grams dehydrated nutrient broth (HiMedia, India) into 1000 ml of distilled water and sterilized by autoclaving at 121ºC under 15 lbs pressure per square inch for 15 minutes. Then the broth was dispensed into test tubes (10 ml/tube) and incubated at 37ºC for overnight to check their sterility, and stored at 4ºC in the refrigerator until used.

3.3.2.2. Nutrient agar (NA)

Nutrient agar prepared based on manufacturer's instruction, 14 grams of NA base (HiMedia, India) was dissolved into 500 ml of distilled water in a conical flask and heated for boiling to dissolve the medium completely. Then, the solution was sterilized by autoclaving at 121ºC under 15 lbs pressure per square inch for 15 minutes. After autoclaving, the medium was then poured into sterile

petridishes and allowed to solidify (3-5 hours). After solidification of the medium, the plates were then kept inverted and incubated at 37ºC for overnight to check their sterility and stored at 4ºC in the refrigerator until used.

3.3.2.3. MacConkey (MC) agar

MacConkey (MC) agar prepared based on manufacturer's instruction, 49.53 grams of dehydrated MC agar (HiMedia, India) was suspended into 1000 ml of distilled water in a conical flask and heated up to boiling to dissolve the medium completely. Then, the solution was sterilized by autoclaving at 121ºC under 15 lbs pressure per square inch for 15 minutes. It was then poured in to sterile petridishes and allowed to solidify (3-5 hours). After solidification of the medium, the plates were kept inverted and incubated at 37ºC for overnight to check their sterility and stored at 4ºC in the refrigerator until used.

3.3.2.4. Eosine Methylene Blue (EMB) agar

Eosine Methylene Blue (EMB) agar prepared based on manufacturer's instruction, 36.0 grams powder of EMB agar base (HiMedia, India) was suspended in 1000 ml of distilled water. The suspension was heated to dissolve for few minutes to dissolve the powder completely with water. On sterilization by autoclaving the medium was poured into sterile glass petridishes to form a thick layer EMB agar plate (3-5 hours). After solidification of the medium, the plates were kept inverted and incubated at 37ºC for overnight to check their sterility, and then stored at 4ºC in the refrigerator until used.

3.3.2.5. Salmonella Shigella (SS) agar

Salmonella Shigella (SS) agar prepared based on manufacturer's instruction, 60 grams of dehydrated SS agar was suspended into 1000 ml distilled water. Then it was heated to boil to dissolve the medium completely .The solution was autoclaved and then after mixing well, it was poured into sterile petridishes and kept to solidify (3-5 hours). After solidification of the medium, the plates were kept inverted and incubated at 37ºC for overnight to check their sterility, and then stored at 4ºC in the refrigerator until further used.

3.3.2.6. Mannitol salt (MS) agar

Mannitol salt (MS) agar prepared based on manufacturer's instruction, 111.02 grams of dehydrated MSA agar (HiMedia, India) was suspended in to 1000 ml of distilled water taken in a conical flask and heated upto boiling to dissolve the medium completely. The solution was sterilized by autoclaving at 121ºC at 15 lb pressure per square inch for 15 minutes. It was then poured onto sterile petridishes and allowed to solidify (3-5 hours). After solidification of the medium, the plates were kept inverted and incubated at 37ºC for overnight to check their sterility, and then stored at 4ºC in the refrigerator until further used.

3.3.2.7. Muller Hinton agar

Muller Hinton agar prepared based on manufacturer's instruction, 38 grams of dehydrated MH agar (HiMedia, India) was suspended in to 1000 ml distilled water. Then it was heated upto boiling to dissolve the medium completely. The solution was sterilized by autoclaving at 121˚C under 15 lbs pressure for 15 minutes and kept for cooling down to 45-50˚C. Then it was shaken well and poured into sterile petridishes for solidification (3-5 hours). After solidification of the medium, the plates were kept inverted and incubated at 37ºC for overnight to check their sterility, and then stored at 4ºC in the refrigerator until further used.

3.3.2.8. Phosphate Buffered Saline (PBS)

To prepare phosphate buffered saline, 8.0 gm of sodium chloride (NaCl), 2.89 gm of disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$), 0.2 gm of potassium chloride (KCl) and 0.2 gm of potassium hydrogen phosphate (KH_2PO_4) were suspended in 1000 ml of distilled water. The solution was heated to dissolve completely and pH was adjusted with the help of pH meter. The solution was then sterilized by autoclaving and stored at 4ºC for future use.

3.3.2.9. Simmon's Citrate (SC) agar

For making a Simmon's Citrate (SC) agar, 5.0 gm sodium chloride (NaCl), 2.0 gm sodium citrate (dehydrate), 1.0 gm ammonium dihydrogen phosphate, 1.0 gm dipotassium phosphate, 0.2 gm magnesium sulfate (heptahydrate) were dissolved in 1000 ml distilled water. The pH was adjusted to 6.9. Then agar and bromothymol blue were added. Gently heat with shaking until agar is dissolved. The media were dispensed 5.0 ml into each test tubes. Autoclave at 121°C under 15 lbs

pressure for 15 minutes. Cooling in slanted position (slant and butt). The un-inoculated medium will be a deep forest green due to the pH of the sample and the bromothymol blue. During inoculation, the surface of the medium is lightly inoculated by streaking and, where slopes are used, the butt of medium is inoculated by stabbing.

3.3.2.10. SIM (Sulfide, Indole, Motility) media

SIM media was prepared by suspending 36.23 grams in 1000 ml distilled water. Heat to dissolve the media completely. Dispensed in tubes. Sterilized by autoclaving at 15 lbs pressure at 121°C for 15 minutes and stored at 4ºC for future use.

3.3.2.11. Methyl Red and Voges–Proskauer (MR-VP) broth

A quantity of 3.4 gm of MR-VP medium (HiMedia, India) was dissolved in 250 ml of distilled water, distributed in 5.0 ml quantities in test tube and then autoclaved. After autoclaving, the tubes containing medium were incubated at 37ºc for overnight to check their sterility and then stored at 4ºC for future use.

3.3.2.12. Sugar solutions

The medium consists of 1% peptone water to which fermentable sugars were added. Peptone water was prepared by adding 1 gram of Bacto peptone (Difco, USA) and 0.5 grams of sodium chloride in 100 ml distilled water, boiled for 5 minutes, adjusted to pH 7.6 by phenol red (0.02%) indicator, cooled and then filtered through filter paper. The solutions were then dispensed in 5 ml amount into cotton plugged test tubes containing invertedly placed Durham's fermentation tubes. Then the sugars, dextrose (MERCK, India), maltose (s.d. fiNE-CHEM Ltd.), lactose (BDH, England), sucrose (MERCK, India) and mannitol (PETERSTOL TENBEG) used for fermentation were prepared separately as 10 percent solutions in distilled water (10 grams sugar was dissolved in 100 ml of distilled water). A little heat was necessary to dissolve the sugar. These were then sterilized by autoclaving for 15 minutes. The sugar solutions were sterilized in Arnold's steam sterilizer at 100ºC for 30 minutes for three consecutive days. An amount of 0.5 ml of sterile sugar solution was added aseptically in each culture tubes containing sterile peptone water. The sugar solutions were incubated at 37ºC for 24 hours to check sterility. These solutions were used for biochemical test.

3.3.3. Isolation of bacteria from bovine raw milk samples

3.3.3.1. Preparation of samples: Serial dilution of samples for bacterial primary culture

Test tubes were autoclaved before use. 10 fold serial dilutions of the raw milk samples with physiological (0.9% NaCl) saline /PBS were prepared upto eight time points (1-8). Initially, 0.1 ml of raw milk was mixed with 0.9 ml of PBS (10^{-1}) in an eppendorf tube and mixed well by repeated pipetting in order to make 10-fold dilution. Then, 0.1 ml of mixed sample was transferred from the 1st tube to 2nd tube and mixed with 0.9 ml PBS solution (10^{-2}) in it by repeated pipetting. This action was repeated up to the last tubes labeled as 10^{-3}, and 10^{-4} upto 10^{-8}.

The already prepared agar plates (Nutrient agar & MacConkey agar) were taken from the refrigerator, kept at room temperature and labeled prior to inoculation. From each dilution (starting with the last dilution), one sterile plates marked with 4 quadrant were inoculated with 25µl in each quadrant of the diluted test sample. With the aid of sterile glass spreader and following the spreading plate technique the diluted samples were spread onto nutrient agar media. All plates were then kept inverted and incubated at 37◦C for 24-48 hours. After the incubation period the plates was showing 30-300 colonies were counted and noted down.

Total viable count: The colonies found in 4 quadrant of nutrient agar plate were made an average. Number of microorganisms is expressed as colony forming unit (CFU) per ml of sample.

CFU/ml=No. of colonies average × Reciprocal of dilution factor /amount of inoculate

Total coliform count: The colonies found in 4 quadrant of MacConkey agar plate were made an average.

CFU/ml=No. of colonies average × Reciprocal of dilution factor /amount of inoculate

3.3.3.2. Isolation and identification of microorganisms

Viable colonies were aseptically picked from nutrient agar plates and purified using prepared sterile nutrient broth. Microscopic examination of the selected colonies was carried out to determine cell morphology and Gram's staining reactions of the bacterial isolates.

3.3.3.2.1. Selective plating and identification of bacterial isolates in culture media

Isolation of specific bacteria was done by streaking onto selective media from primary culture. Overnight cultures were grown onto nutrient broth and a loopful of inoculum from nutrient broth

was streaked onto selective media and incubated at 37°C for 24 hours to get pure culture for characterization of their physical properties. Eosin Methylene Blue and MacConkey agar for *E. coli*, Salmonella-Shigella (SS) and MacConkey agar for *Salmonella spp*.; Mannitol salt agar (MSA) and 5% sheep blood agar (HiMedia, India) was used for isolation of *Staphylococcus aureus*. Cultural (colony) characteristics (e.g. shape, size, surface texture, edge and elevation, color, opacity etc.) of the suspected colonies of test organisms onto different selective media were carefully recorded [87].

3.3.3.2.2. Microscopic study for identification of *E. coli, Salmonella spp.* and *Staphylococcus aureus* from the suspected colonies by Gram's staining method

The morphology of the bacteria in the suspected colonies from pure culture were confirmed using Gram's staining [87, 88] to determine the size, shape and arrangement of isolated bacteria. Gram's staining was performed using a Gram's staining kit (BD Biosciences) according to the manufacturer's instructions. Shortly, a single colony was picked up with a bacteriological loop, smeared on a glass slide and fixed by gentle heating. Crystal violet solution was then applied on the smear to stain for 2 minutes, and then washed through running tap water. A few drops of Gram's iodine was then added to act as a mordent for one minute and then again washed with running tap water. Acetone alcohol was then added, which act as a decolorizer. After washing with water, safranine was added as counter stain and allowed to stain for 2 minutes. The slide was then washed with water, blotted and air dried, and then examined under light microscope with high power objective (100X) using immersion oil.

3.3.3.2.3. Motility test for isolated bacterial species

The motility test was performed according to the method described elsewhere [89] to differentiate the motile bacteria from the non-motile one. Before performing the test, a pure culture of the test organism was allowed to grow in nutrient broth. One drop of cultured broth was placed on the cover slip and was placed inverted condition over the concave depression of the hanging drop slide to make hanging drop preparation. Vaseline was used around the concave depression of the hanging drop slide for better attachment of the cover slip to prevent air current and evaporation of the fluid. The hanging drop slide was then examined carefully under 100X power objective of a

compound microscope using immersion oil. The motile and non-motile organisms were identified by observing motility in contrasting with to and fro movement of bacteria.

3.3.3.2.4. Biochemical tests (identification of isolated bacteria)

Several biochemical tests were performed for confirmation of the isolated strains of *E. coli, Salmonella* spp.*, S. aureus.*

3.3.3.2.4.1. Carbohydrate fermentation test

The carbohydrate fermentation test was performed by inoculating 0.2 ml of nutrient broth culture of the isolated organisms into the tubes containing different sugar media (five basic sugars such as dextrose, maltose, lactose, sucrose and mannitol) and incubated for 24 hours at 37ºC. Acid production was indicated by the color change from red to yellow and gas production was noted by the accumulation of gas bubbles in the inverted Durham's tube [87].

3.3.3.2.4.2. Methyl Red test

The test was conducted by inoculating single colony from the pure culture of the test organism in 5 ml sterile MR-VP broth. After 5 days incubation at 37ºC, 5 drops of methyl red solution was added and observed for color formation. Development of red color was positive and indicated an acid pH of 4.5-6 resulting from the fermentation of glucose. Development of yellow color indicated negative result [87].

3.3.3.2.4.3. Voges-Proskauer (V-P) test

The test *E. coli* organisms were grown in 3 ml of sterile MR-VP broth at 37ºC for 48 hours. Then 0.6 ml of 5% alpha-napthol and 0.2 ml of 40% potassium hydroxide containing 0.3% creatine was added per ml of broth culture of the test organism. Then shaking well and allowed to stand for 5-10 minutes to observe the color formation. Positive case was indicated by the development of a bright orange red color. In negative cases there was no development of pink color [87].

3.3.3.2.4.4. Indole test

The test organisms were cultured in test tubes having 3 ml of peptone water containing tryptophan at 37ºC for 48 hours. Then 1 ml of diethyl ether was added, shaked well and allowed to stand until the ether rises to the top. Then 0.5 ml of Kovac's reagent was gently run down the side of the color of the ring. Development of a brilliant red colored ring indicated indole production test tube so that it forms a ring in between the medium and the ether layer and observed for the development. In negative case there is no development of red color (Cheesbrough, 1985).

3.3.3.2.4.5. Citrate utilization test

This test uses Simmon's citrate agar to determine the ability of a bacteria to use citrate as its sole carbon source. Bacteria colonies are picked up by a straight wire and inoculated into slope of Simmon's citrate agar and incubated overnight at 37 °C. If the organism has the ability to use citrate by producing an alkaline reaction and changes the color of medium from green to bright blue. In case of negative test, (i.e. no citrate utilization) the color of the medium remains unchanged [90].

3.3.3.2.4.6. Catalase test

Catalase test was done to determine the ability of the bacteria to degrade hydrogen peroxide by producing the enzyme catalase. A drop of 3% hydrogen peroxide solution was placed on a glass slide. Using a sterile inoculating loop, a small amount of bacteria from 24-hour pure culture was emulsified in the hydrogen peroxide. A positive test was indicated by immediate bubble formation [90].

3.3.3.2.4.7. MIU (Motility- Indole- Urease) test

MIU test was done to simultaneously determine the ability of the bacteria to produce indole, check motility and degrade urea by means of the enzyme urease. MIU media was prepared by autoclaving at 15 psi at 121°C. The media was cooled to about 50-55◦C and 100 ml of urea glucose solution was added aseptically to 900 ml base medium. After that, 6ml solution was transferred to each sterile test tube and allowed to form a semi solid medium. Using sterile technique, small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a stab inoculation method with an inoculating needle and the tubes were then incubated for 24 hours at 37◦C [90].

3.3.3.2.4.8. Oxidase test

Two drops of 1% freshly prepared oxidase reagent (phenylenediamine) was placed on a filter paper in a clean Petridish. The test organism was smeared on it with a glass rod. A positive result showed deep purple colour appearing within 5-30 secs. The absence of deep purple colour indicates a negative result.

3.3.3.2.4.9. Urease test

10 gm Urea agar base was diluted with 90 ml distilled water. Then phenol red was added as a indicator. The broth was poured in each test tubes. A heavy inoculum from an 18 hour pure culture was streaked on the entire slant surface. The slant was incubated at 35-37°C for 48 hours to 7 days. The development of a pink color indicates a positive reaction.

3.3.3.2.4.10. Coagulase test

A drop of physiological saline was placed on a clean glass slide and a colony picked from the solid medium was emulsified in the saline. A loopful of citrated human plasma was added to the bacterial suspension and mixed using the wire loop. The slide was then held up and tilted back and forth for one minute. A positive test is indicated by clumping of cells in the mixed suspension.

3.3.3.2.5. Molecular detection and characterization by PCR

Molecular detection and characterization was done on the isolated strains of *E. coli* and *S. aureus*.

3.3.3.2.5.1. Extraction of bacterial genomic DNA

E. coli and *S. aureus*: Genomic DNA was extracted from pure-culture by boiling-centrifugation method as described previously [91, 92] with a little modification.

i. Materials

- Deionized water
- 1.5 ml Eppendorf tube
- Microcentrifuge
- Waterbath
- Ice

- Pipette
- PCR machine and PCR reagents
- Gel documentation systems

ii. Procedure

Shortly, 100µl of deionized water was taken into an Eppendorf tube. A pure bacterial colony from overnight culture at 37°C onto Eosin Methylene Blue Agar (*E. coli*) and Mannitol Salt Agar *(S. aureus*) was gently mixed with deionized water. The tube was then transferred into boiling water bath at 100°C and boiled for 8~10 min, then immediately transferred onto ice for cold shock for about 5~10 min, and finally centrifuged at 13,000 rpm for 5 min. Supernatant from each tube was collected and used as DNA template for conducting PCR assays. The extracted DNA was stored at 4°C for (for short time ~7days) and at -20°C (for long time) until use.

3.3.3.2.5.2. Primers used for PCR amplification

The 16S rRNA sequence has been used to detect genetic relatedness between different species of bacterial. The gene-specific *E. coli* and *S. aureus* was amplified based on *16S rRNA* and *nuc* (highly specific gene for *S. aureus)* genes, respectively with the gene specific primer-sets (Table-2).

3.3.3.2.5.3. PCR amplification (preparation of PCR reaction and thermocycler conditions) and electrophoresis

PCR amplification was carried out with a reaction volume was [using a commercial PCR kit (PCR Master Mix, 2x, Promega, Madison, WI, USA) according to the manufacturer's recommendations] in a total of 25µL including 12.5µL of PCR Master Mix (2x), 2.5µL of each primer (10 pm), 1µL of DNA template (100 ng) and 6.5µL of dH_2O. PCR amplification were carried out in a thermo-cycler (GeneAtlas, Model: G02, Japan). In brief, the reaction condition was 1 cycle of initial-denaturation at 95ºC for 5 min followed by 35 cycles of PCR amplification at 94ºC for 1 min (denaturation), 55ºC for 1 min (annealing), and 72ºC for 1 min (extension). Then, a final extension of 1 cycle was performed at 72ºC for 10 min.

Table-2: Primers and sequences used in PCR amplification.

Target Gene	Primer Sequences (5´- 3´)	Size (bp)	Reference
E. coli 16E1 (F)	GGGAGTAAAGTTAATCCTTTGCTC		
E. coli 16E2 (R)	TTCCCGAAGGCACATTCT	584	[93, 94]
E. coli 16E3 (R)	TTCCCGAAGGCACCAATC		
S. aureus Nuc (F)	CGATTGATGGTGATACGGTT	279	[95]
S. aureus Nuc (R)	ACGCAAGCCTTGACGAACTAAAGC		

Note: F = Forward, R = Reverse, bp = base pair; Primer E1 and E2 can amplify 584bp fragments from the pathogenic *E. coli.* Primer E1 and E3 can amplify same from nonpathogenic organism.

Electrophoresis: PCR amplicons (products) were analyzed by electrophoresis in 2% agarose (Sigma). Positive and negative controls were also used along with test samples. The procedure of gel preparation and electrophoresis is given below:

- **i.** Gel casting tray was assembled with gel comb of appropriate teeth size and number.
- **ii.** 2% agarose solution was prepared in TBE buffer by melting in a microwave oven.
- **iii.** Molten agarose was poured onto the casting tray and allowed to solidify on the bench.
- **iv.** The hardened gel in its tray was transferred to the electrophoresis tank containing sufficient TBE buffer to cover the gel 1 mm. The comb was gently removed.
- **v.** Seven microliter (7µl) of each PCR product was mixed with 2-3µl loading buffer and the sample was loaded to the appropriate well of the gel.
- **vi.** Five microliter (5 µl) DNA size marker was loaded in one well.
- **vii.** The leads of the electrophoresis apparatus were connected to the power supply and the electrophoresis was run at 100V.
- **viii.** When DNA migrated sufficiently, as judged from the migration of bromphenicol blue of loading buffer, the power supply was switched off.
- **ix.** The gel stained in ethidium bromide (0.5µg/ml) for 10 minutes, in a dark place.
- **x.** The gel was detained in distilled water for 10 minutes.
- **xi.** The detained gel was placed on the UV transilluminator in the dark chamber of the image documentation system.
- **xii.** The UV light of the system was switched on, the image was viewed on the monitor, focused, acquired and saved in the USB flash drive.

3.3.3.3. Maintenance of stock culture

1ml of 80% sterile buffered glycerol was made by mixing 800µl of pure glycerol and 200µl of PBS. Then 0.5 ml of pure bacterial culture was mixed with 80% sterile buffered glycerol in 1.5 ml cryo-vials and was preserved at -80^{0}C for future use.

3.3.3.4. Antibiotic/ Antimicrobial sensitivity test

The in vitro susceptibility and resistance of the isolated bacteria was done against 9 different types of commonly used antibiotics followed by the Kirby-Bauer disc diffusion method as described earlier [96] according to National Committee for Clinical Laboratory Standards (NCCLS) procedures. The antibiotics discs such as Gentamicin (GM, 10µg), Azithromycin (AZM, 15µg), Levofloxacin (LEV, 5µg), Tetracycline (TET, 30µg), Ampicillin (AMP, 10µg), Erythromycin (ERY, 15µg), Amoxicillin (AMX, 10µg), Nalidixic acid (NA, 30µg) and Ciprofloxacin (CIP, 5µg) were used. This method allowed for the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that results from diffusion of the agent in to the medium surrounding the disc (Table-1).

Briefly, the suspension of the test microorganism was prepared in nutrient broth by overnight culture. By using sterile tips in pipette 0.1ml of broth culture of the test organism was poured on Muller Hinton agar (Merck, Darmstadt, Germany). Sterile glass spreader was used to spread the culture homogenously on the medium. Inoculated plates were closed and allowed to dry for approximately 3-5 minutes. Then the antibiotic discs were applied aseptically to the surface of the inoculated agar plates at a special arrangement with the help of a sterile forceps. The plates were then inverted and incubated at 37ºC for 24 hours. After incubation at 37ºC for 24 hours, the zones of growth inhibition for individual isolates were recorded in millimeter scale. The results was interpreted into the categories of susceptible/sensitive, intermediate and resistant according to the guidelines of the National Committee of Clinical and Laboratory Standards Institute [86]. The isolates were resistant against 3 (at least) types of antibiotics considered as multidrug resistance (MDR) [97, 98].

3.4. Data analysis

Microsoft excel spread sheet was used for raw data entry. A nonparametric *t*-test (Mann-Whitney *U* test) was used to compare the significance difference within groups. The differences were considered statistically significant at $p < 0.05$. GraphPad Prism 5.0.2 (GraphPad Software, Inc., CA, USA) was used to generate the graphs and the statistical analysis was done using SPSS Advanced Statistics 17.0 software (SPSS, Inc., Chicago, USA).

CHAPTER 4
RESULTS AND DISCUSSION

The results presented below was demonstrated on the isolation, identification and molecular characterization of milk-borne bacterial pathogens in bovine raw milk samples collected from local markets, small scale farms and households in and around the Dhaka city, Bangladesh. The results also focused on the antibiotics sensitivity and resistance pattern of the isolated bacterial species against different drugs.

4.1. Results

4.1.1. Enumeration of bacterial loads (total viable count)

The bacterial load was counted on all the milk (raw) samples collected from the outlets (n=27), households (n=15) and small farms (n=12) in and around the Dhaka city, and summarized in Figure-2 and Table-3. The results revealed that all the collected samples (100%, n=54/54) were found positive for total viable bacterial count (TVBC). However, the lowest average TVBC of 1.216×10^9 CFU/mL was recorded in the samples collected from the households compared to the highest average TVBC of 1.978×10^9 CFU/mL found in the samples collected from the small farms. While the samples collected from the outlets had an average TVBC of 1.637×10^9 CFU/mL (Figure-2A). In addition, a total of 20.37 % (n=11/54) samples were positive for *E. coli* While, the lowest average total *E. coli* count (T*Ec*C) was recorded as of 8.390×10^4 CFU/mL found in the samples of households than the highest average T*Ec*C of 2.194×10^5 CFU/mL recorded in the samples of outlets, and the samples collected from small farms had an average T*Ec*C as of 9.825×10^4 CFU/mL (Figure-2B). Similarly, a total of 3.70% (n=2/54) samples were positive for *Salmonella spp.* found only in the samples collected from the outlets having an average total *Salmonella spp.* count (T*S*C) as of 4.204×10^2. However, there no positive samples were found for Salmonella spp. in the samples collected from the households and small farms (Figure-2C). Furthermore, a total 35.18 % (n=19/54) samples were positive for *S. aureus.* Wherein, the lowest average total *S. aureus* count (T*Staph*C) was recorded as of 3.000×10^3 CFU/mL found in the samples of households than the highest average T*Staph*C of 7.325×10^3 CFU/mL found in the samples of small farms, and the samples collected from the outlets had an average T*Staph*C as of 5.567×10^3 CFU/mL (Figure-2D).

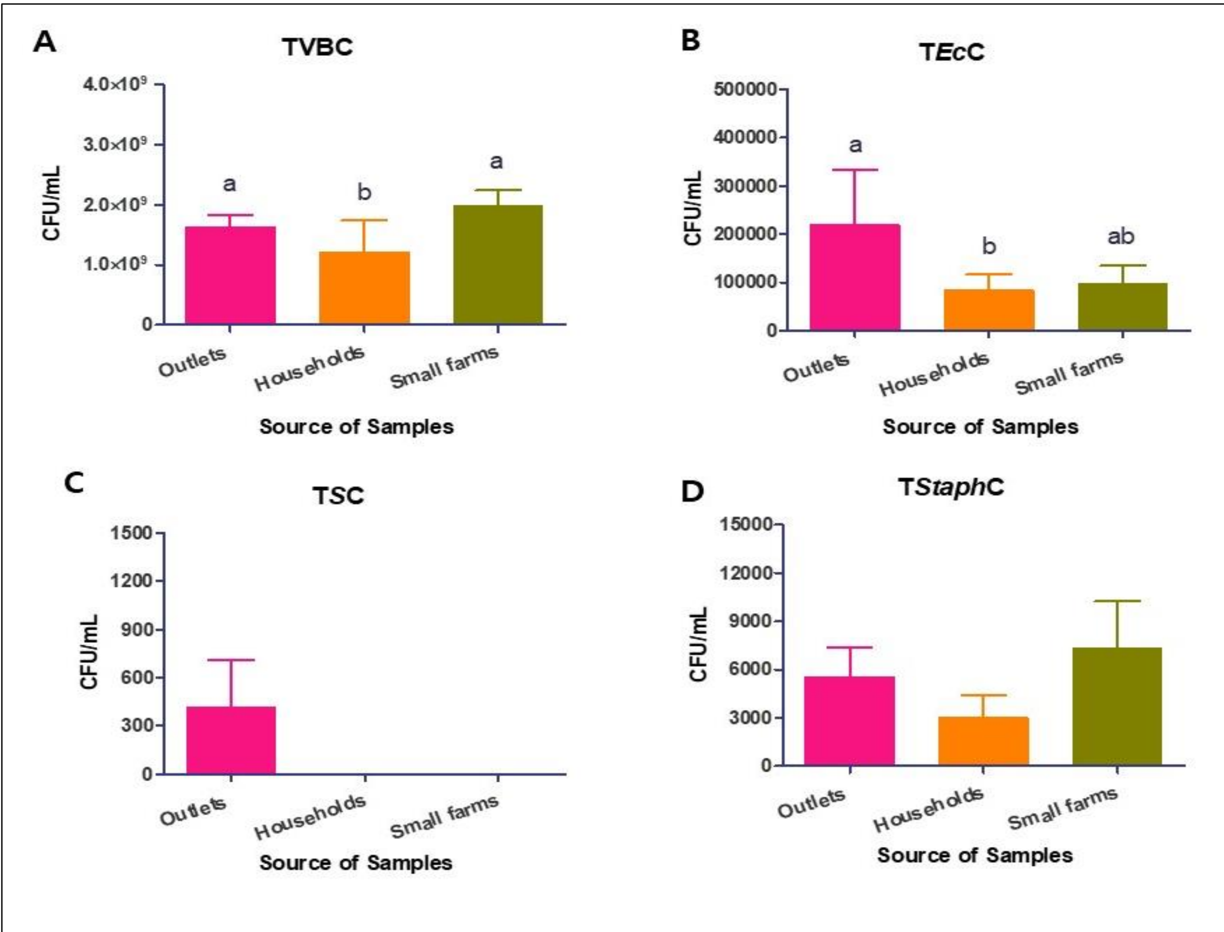


Figure-2: Total Bacterial count. **A**. Total viable bacteria count (TVBC), **B**. Total *E. coli* count (T*Ec*C), **C**. Total *Salmonella* count (T*S*C); and **D**. Total *Staphylococcus* count (T*Staph*C). The bar represent the means, and the error bars represent the standard errors of the mean (SEM). Bars showing different letters represent the values significantly different from each other ($p < 0.05$).

Table-3: Total Bacterial count and prevalence of isolated and identified bacterial species in milk.

Source of collected milk (raw) samples	Total no. of samples tested (n)	Total viable bacterial count (TVBC)		Isolated bacterial species with total count					
				Escherichia coli		*Salmonella spp.*		*Staphylococcus aureus*	
		No. of growth (%)	**Bacterial Count [Mean (SEM) CFU/mL]**	***No. of* positive sample (%)**	**T*EcC* [Mean (SEM) CFU/mL]**	*No. of* positive Sample (%)	**T*SC* [Mean (SEM) CFU/mL]**	***No. of* positive sample (%)**	**T*StaphC* [Mean (SEM) CFU/mL]**
Outlets	27	27 (100)	1.637×10^{9} **a**	8 (29.63)	2.194×10^{5} **a**	2 (7.41)	4.204×10^{2}	10 (37.04)	5.567×10^{3}
Households	15	15 (100)	1.216×10^{9} **b**	1 (6.67)	8.390×10^{4} **b**	0 (0)	-	4 (26.67)	3.000×10^{3}
Small farms	12	12 (100)	1.978×10^{9} **a**	2 (16.67)	9.825×10^{4} **ab**	0 (0)	-	5 (41.67)	7.325×10^{3}
Total	**n=54**	**(54)** (100)		**11 (20.37)**		**2 (3.70)**		**19 (35.18)**	

Note: Different letter indicate the values are significantly different from each other ($p < 0.05$).

4.1.2. Isolation and Identification of the bacterial species

A number of bacterial species were isolated and identified based on the cultural (colony), morphological and biochemical characterization based the previous studies [99].

For *E. coli*, the samples produced a bright, pink-colored, transparent, smooth and raised colonies on MacConkey's agar (due to their ability to ferment lactose); whereas formed greenish/green black colonies with metallic sheen on EMB agar (is the typical feature of *E. coli*); whereas,; after overnight incubation at 37ºC, which suspected as *E. coli* (Figure-3A & Table-4). Then, the Gram's staining results showed a Gram negative, pink-colored, small, rod-shaped organisms that were in single or paired or arranged in a short chain under the light microscope (Figure-4A). All the suspected isolates of *E. coli* fermented five basic sugars with the production of acid and gas. Acid production was indicated by a color change of the sugar media from reddish to yellow, and the gas production was noted by the accumulation of gas bubbles in the inverted Durham's tube. Furthermore, all the isolates of *E. coli* showed positive reaction in Methyl red (MR) and Indole test positive, and Voges- Proskauer (V-P), oxidase, citrate utilization and Urease test negative (Figure-5 and Table-5), which were indicated as *E. coli*. As the overall prevalence, a total of 20.37 % (n=11/54) samples were positive for *E. coli,* while, the highest prevalence was 29.63% (n=8/27) found in the samples collected from the outlets compared to the prevalence of 6.67% (n=1/15) and 16.67% (n=2/12) recorded in the samples of households and small farms, respectively (Figure-2A and Table-3). Then, PCR test was conducted on the isolated strains of *E. coli* for the molecular detection. All the isolated strains of *E. coli* (100%, n=11/11) were positive by PCR amplification using a gene specific primer-sets based on a fragment of 584bp in the *16S rRNA* gene and the results are summarized in Figure-8A and Table-6. Among them, a total of 63.64% (n=7/11) isolated strains of *E. coli* were found to be pathogenic described in the previous studies [93, 94]; whereas, the pathogenic strains of 62.5% (n=5/8), 0% (n=0/1) and 100% (n=2/2) were found in the samples of the outlets, households and small farms, respectively. On the other hand, a total of 36.36% (n=4/11) isolated strains of *E. coli* were found to be non-pathogenic based on the previous reports [93, 94]; whereas, these non-pathogenic strains of 37.5% (n=3/8), 100% (n=1/1) and 0% (n=0/2) were found in the samples of the outlets, households and small farms, respectively. However, the isolated *E. coli* could not serotyped in this study.

Besides, for *Salmonella spp*., the samples produced the characteristics black-centered, smooth and rounded colonies on the Salmonella-Shigella (SS) agar and also formed a non-lactose fermenting, colorless, smooth and transparent colonies on the MacConkey's agar after overnight incubation, which was indicated as *Salmonella spp*. (Figure-3B and Table-4). Subsequently, the Gram's staining results revealed a Gram-negative, pink-colored, small, rod-shaped organisms that were in single or paired or arranged in a short chain under the light microscope (Figure-4B). Based on the cultural and morphological properties, all the suspected isolates of *Salmonella spp*. were subjected to perform the selected biochemical tests. Among the basic five sugars, all the suspected isolates of *Salmonella spp.* fermented maltose, dextrose, and mannitol producing both acid and gas (H_2S) but they did not ferment lactose and sucrose based on the carbohydrate fermentation test (Table-5). The Methyl Red (MR) test and citrate utilization test was positive for all *Salmonella spp.* (Figure-6); whereas the Voges–Proskauer (VP) reaction and indole tests were negative (Table-5), which were indicative of *Salmonella spp*. As the prevalence, a total of 3.70% (n=2/54) samples were showed positive for *Salmonella spp.,* which were only found in the samples collected from the outlets with the highest prevalence rate as of 7.41% (n=2/27) compared to the samples collected from the households and small farms as of 0% (Table-3).

Furthermore, for *S. aureus*, the samples produced yellow colored colonies with yellow zones on MSA agar, and subsequently showed β-hemolysis on 5% sheep blood agar (Figure-3C and Table-4), which were suspected as *S. aureus*. After Gram's staining, these *suspected S. aureus* showed a gram-positive, purple-colored, and cocci-shaped appeared as grapes like cluster under light microscope (Figure-4C). Furthermore, all the suspected *S. aureus* isolates gave the positive reaction in catalase test [indicated that these isolates were well differentiated than the non-catalase producers (*Streptococci*)] (Figure-7) and coagulase test (indicated as pathogenic *S. aureus*) (Table-5). As the prevalence, a total 35.18 % (n=19/54) samples were positive for *S. aureus.* While, the highest prevalence was 41.67% (n=5/12) found in the samples collected from the smallfarms compared to the prevalence of 37.04% (n=10/27) and 26.67% (n=4/15) found in the samples of the outlets and households, respectively (Table-3). All the isolated strains of *S. aureus* (100%, n=19/19) were positive by PCR amplification using a gene specific primer-set based on a fragment of 279 bp in the *nuc* gene (highly specific for *S. aureus)* (Figure-8B and Table-6).

Figure-3: Cultural (colony) characteristics of the suspected bacterial species isolated from bovine raw milk samples onto selective media.

Table-4: Demonstration of the cultural (colony) characteristics of *E. coli, Salmonella spp.*, *and S. aureus* in different selective media

Isolated Bacterial species	MacConkey Agar	EMB Agar	SS agar	MSA	Blood agar
E. coli	Bright pink colored colonies	Greenish colonies with metallic sheen	Pinkish colony	-	-
S. aureus	-	-	-	Yellow colored colonies	β-hemolysis
Salmonella spp.	Colorless colonies	-	Black centered colony	-	-

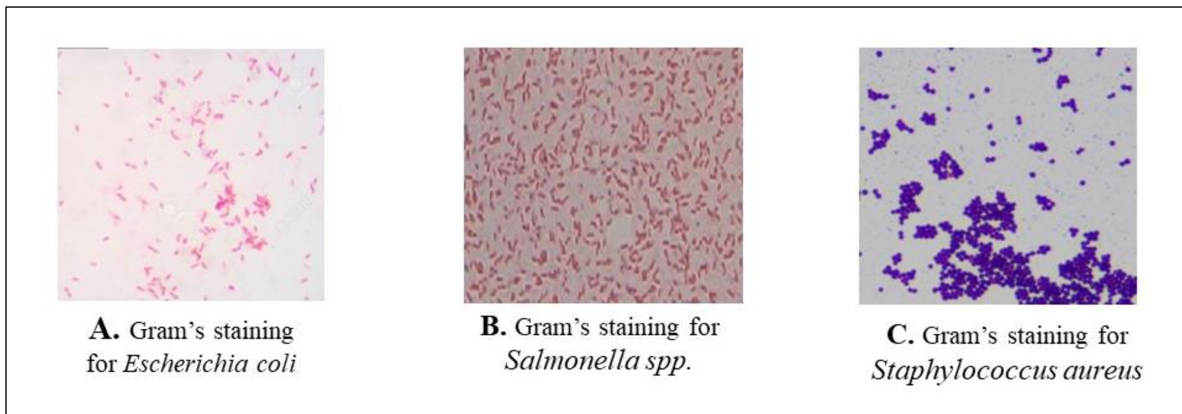


Figure-4: The morphological characterization of different bacterial species from the suspected colonies isolated from bovine raw milk was confirmed by Gram's staining.

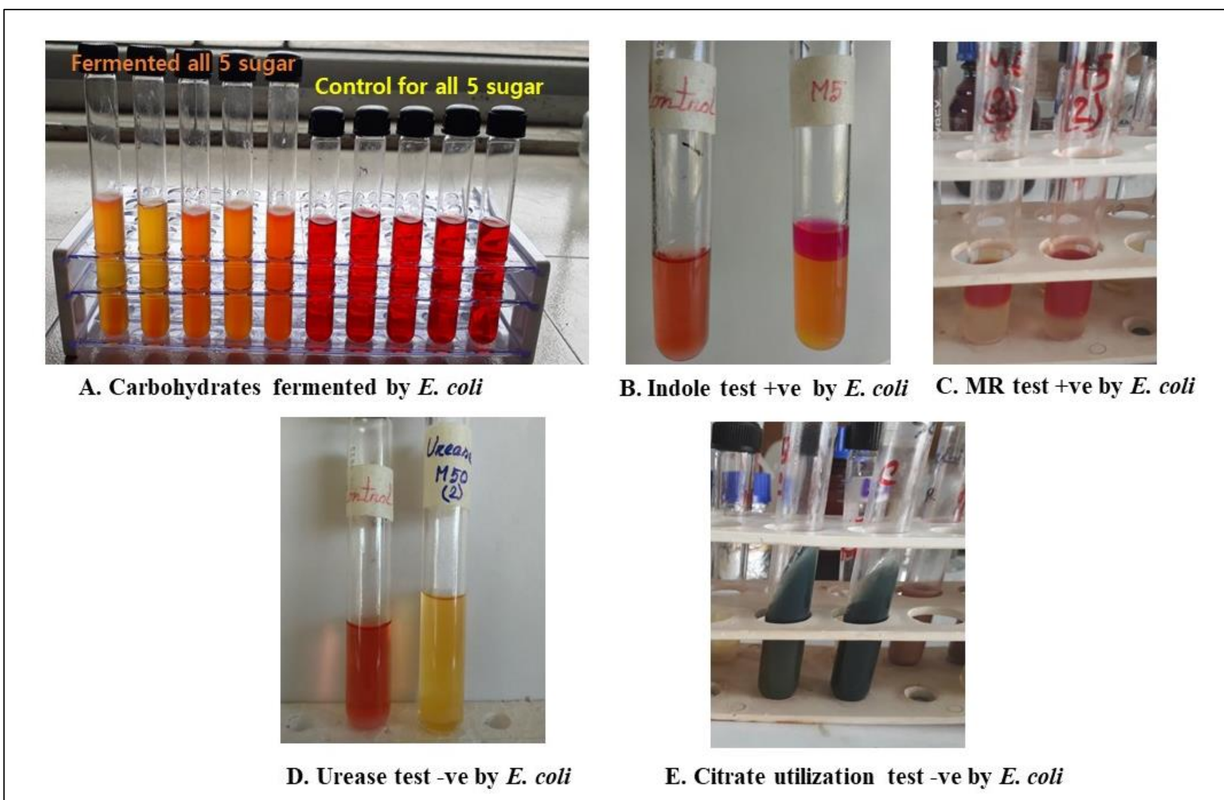
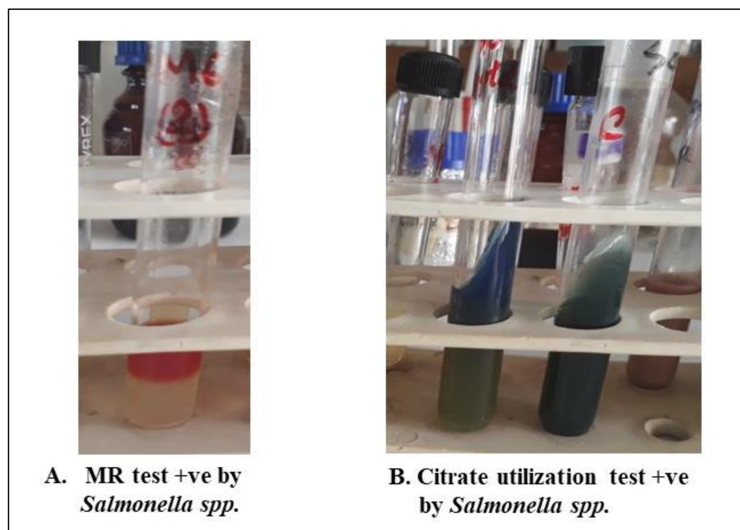


Figure-5: Biochemical characterization of isolated *E. coli.* A. Sugars (dextrose, maltose, lactose, sucrose and mannitol) **fermentation test** (left ~ right)**; B. Indole test; C. MR test; D. Urease test; and E. Citrate utilization test.**

Table-5: Biochemical tests for the isolated bacterial species (*E.coli, Salmonella spp., and S. aureus)* from bovine raw milk.

Name of biochemical tests		***E.coli***	***Salmonella spp.***	***S. aureus***
Carbohydrate (CHO) fermentation test	Glucose	**AG (Acid, Gas)**	**AG**	
	Lactose	**AG**	**-**	
	Maltose	**AG**	**AG**	
	Sucrose	**AG**	**-**	
	Mannitol	**AG**	**AG**	
Methyl red (MR) test		+	+	
Voges- Proskauer (V-P) test		**-**	**-**	
Indole test		+	**-**	
Motility test		+	+	**-**
Oxidase test		**-**		
Citrate utilization test		**-**	+	
Urease test		**-**		
Catalase test				+
Coagulase test				+

Note: "+" = positive, "**-**"= negative.



A. MR test +ve by *Salmonella spp.*

B. Citrate utilization test +ve by *Salmonella spp.*

Figure-6: Biochemical characterization of isolated *Salmonella spp*.
A. MR test, B. Citrate utilization test.

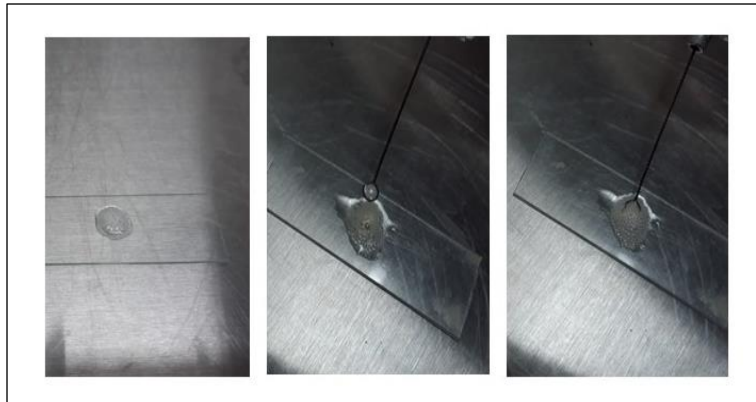


Figure-7: Biochemical characterization (catalase test +ve by *S. aureus*) of isolated *S. aureus*.

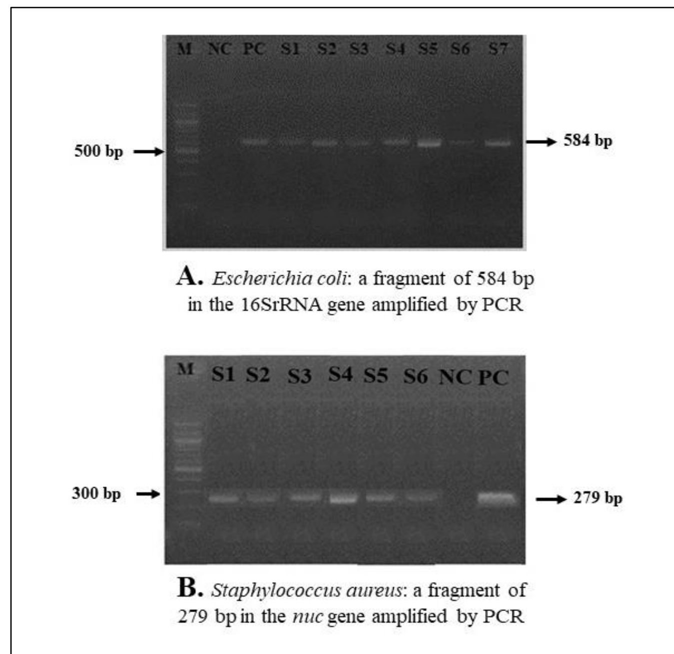


Figure-8: Molecular characterization of isolated bacteria from bovine milk (raw) by PCR. **A.** Detection and differentiation of *E. coli*. Lane M: DNA marker; lane 1-2: NC-PC; Lane 3-5: Test samples 1~3 (PCR amplification with 16*E*1+16*E*3 primer), Lane 6-9: test samples 4~7 (PCR amplification with 16*E*1+16*E*2 primer; **B.** Detection of *S. aureus* by the primer sets based on *nuc* gene.

Table-6: Molecular detection of bacterial isolates from bovine milk (raw) samples by *PCR*.

Source of Collected Samples (raw milk)	***Escherichia coli***				***Staphylococcus aureus***	
	No. of isolates *E. coli* (n)	**PCR (+ve) for Pathogenic (%)**	**PCR (+ve) for Non-pathogenic (%)**	**PCR (+ve) for both type (%)**	**No. of isolates *S. aureus* (n)**	**PCR (+ve) (%)**
Outlets	8	5 (62.5)	3 (37.5)	8 (100)	10	10 (100)
Households	1	0 (0)	1 (100)	1 (100)	4	4 (100)
Small farms	2	2 (100)	0 (0)	2 (100)	5	5 (100)
Total	**n=11**	**7 (63.64)**	**4 (36.36)**	**11 (100)**	**n=19**	**19 (100)**

Note: Pathogenic *E. coli* (PCR amplified the test samples with 16*E*1+16*E*2 primer) and Non-pathogenic *E. coli* (PCR amplified the test samples with 16*E*1+16*E*3 primer) [93, 94].

4.1.3. Antimicrobial susceptibility test

All the isolated bacterial strains were subjected to evaluate the antimicrobial susceptibility profile using antibiotics disk diffusion test against nine antibiotics and the results are summarized in the Table-7. Among 11 isolated strains of *E. coli*, a large number of isolates showed a high resistance to erythromycin (81.82%), amoxicillin (72.73%), tetracycline and ampicillin (63.64%) followed to azithromycin (54.55%), nalidixic acid (45.45%), gentamycin (36.36%), and ciprofloxacin (27.27%) and. In addition, a number of *E. coli* isolates also showed intermediate resistance to nalidixic acid and tetracycline (36.36%), amoxicillin and ciprofloxacin (27.27%), gentamycin and erythromycin (18.18%) followed to azithromycin and levofloxacin (9.09%). However, a big number strains of *E.coli* were also found to be sensitive to levofloxacin (90.90%) followed to gentamycin and ciprofloxacin (45.45%), ampicillin and azithromycin (36.36%) followed to nalidixic acid (18.18%). Besides, within 2 isolated strains of *Salmonella spp*., both showed complete resistance (100%) to amoxicillin, ampicillin and erythromycin followed to azithromycin, nalidixic acid and tetracycline (50.00%), and one isolates also had an intermediate resistance to gentamycin and tetracycline (50.00%). However, both isolated *Salmonella spp*. were found to be highly sensitive to levofloxacin and ciprofloxacin (100%) followed to azithromycin, nalidixic acid and gentamycin (50.00%). In addition, 100% of the isolated *Salmonella* spp. (n=2/2) were found to be resistant against multi-drug (resistant to more than three drugs). Similarly, among 19 isolated strains of *S. aureus*, a large number of isolates showed a high resistance to erythromycin (73.68%), tetracycline (68.42%), nalidixic acid (63.16%), ampicillin (57.89%), azithromycin (52.63%) followed to amoxicillin (47.37%) and gentamycin (31.58%). And, a few number of isolates showed intermediate resistance to tetracycline (31.58%), ampicillin (26.31%), and amoxicillin (21.05%) followed to erythromycin and nalidixic acid (15.79%); and azithromycin, gentamycin and levofloxacin and (10.53%). Though, all the isolates were found to be fully sensitive to ciprofloxacin (100%) and a big number of isolates were also found to be sensitive to levofloxacin (89.47%), gentamycin (57.89%) followed to azithromycin (36.84%), amoxicillin (31.58%), nalidixic acid (21.05%), ampicillin (15.79%) and erythromycin (10.53%). A big number of isolated *E. coli, Salmonella spp*. and *S. aureus* strains were found to be resistant to more than one of the antibiotics used in this study (Table-7). Microorganisms that showed resistance to three or more antibiotics of three different classes were considered to be MDR. The overall prevalence of

MDR traits was approximately of 27.27%, 50% and 42.11% found against *E. coli, Salmonella spp*. and *S. aureus* isolates, respectively (Table-8).

Table 7: Antibiotics-resistance/ susceptibility profile of the isolated bacterial species from bovine raw milk.

Antimicrobial disc		**Disk potency (µg)**	***E. coli* isolates (n=11)** (Percentage %)			***Salmonella spp. isolate* (n=2)** (Percentage %)			***S. aureus isolates* (n=19)** (Percentage %)		
			R	***I***	***S***	***R***	***I***	***S***	***R***	***I***	***S***
Amoxicillin	AMX	10	8 (72.73)	3 (27.27)	0 (0)	2 (100)	0 (0)	0 (0)	9 (47.37)	4 (21.05)	6 (31.58)
Ampicillin	AMP	10	7 (63.64)	0 (0)	4 (36.36)	2 (100)	0 (0)	0 (0)	11 (57.89)	5 (26.31)	3 (15.79)
Azithromycin	AZM	15	6 (54.54)	1 (9.09)	4 (36.36)	1 (50)	0 (0)	1 (50)	10 (52.63)	2 (10.52)	7 (36.84)
Erythromycin	ERY	15	9 (81.82)	2 (18.18)	0 (0)	2 (100)	0 (0)	0 (0)	14 (73.68)	3 (15.79)	2 (10.53)
Nalidixic acid	NA	30	5 (45.45)	4 (36.36)	2 (18.18)	1 (50)	0 (0)	1 (50)	12 (63.16)	3 (15.79)	4 (21.05)
Gentamycin	GM	10	4 (36.36)	2 (18.18)	5 (45.45)	0 (0)	1 (50)	1 (50)	6 (31.58)	2 (10.53)	11 (57.89)
Tetracycline	TET	30	7 (63.64)	4 (36.36)	0 (0)	1 (50)	1 (50)	0 (0)	13 (68.42)	6 (31.58)	0 (0)
Levofloxacin	LEV	5	0 (0)	1 (9.09)	10 (90.9)	0 (0)	0 (0)	2 (100)	0 (0)	2 (10.53)	17 (89.47)
Ciprofloxacin	CIP	5	3 (27.27)	3 (27.27	5 (45.45)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	19 (100)

Table 8: Prevalence of multi-drug resistant (MDR) bacterial species isolated from bovine raw milk.

Source of samples	***E. coli***			***Salmonella spp.***			***S. aureus***		
	Total No. of isolated *E. coli* (n)	***No. of MDR E.coli***	**Prevalence (%)**	**Total No. of isolated *Salmonella spp.* (n)**	***No. of MDR Salmonella spp.* (n)**	**Prevalence (%)**	**Total No. of isolated *S. aureus.* (n)**	***No. of MDR S. aureus.* (n)**	**Prevalence (%)**
Outlets	8	2	25	2	1	50	10	4	40
Households	1	1	0	0	0	0	4	1	25
Small farms	2	0	50	0	0	0	5	3	60
Total	**n=11**	**3**	**27.27**	**n=02**	**1**	**50**	**n=19**	**8**	**42.11**

4.2. **Discussion**

Raw milk in the value chain is dispersed locally to the consumers without controlling strategies to maintain the safety and quality before reaching to the consumers usually practiced in the low and middle income countries including Bangladesh. In addition, the outlets of raw milk are continuously increasing in the local markets found in Bangladesh. These outlets mostly buy raw milk from the local households/or small farms, and collected them together and stored until sale. They retail these milk to the majority of population with poor economic capacity to purchase high quality processed milk, and these people are at high risk of the illnesses from milk-borne pathogens since raw or unprocessed milk works as an excellent medium for different bacterial growth. So, milk could have the major public health concerns due to poses of the pathogenic bacteria for the communities who still consume raw or unprocessed milk [3, 28].

Dairy cattle could be a potential source for the contamination of the farm environment and farm products with *E. coli* and *Salmonella* spp., which normally shed in cow dung [21]. The presence of these bacterial pathogens in milk and milk products are not only the indicator of fecal contamination but also likely as an indicator of poor hygiene and sanitary practices during milking and processing. Furthermore, antibiotic-resistance of these bacterial components could also be transferred to the persons working directly in the farms through contaminated milk, meat, water, soil, direct contact with animals, or their environmental equipment as well cause serious public health hazards [47, 100]. While, *E. coli* is found to be one of the dangerous pathogens in the dairy industries worldwide has significant economic impact [5, 101, 102]. In this study, the overall prevalence of *E. coli* contamination in raw milk samples was 20.37 % (n=11/54). Whereas, the highest prevalence [29.63% (n=8/27)] was found in the samples collected from the outlets compared to the prevalence of 6.67% (n=1/15) and 16.67% (n=2/12) recorded in the samples of households and small farms, respectively (Table-3). The pathogenic strains of *E. coli* can also get access to milk and milk products as they are the most commonly contaminating organisms and considered a reliable indicator of the source of contamination by manure, soil and contaminated water [49, 102]. All the isolated *E. coli* (100%, n=11/11) were also found to be positive by PCR (Table-3). The overall prevalence of pathogenic *E. coli* was found as of 63.64% (n=7/11); whereas, the highest prevalence of pathogenic *E. coli* was 62.5% (n=5/8) found in the samples collected from the outlets compared to the prevalence of 0% (n=0/1) and 100% (n=2/2) recorded in the

samples collected from the households and small farms, respectively (Table-6). On the other hand, a total of 36.36% (n=4/11) isolated *E. coli* were found to be non-pathogenic, which were as of 37.5% (n=3/8), 100% (n=1/1) and 0% (n=0/2) found in the samples of the outlets, households and small farms, respectively (Table-6). The results demonstrated in this study are supported by the previous studies as well; whereas, the prevalence of *E. coli* contamination in raw milk samples were found as of 10% in BAU dairy farm and 20% in a village of Mymensingh, Bangladesh [54], 67.5% [103] in four different dairy farms in Mymensingh, Bangladesh, 8.75% [55] in Malaysia, 26% in vendors followed by in dairy farm (20%) and in house milk (6.6%) [56] in India, 15.3% [51], 27.91% [4], 25% [104] and 33.9% [105] in Ethiopia, 66.67% [52] in Brazil etc. These are indicated that the prevalence of *E. coli* was different in raw milk supply chain possibly of many factors such as topographical location, period of transportation, and size of the farm, environmental sanitation, farm management practices, and also the different methodologies used in the studies. In this study, the highest prevalence was found in the samples of outlets, which could be the reason of longer time of milk transportation to the collectors with high ambient temperature under poor hygienic condition. Additionally, these collectors usually receive milk from several individual dairy farms while there not such well-organized checking systems are practiced for quality milk handling [105]. Antimicrobial drugs are indiscriminately used in animal production system for the prevention and control of infectious diseases that cause the development of resistance against these drugs, particularly in zoonotic bacteria that can easily transfer to human through food chains [102]. Antibiotic resistance is a serious public health issue globally. Antibiotic-resistance of these components could be transferred to the persons working directly in the farm through contaminated milk, meat, water, soil, direct contact with animals, or their environmental equipment [47, 100, 102]. Nine commonly used antibiotics were tested in this study in the antibiogram profiling assay. A study reported that *E. coli* from milk in Bangladesh were found 100% resistant to azithromycin and highly effective against and gentamycin and ciprofloxacin [103]. In this study, a large number of isolates of *E. coli* showed higher resistance to erythromycin (81.82%), amoxicillin (72.73%), tetracycline and ampicillin (63.64%) followed to azithromycin (54.55%), nalidixic acid (45.45%), gentamycin (36.36%), and ciprofloxacin (27.27%) supported by the studies elsewhere [4, 104, 105]. However, levofloxacin was found to be largely effective (90.90%) as antimicrobial agent against *E. coli,* but gentamycin and ciprofloxacin was less effective (45.45%) [71, 106]. Besides, the recorded colony characteristics of *E. coli* isolates found onto EMB agar, MC agar, and SS agar,

as well as Gram's staining, and biochemical properties were also supported by the findings reported elsewhere [107-109].

Salmonella spp. is a ubiquitous pathogen in nature and most important foodborne zoonotic bacteria, which is one of the priority pathogens listed by WHO [110]. Dairy cattle serve as a reservoir of *Salmonella spp.* causes human salmonellosis [111]. *Salmonella spp.* can transmit through contaminated feces of infected cattle and their environment. In recent years, *Salmonella* serotypes have frequently become the issue of resistance to commonly used antibiotics that increases the treatment cost in food animal production and underscores a significant food safety hazard [21]. In the present study, the overall prevalence of *Salmonella spp*. found in milk (raw) samples was 3.70% (n=2/54), while the highest (only) prevalence (7.41%, n=2/27) was in the samples collected from outlets compared to the samples collected from households (0%, n=0/15) and smallfarms (0%, n=0/12). The high prevalence of *Salmonella spp*. in milk samples collected from the outlets could be the reason of milk adulteration or poor handling during milking and distribution, used contaminated utensils as these outlets usually buy milk from different farmers and aggregated together in big containers for retail in the outlets [5]. This data is also supported by the previous studies where they reported that raw milk contamination with *Salmonella spp.* were found with a wide range of prevalence as of 1.85 % [112], 35.71% [113, 114], 20% [54], 45% [103] in Bangladesh,, 1.4% in Malaysia [64], ~ 2.9% in Europe [115], 7.61% in India [61], 9.35% in Ethiopia [17] and in other study in Ethiopia was found 20% [116], 10% in Nigeria [62]. These studies reported a different prevalence of *Salmonella spp.* might be the reason of many factors, such as topographical location, period, size of the farms, environmental sanitation, farm management practices, variation of using different methodologies etc. Though the variation, all these studies reported quite clearly that milk can be a significant source of foodborne pathogens for humans. Furthermore, the above demonstrated results indicate clearly that milk can get also spoiled with *salmonella spp.* when the post-processing contaminations are present in the farms commencing from the milking system to the supply chains [5, 59]. The CDC reported that dairy cattle are the vital reservoir for *Salmonella* and highly associated with human salmonellosis as well [117]. The US National Animal Health Monitoring System reported that about 5.4% of dairy cows shed Salmonella and about 27.5% of dairy operations had at least one cow shedding Salmonella [118]. *Salmonella spp.* was isolated from dairy cattle at all ages throughout the production process [119]. Moreover, alongside the contamination of milk, these isolated

Salmonella s*pp.* were found to be highly resistant (100%) to amoxicillin, ampicillin and erythromycin followed to other commonly used antibiotics (50%) in this study (Table-7). These antibiotics are extensively used for the treatment of infections in animals and humans [17, 62, 113]. Though the isolated *Salmonella* spp. strains were also found to be highly sensitive (100%) to levofloxacin and ciprofloxacin, which was also supported by the previous studies [17, 62, 113]. In addition, multi-drug resistant *Salmonella* isolated from milk samples were also found in different studies [17, 112, 120].

S. aureus, a facultatively anaerobic, non-spore forming cocci, an usual causative agent of bovine mastitis [69] in dairy farms worldwide, which has a wide range of pathogenicity and virulence factors like staphylokinase (SAK), hyaluronidase, coagulase and haemolysin [65], and causes superficial skin infections to life-threatening diseases [121]. *S. aureus* may get entry to milk samples either by direct excretion from udders with clinical and subclinical staphylococcal mastitis or by environmental contamination during the handling and processing of raw milk [66, 67]. In this study, the overall prevalence of *S. aureus* found in milk (raw) samples was 35.18% (n=19/54), while the highest prevalence was 41.66% (n=5/12) found in the samples collected from smallfarms compared to the samples collected from outlets (37.03%, n=10/27) and households (26.66%, n=4/15). The thermostable nuclease-encoding *nuc* gene is highly specific for *S. aureus* while nuclease production could be suggested as an indicator of potentially pathogenic staphylococci [122]. PCR amplification using gene specific primer of the *nuc* gene on isolated strains from raw milk samples that yielded a 279 bp amplicon of genomic DNA (Figure-8) has indicated that the isolated strains are *S. aureus.* All the isolated strains of *S. aureus* (100%, n=19/19) were positive by PCR amplification (Table-6). This result is supported by many previous studies wherein they reported that raw milk samples were found frequently contaminated with *S. aureus*, which are highly associated with subclinical mastitis with a varying range of prevalence. They reported that the overall prevalence rate of *S. aureus.* contamination was found as of 75% in retail outlets of raw milk and 29% in bulk milk [73] in South Africa, 45% [75], 38.6% [68] and 23.4% [70] raw milk in Ethiopia, 61% raw milk [74] in India, 75% raw milk [72] in Egypt, 35.29% [71] and 30% [54] raw milk in Bangladesh. Contamination of raw milk with antibiotic resistant bacteria may pose serious threat to the consumers leading to public health hazard. In Bangladesh, the dairy sectors are progressively extended which lead to widespread use of antibiotics for the betterment of health and productivity of animals. Consequently, the pathogenic *S. aureus* causing mastitis has

concerned the issue as of becoming more resistant to the commercially available antibiotics. In this study, a large number of isolates of *S. aureus* showed highly resistance to erythromycin (73.68%), tetracycline (68.42%), nalidixic acid (63.16%), ampicillin (57.89%), azithromycin (52.63%) followed to amoxicillin (47.37%) and gentamycin (31.58%) supported by the previous studies [69, 106, 123]. These antimicrobial residues such and other antibiotics could be found in milk as leftovers after the drugs have been administered in animals. These residues in raw milk are often due to farmers failing to adhere to the specified milk withdrawal periods after antibiotic use to sick lactating cows, illegal or extra label use of drugs and incorrect dosage levels and route of administration [84, 85]. Though, all the isolates were found to be sensitive to ciprofloxacin (100%) and a big number of isolates were also found to be sensitive to levofloxacin (89.47%), gentamycin (57.89%) followed to azithromycin (36.84%), amoxicillin (31.58%), nalidixic acid (21.05%), ampicillin (15.79%) and erythromycin (10.53%). All recorded colony characteristics of the isolated *E. coli*, *Salmonella spp*., and *S. aureus* strains found onto the different selective media, as well as the staining and biochemical properties were also supported by the findings reported elsewhere [107-109].

CHAPTER 5

SUMMARY AND CONCLUSION

This study was conducted together at the laboratory of Pathology and Environmental Biotechnology under the department of Pathology and APMA, respectively, Sher-e-Bangla Agricultural University (SAU), Dhaka-1207; and the laboratory of Animal Biotechnology at National Institute of Biotechnology (NIB), Savar, Dhaka. This study was carried out between the periods of October'2019 to November'2020. A total of 54 bovine raw milk samples were collected from local market, small farms and household to assess the quality of raw milk in our country. Raw milk posed pathogenic organisms has played serious concern in terms of public health safety issue. After collection samples, total viable bacterial count was done and subsequently a couple of pathogenic bacteria was also isolated and characterized based on their colony characteristics (physical properties), morphological characterization, biochemical properties and also molecular detection and characterization was done by PCR test. Side by side, antibiogram profiling was also conducted on the isolated bacterial species to see the antibiotic sensitivity and resistance pattern against those isolated bacteria. Based on the data demonstrated in this study, it could be concluded that proper hygienic maintenance of both milking and farm husbandry practices might reduce the chances of contamination. Besides, it is highly suggested that there is the urgency of increase adequate public awareness about the importance of hygienic milk production and also consumption of pasteurized/ processed milk to prevent milk borne infections.

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