ASSESSMENT OF BACTERIOLOGICAL QUALITY AND HYGIENIC PRACTICES OF RAW MILK FROM DAIRY FARMS AND VENDOR SHOPS IN DINAJPUR DISTRICT OF BANGLADESH

A THESIS

BY

AHMED ALI FARAH
REGISTRATION NO. 1905307
SEMESTER: JANUARY-JUNE, 2020
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MASTER OF SCIENCE (M.S.)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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JUNE, 2020

DEDICATED

TO

MY BELOVED PARENTS, SISTER AND BROTHERS

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ABSTRACT

Milk is a highly nutritious food to human and animals, but serves as an excellent growth medium for a wide range of microorganisms. The present cross sectional study was conducted from January to June 2020 with objective of assessing the hygienic practices and bacteriological quality of raw milk from dairy farms, and vendor shops in different settings of Dinajpur district. During the assessment a total of 45 respondents were interviewed to collect the required information from farmers and vendors about owner and workers 'awareness about pre and post-harvest milk handling practices. The Physico-chemical analysis parameters were considered to evaluate the quality of milk samples. The organoleptic properties of milk such as color, flavor and texture were evaluated with the help of eyes, nose and mouth. Similarly, to clot on boiling test and Alcohol test was employed to test the quality of milk. Also, Methylene blue reduction test was used to grading milk from two sources and in all milk sample from vendor shops were grading as poor quality milk compared the milk from dairy producers. At the same time, milk samples were collected for laboratory analysis including bacterial load assessment with isolation and identification. The mean value of bacterial load was found higher in vending shops ($8.1x10^9$ (log 9.9 CFU/mL) followed 6×10^6 (log 6.4) CFU/mL Within dairy farms the mean values of bacterial load were highest value in small scale farms. Among current the milk samples collected, (48%) of the farm settings and (60%) of milk vending shops were graded as poor quality. In the course of this study, out of 45 samples 30 were found to be positive among bacteria belong to the five genera isolated. Among them the most frequent isolate was of *Staphylococcus aureus* (33.3%), *E. coli* (23.3%), *Salmonella* spp. (20%), *Klebsiella* spp. (14.2%) and *Shigella* spp. (10.7%). Antimicrobial susceptibility pattern showed that all the isolated bacteria were sensitive to Ciprofloxacin (CIP), Chloramphenicol (C), Levofloxacin (LE) followed by Cefixime(CFM) but resistant to Ampicillin(AMP) Azithromycin, Tetracycline (TE) which were showed very poor efficacies resistance on many isolates. Only Gentamycin (GEN) was intermediate antibiotic to *Shigella* spp. According to international standards of raw milk quality both of the above counts found to have values above the upper limits. The quality of milk consumed in the study area was found inferior quality according to the standard level. Thus, awareness should be strengthened on hygienic methods of production, handling, transportation and distribution of milk among all level of producers, milk vending shops and consumers in the town.

Key words: Dinajpur, Dairy farms, Vendor shops, Milk, Bacteriological quality.

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LIST OF ABBREVIATIONS

FDA	: Food and Drug Administration
ICMSF	: International Commission on Microbiological Specifications for Foods
ISO	: International Organization for Standardization
ILRI	: International Livestock Research Institute
IDF	: International Dairy Federation
DLS	: Department of Livestock Services
CLSI	: Clinical and Laboratory Standards Institute
TVC	: Total Viable Count
TCC	: Total Coliform Count
TSC	: Total *Staphylococcus* Count
TSSC	: Total *Salmonella* and *Shigella* Count
DLS	: Department of Livestock Services
BBS	: Bangladesh Bureau of Statistic
ILFR	: Institute of Land and Food Resources
BSTI	: Bangladesh Standards and Testing Institution
BER	: Bangladesh Economic Review
CFU	: Colony Forming Units
KOH	: Potassium hydroxide
Log	: Logarithms
SSA	: *Salmonella- Shigella* agar
MSA	: Mannitol Salt Agar
BPW	: Buffer peptone water (BPW)
BPA	: Bair pairk agar
SPSS	: Statistical package for social sciences
GDP	: Gross Domestic Product
AOAC	: Association of Official Analytical Chemists
HSTU	: Hajee Mohammad Danesh Science and Technology University

CHAPTER I

INTRODUCTION

Milk is a highly nutritious food that serves as an excellent growth medium for a wide range of microorganisms (Pathot, 2019). Globally, billions of people consume milk every day. Milk and milk products have great nutritional qualities and hence, their consumption is increasing worldwide. Due to its highly nutritious nature of the milk, it serves as an excellent growth medium for a wide range of microbes. Microbial contamination of milk is a universal problem (Regasa *et al.,* 2019). Milk contains many a complex mixture of fat, protein, carbohydrates, minerals, vitamins, and other miscellaneous constituents dispersed in water, making it a complete diet. Milk is a sterile fluid when secreted into alveoli of udder; however, after secretion bacterial contamination can generally occur from three main sources; within the udder, outside the udder and from the surface of equipment used for milk handling, transport and storage could serve as source of contamination and causing several diseases outbreaks (Oumer *et al.*, 2017). Similarly, the surrounding air, soil, feed, grass, feces, chemicals used during treatment of animal and from water used for adulteration by unscrupulous and unfaithful workers/sellers are also possible sources of contamination (Abebe and Zelalem, 2012). Milk and milk products have a high value in feeding the population in both rural and urban areas as well milk have a refreshing, potable, economical and nutritious food for human being (Torkar and Teger, 2008).

The demand of consumers for safe and high-quality milk has placed a significant responsibility on dairy producers, vendor shops, retailers and manufacturers to produce and market safe milk and milk products (Mennane *et al.,* 2007). For this reason, bacteriological quality of milk is important to in ensuring the safety milk to the consumer and profit to producers (Korma *et at.*, 2018). The cow health status and its environment, uncleaned and non-hygienic milking equipment, and unhygienic milk workers could serve as sources of contamination. It is a vital type of food for over 6 billion human beings all over the world and a major contributor to food security as it alleviates poverty and mitigates malnutrition (Belewu, 2006).

Besides its benefit, it serves as an excellent growth medium for a wide range of microorganisms. The microbial contamination of milk products is a universal problem.

(Walstra *et al.,* 2006). The nature of milk and its chemical composition renders it one of the ideal culture media for microbial growth and multiplication of diverse microorganisms resulting in its early deterioration (Woldemariam and Asres, 2017). Raw milk is an important vehicle for the transmission of milk borne pathogens to humans, as can be easily contaminated during milking and handling (Addo *et al.,* 2011). A recent study in Bangladesh showed many farmers do not properly clean teats and equipment prior to milking. This practice can clearly lead to the spread of contagious pathogens (Pal *et al*., 2012).

A variety of pathogenic bacteria have been isolated from raw milk including *Staphylococcus sp., Escherichia coli, Salmonella* sp., *Shigella* sp., *Streptococcus* sp., *Klebsiella* sp., *Mycobacterium, Clostridium botulinum, Brucella, Corynebacterium, Acinetobacter* sp., *Lactobacillus* sp., *Corynebacterium* sp., *Streptococcus* sp., *Listeria* sp., *Lactobacillus* sp., *Enterobacter* sp. *Pseudomonas* sp., *Yersinia enterolitica* and *Listeria monocytogenes* (Swai and Schoonman, 2011). The presence of these pathogens in raw milk is a major public health concern, especially for those individuals who drink raw milk frequently). Milk contamination by zoonotic pathogens is often natural but can occur through handling milk in unhygienic conditions (Ali, 2010). Consequently, regular assessment of the bacteriological quality and safety of milk at all levels of value chain is important to safeguard the health of the community (Reda *et al.,* 2014).

Bangladesh is one of the developing countries, urban and peri-urban dairying constitutes an important sector of the agricultural production system. Trend of rapidly increasing human population together with growing urbanization creates increased demand for milk and milk products (DLS, 2018). Milk is a key contributor to improving nutrition and food security particularly in developing countries. Improvements in livestock and dairy technology offer significant promise in reducing poverty and malnutrition in the world (Hemme and Otte, 2010). Livestock is a vital component of agriculture and contributing about 3.47% to gross domestic products (GDP) and this is also contributing more than 6% of total foreign exchange earnings in Bangladesh (BBS, 2016). In Bangladesh, cattle, buffalo and goat are considered as dairy animals. Out of total milk production, about 90% share is from cattle, 8% from goat and the remaining 2% from buffalo (DLS, 2015). According to the data of Department of livestock serve (DLS), there are about 242.38 million cattle, 262.67 million goats, 35.37 sheep million and buffaloes in 14.86 million respectively in the country (DLS, 2018).

Additionally, Bangladesh possess more than 70% of the dairy farmers are smallholders producing 70–80% of the country's total milk. The demand of milk per capital is 250 ml/day while the availability of milk per capital is 165.07 (ml/day/head, which increased per capita by 4% however, the annual production of milk is 99.23 million tons in Bangladesh (DLS, 2018-19). So that the demand for dairy products in the country exceeds supply, which is expected to induce rapid growth in the dairy sector. Factors contributing to this include rapid population growth, increased urbanization and expected growth in incomes creates an increased production demand for milk and milk products (BER, 2018).

The consumption of raw milk and its products is common in Bangladesh (BER, 2018), which is not safe from consumer health point of view as it is good media for the growth of microorganisms. Provision of milk and milk products of good hygienic quality is desirable for consumers. This is one reason why milk quality testing and quality control include hygiene as well as microbial qualities in addition to testing for alcohol, methylene blue reduction test content and heat stability is essential steps (Yilma, 2010).

Though, milk is the most easily contaminated and perishable product of animal origin. This is mainly due to its high nutritional value creating an ideal medium for the growth of spoilage as well as pathogenic microorganisms. The handling and safety of milk and milk products is of great concern around the world. This is especially true in developing countries where production of milk and various dairy products takes place under rather unsanitary conditions and poor production practices *(*Amentie *et al.,* 2016**)**. Even though many countries have milk quality regulations, including limits on the total number of bacteria in raw milk, to ensure the quality and safety of the final product. However, hygienic quality control of milk and milk products in Bangladesh is not usually conducted on routine basis. There is little information on the bacterial quality of raw milk especially in the pastoral and Agro-pastoral area of Bangladesh, where milk consumption plays a significant role in the diet of the community (Ahmed *et al.*, 2019).

Consequently, there is limited data on hygienic practices throughout the dairy production system in Bangladesh and standard milking procedures do not exist. A recent study in Bangladesh showed many farmers, vendor shops do not properly clean teats and storage equipment prior to milking and selling. In Bangladesh milk is subjected to more contamination during long distance transportation under high ambient temperature and

without cold-chain facility and improper handling of milk can exert both a public health and economic constraints thus requiring hygienic vigilance throughout the milk value chain (Swai and Schoonman, 2011).

Despite milk placed in an important role in the nutrition of consumer's as well as in the nutrition and as an income of producers, limited work so far undertaken on the assessment of bacteriological quality and hygienic practices of raw milk from dairy farms and vendor in Dinajpur town. In addition, there has been no established milk quality control system. Therefore, it is important to establish milk quality standards that focus on food safety measures in order to improve public health and eventually to check the quality of milk.

Therefore, present study is undertaken with the following objectives.

Research Objectives

1. To assess bacteriological quality and hygienic practices of raw milk at the study dairy farms and vendor shops.
2. To isolate and identify the major bacterial species from milk samples having high bacterial load among dairy farms and vendor shops.

CHAPTER II

LITERATURE REVIEW

2.1. Bacteriological Quality of Milk and Hygienic Practices

Megersa *et al.* (2019) Assessed that bacteriological quality of milk refers to the cleanness of milk. This is defined by a number of bacteria present in milk. The high bacterial count as well as the presence of pathogenic bacteria in milk not only degrades the milk quality and shelf-life of milk or milk related products but also poses a serious health threat to consumers. Milk being a wholesome food with high nutritive value is often prone to early contamination and spoilage if not handled properly. The microorganism may originate from the cow, utensils, personnel or the environment. The handling and hygienic practice of milk strongly affects the quality of the finished product. This hygienic of milk deals with milk quality and hygiene under smallholder dairy production system. Consequently, milk quality and hygiene activities are pay vital role under dairy production system, market centers of milk, vendor shops and should not be under estimated. Milking activity, transportation, storage and processing activities can have determined milk quality and hygiene. The bacteriological quality of cow milk from dairy farms has received a big attention to the world. For this reason, utilization of both raw untreated milk and raw milk has frequently been associated with food-borne illness. Especially, developing countries included Bangladesh are mostly affected by food borne infections because of the prevailing poor food handling and sanitation practices, inadequate food safety regulatory systems, lack of financial resources to invest in safer equipment, and lack of education for food-handlers can result contamination.

Nuhriawangsa *et al.* (2019) were detailed an efficient hygiene program should begin at the farm and hygienic production of milk is important for the safety of consumers. Essentially milk hygiene practice has interests in preventing the transmission of disease from animals to man, preventing the transmission of communicable diseases of man through milk, preventing diseases or physical defects that may arise from malnutrition and improving the nutritional status of man in general and of infants, children, and mother in particular. In Bangladesh, there is no standard hygienic condition followed by producers during milk production. The hygienic conditions are different according to the production system, adapted practices, level of awareness, and availability of resources.

The good hygienic quality of milk for consumers requires good hygienic practices and the levels exercised during milk handling practices. To ensure that raw milk remains fresh for a longer time, you need to practice good hygiene during milking and when handling the milk afterwards. So the production of quality milk is a complicated process.

Bekuma and Galmessa. (2018) have stated that hygienic practice of the milk with respect to quality has received a great concern in developing countries, where production of milk and various milk products usually takes place under unsanitary conditions and poor production. Thus emerging economies have often poor hygiene practices in traditional milk and dairy production all over the world. It is essential to produce best quality raw milk in the dairy farm in order to manufacture milk products of acceptable quality. In Bangladesh milk produced at smallholder farm is marketed without quality control measures. Hygienic control of milk and milk products is not usually conducted on routine bases. Apart from this, door-to-door raw milk delivery in the urban and peri-urban areas is commonly practiced with virtually no quality control at all levels. So, consumers need clean, wholesome and nutritious food that is produced and processed in a sound sanitary manner and free from pathogens. Preventing the growth of contaminating bacteria in milk involves limiting contamination levels, cooling immediately after milking, and maintenance of cold storage temperatures. Limitation of bacteria primarily includes cleaning, sanitizing and drying cow's teats and udder before milking and using sanitized milking equipments. The common predisposing factors of milk contamination by microorganisms are milking environment, cow's udder, milking personnel, milking equipment and storage milk transportation and water.

Velázquez-Ordoñez *et al.* (2019) indicated that the maintaining of milking house environmental and sanitary condition of the milking area is important for the production of good quality milk. The milking barn should have a good floor that is easy to clean and drain. There should be good ventilation, lighting, and facilities for manure disposal and washing cows. A good supply of clean water is required. Clean regularly the milk house. Dirty milking places tend to breed flies, which may fall in milk causing contamination and thus spoilage may occur. When a cow urinates or defecates in the course of milking some of its urine or dung particles may drop into the milk. Milk quality should not be ignored at all stages of the dairy value chain from farm to table. As the bacterial quality of raw milk is important to product shelf-life, flavor and product

yield, it is important that dairy enterprises should strive to obtain the highest quality raw material possible from their own farm as well as their suppliers. It is therefore essential to produce best quality raw milk in the dairy farm in order to manufacture milk products of acceptable quality. In Bangladesh milk produced at smallholder farm is marketed without quality control measures. Hygienic control of milk and milk products is not usually conducted on routine bases. Hence, quality milk production is necessary for fulfilling consumers' demand.

Talukder *et al.* (2019*)* examined that milk from the udder of a healthy cow contains very few bacteria. Cleaning the udder of cows before milking is one of the most important hygienic practices required to ensure clean milk production. This is important since the udder of the milking cows could have direct contact with the ground, urine, dung and feed refusals. Cleaning and removal of soil particles, bedding material and manure from the udder and flanks is necessary to prevent the entry of many types of bacteria into the milk. Udder washing with clean water and drying using hand towels reduces milk contamination by transient bacteria located on the udder. Special care must be given to the cloths used for cleaning the udder. The re-use of cloths for cleaning and sanitizing may result in recontamination of the udder. It is therefore recommended that separate cloths be used for cleaning and sanitizing and, if possible, each cloth should be used for one cow only. Not washing the udder before milking can impart possible contaminants into the milk. A maximum reduction of teat contamination of 90 % can be achieved with good udder preparation before milking. This depends on the initial level of contamination and the way of udder preparation. So with high initial contamination levels this 90 % reduction might not be reached. The health of the cow and its environment, improperly cleaned and sanitized milk handling equipment, and workers who milk cows come in contact with milk due to a number of reasons could serve as sources of microbial contamination of milk. Consequently, good hygiene is essential whether the animals are milked by hand or machine.

Pathot. (2019) reported that the health of milker and personnel handling milk is of considerable importance of milk quality and hygienic matter. The health of milker and personnel handling milk, is of considerable importance. The milker should be healthy, clean, have short and clean finger nails and wear clean clothes. He or she should milk the cow paying full attention to the task and not smoke, spit or cough while milking. The cow should be milked as quickly and completely as possible, and preferably always

milked by the same person. By calm and gentle handling, touching the cow, talking to her and maintaining routine actions during milking, she will feel at ease. Wash hands with clean water and soap before milking is key essential. The milker may contribute various organisms including pathogens especially when they are careless, uninformed, or willfully negligent, directly to milk. Organisms may drop from hands, clothing, nose, and mouth and from sneezing and coughing. It is important for milk men to be in good health so that they can be a source of infectious diseases such as tuberculosis. Thus, the effective handling practice during milking is important and necessary element to produce safe and suitable milk and milk products.

Bekuma and Galmessa. (2018) evaluated that many milk equipments, milking utensils, and storage tanks are the major source for bacterial contamination of raw milk. Milk producers and handlers use plastic containers along the informal value chains, including jerry cans and buckets during milk handling practices such as milking, farm bulking, and its distribution. The plastic containers in comparison with aluminum cans are cheap; therefore, they have widespread usage by the dairy actors in emerging economic world for milk handling. Plastic jerry cans are difficult to properly clean and this result in unhygienic handling, which contributes to milk quality deterioration compared with the use of aluminum can stainless steel and that are easy to clean are mostly preferred. Using plastic jerry cans for milk handling has been reported in many emerging economies. So, plastic containers are not recommended for handling milk as they are known to be vulnerable to bacterial contamination. The equipment used for milking, transportation and storage determine the quality of milk and milk products. Of this, types of milk containers especially during transportations of milk to the selling point greatly determine the qualities of milk. Producers need to pay attention for the type as well as cleanliness of milk equipment. Unclean milk utensils play a vital role in affecting the quality of milk. Thus, it is important that the utensils are properly cleaned and dried before and after milking.

Wanjala *et al.* (2018) studied that an effective milk cooling and storage is essential to ensure the quality of the product and hygiene. The rate of cooling and milk handling procedures during and after milking are also important in determining the quality of milk. So, cooling milk is essential to prevent an increase in bacterial numbers and spoilage of the milk. If cooling facilities are lacking on the farm, the milk should be brought to the collection centre, vendor shops at least two hours after the start of milking.

Milk when it emerges from a healthy udder contains only a very few bacteria. Milk contains a natural inhibitory system, which prevents a significant rise in the bacteria count during the first 2 - 3 hours. If milk is cooled within this period to 4℃, it maintains nearly its original quality and remains good for processing and consumption. However, in rural areas it is hardly possible to achieve this. Simple alternatives are putting the container with milk in water or placing a moist cloth around the metallic milk containers. During milk storage having limited the number of bacteria entering milk during milking, it is essential that contamination from equipment situated between the cow and the refrigerated storage unit is kept to a minimum. Prompt cooling or chilling of milk at a temperature of 5°C or below is necessary to minimize microbial growth and prevent milk quality deterioration during handling, storing and transporting before the raw milk being processed. In order to facilitate bulking of raw milk supply and transport the incoming milk, refrigeration facilities are provided at points of collection and transport means to maintain the temperature as much as possible. In the tropical or subtropical countries included Bangladesh with high ambient temperatures, lack of refrigeration facilities at the farm and household level imply that raw milk will acidify very fast. Therefore, the collection systems must be designed to move the milk to the cooling and/or processing center in shortest possible time.

Gashaw and Gebrehiwot. (2018) were analyzed that the bacteriological quality of milk is affected by time taken in milk transporting. As bacterial load of milk increases during transportation and if the transportation equipment is not appropriate the bacterial counts increase causing spoilage before milk reaches its destination. Accordingly, milk must be transported from producers to consumers. Because milk is a very perishable product, transporters must ensure high levels of hygiene, speedy transport and careful handling. The longer the time taken to transport the milk, the more likely the milk is going to spoil. Milk transported and handled under such conditions would have poor quality and may contain pathogenic microorganisms of public health concern. The bacterial quality of milk is determined by the distance transport between the farm and until the consumers, the time lapsed during transportation of milk from the farm to the consumers and the temperature of milk during transportation which gives bacteria the chance to adapt and grow in this nutritious liquid. So that milk must be transported from producers to processors to consumers. Because milk is a very perishable product, transporters must ensure high levels of hygiene, speedy transport and careful handling. This will minimize

losses due to spillage and spoilage, avoid contamination of milk by pathogens, and also increase the profits from your milk transportation business. Then, these containers are easier to handle especially transporting by Motorbikes, on foot, by car and Auto-rickshaw which is the most common mode of milk transportation in emerging economies such as Bangladesh studies have found that milk producers who use plastic containers have high coliform counts in their milk. The safety of dairy products with respect to food-borne diseases is a great concern around the world.

Oumer *et al.* (2018) reported that water used serves as primary sources of microorganism's contamination. If Water is obtained from an open water supply care should be taken to prevent drainage that may contain human feces and other contaminants gaining entry into the source. The previous results have shown that bovine feces are not an important source for coliforms contamination in raw milk but the water used in sanitation and the milking environments are considered as one of the critical source. Lack of enough water sources for cleaning the milk handling equipments may result in milk remaining on the surfaces of the equipment, providing nutrients for bacterial growth, and then milk contamination. Hence, usage of low quality and unhygienic water during sanitation procedures can indirectly contaminate the milk.

Lemma *et al.* (2018) tested that cleaning and disinfections of equipment after each milking is important for reduction of contamination of milk from the equipment and with rinsing, about 10 % of the number of bacteria found in milk can be reduced. In most cases not all bacteria are removed and killed during cleaning and disinfections. First wash the utensils with hot water and a detergent. A clean brush with good bristles should be used, which is only designated for the cleaning of the milk equipment. Detergents are necessary to clean milking equipment effectively before disinfection. The effectiveness is increased when warm water is used. This helps to displace milk deposits and to remove dirt, dissolve milk protein and emulsify the fat. Disinfectants are required to destroy the bacteria remaining after washing and to prevent these subsequently from multiplying on the cleaned surfaces. Hence, using detergents/disinfectants as part of the cleaning process at temperatures between 45-60° C in manual cleaning and for cold milk lines, storage tanks and tankers. Despite bacteria may enter milk through the udder and most of the organisms in raw milk are contaminants from the external surface of udder, milking utensils and handlers. Various types of equipment and utensils, such as milking machines, pails, cans and milk churns are used in handling milk on the farm. In order to

reduce contamination of milk, utensils used for milking should be rinsed, cleaned using detergent and disinfected immediately after use. As well as personnel connected with the milking and handling of milk should be healthy and should acknowledge the importance of cleanliness by wearing clean overalls and wash hands with soap and clean water prior to milking.

Abunna *et al.* (2017) investigated that bacterial quality tests of raw milk from different farmer groups and operators of milk collection points and centers need systems of quality control for the milk they receive from individual farmers. This enables segregation of poor-quality milk at collection centres. Several simple tests, if carried out judiciously and consistently, will enable the milk collection centre to ensure that only good quality milk is accepted for onward transportation to milk processing factories, milk bars or retailers of raw milk in urban centers. These tests are routinely carried out at milk collection points to ensure that only milk of acceptable quality is received. Because the consumer has no way of knowing whether or not the milk delivered to the home or purchased in the store is contaminated, a number of standard tests are carried out periodically on milk in that area. Usually during testing, only a small amount (sample) of milk from each container is assessed. These tests are less precise criterion for classifying raw milk according to its bacteriological quality. This calls for the need to periodically verify the quality of milk with more precise bacteriological tests. The tests commonly employed to determine the quality of milk include dye-reduction (Methylene blue reduction and resazurine reduction), Alcohol test, Standard plate count, Coliform count, Somatic cell count, Titrable acidity, and phosphatase tests. Using these have encouraged the producers and center distributors of milk to improve the hygiene conditions, storage and transportation of the milk in order to avoid rejection of the product on delivery to the collection centre.

Abdirahman *et al.* (2017) investigated that good milk hygiene produces dairy products that are safe for human consumption, and that have good keeping quality. On the other hand, poor milk hygiene leads to spoiled products, food-borne diseases and unsatisfactory or declining product image. This all leads to reduced consumer confidence in the integrity of the dairy value chain. Milk hygienic quality, on the other hand, refers to the levels of various contaminants in milk, whether bacterial, chemical or any other adulterants those are detected. Good milk hygiene produces dairy products that are safe for human consumption, and that have good keeping quality. A quality control

system will test milk and milk products for quality, and ensure that milk collectors, processors and marketing agencies follow the correct methods. Having such a system will cost a lot of money. But it is important to have a good system, because it will provide benefits to everyone involved in the dairy industry such as milk producers, milk processors, consumers, government agencies. In Bangladesh, around 97% of the annual milk production is accounted by the traditional milk production system, which is likewise dominated by indigenous breeds. Therefore, proper milking, cleaning and sanitizing procedures of equipments and environments are essential tool to ensure quality of milk.

Sarkar. (2016) reported that Hazard Analysis and Critical Control Point (HACCP) has become the internationally recognized system for the management of food safety for all companies involved in the production, processing, storage, and distribution of food for human consumption. HACCP system during milk collection, processing and storage and microbial exposure assessments and risk analysis should be implemented to ensure safe and healthy milk products. Raw milk was recognized as a source of food-borne illness and disease and epidemiological reports on food borne outbreaks due to consumption of raw milk infected with potential pathogens have been reported. Since the HACCP is a science based analytical tool that enables management to introduce and maintain a cost-effective ongoing food safety program involving systematic assessment of all steps involved in a food operation for identification of those steps that are critical to the safety of the product. Implementation of HACCP system at the dairy farm and during milk handling resulted in a significant improvement in the bacteriological quality of raw milk which in turn resulted in a decline in total viable count (log cfu/ml) of pasteurized milk epidemiological reports on food-borne outbreaks due to consumption of raw milk infected with potential pathogens have been reported.

Debela. (2015) evaluated fresh milk contains bacteria that undergo multiplication when improperly handled. The microbial content of milk is a major feature in determining its quality. Milk from a healthy cow contains rare bacteria. It picks many bacteria from the time it leaves the teat of the cow until consumption or further processing. These bacteria are indicators of both the manner of handling milk from milking till consumption and the quality of the milk. Milk produced under hygienic conditions from healthy animals should not contain more than 5 × 105 bacteria per milliliter. However, counts may reach several millions of bacteria per ml. That indicates a very poor hygienic standard during

milking and the handling of the milk or milk of a diseased animal with i.e. mastitis. Moreover, health of the animal, cleanliness of the housing area, the nature of feed, the water used at farm, the milk vessels / utensils for storage used for dairy farms and vendor shops, are essentially hygiene of the milker / handler are major factors that increase microbial deterioration of raw milk. To prevent a too high multiplication of bacteria, the milk has to be produced as hygienic as possible and should be cooled or heated at the earliest.

2.2. Major Bacterial Species Isolated from Raw Milk

Matin *et al.* (2019) determined the commonly bacteria spread through consumption of contaminated milk to human beings are bovine tuberculosis, brucellosis, salmonellosis, listeriosis, Q fever, campylobacteriosis, yersinoses, and other bacterial pathogens transmitted to humans include streptococcus agalactaciae, *Staphylococcus aureus* and *E. coli*. These are zoonotic diseases which are transmitted to consumers and pose a risk to public health. Milk may contain both pathogenic and nonpathogenic organisms. Pathogenic organisms, which may come directly from the cow's udder, are species of *Staphylococcus* , Streptococcus, Mycobacterium, Brucella, *Escherichia*, Corynebacterium could be isolated from raw cow's milk and some of these have been determined to be pathogenic and toxicogenic, and implicated in milk borne gastroenteritis. Numerous other pathogenic causing diseases like cholera and typhoid may find access in the milk from various other sources, which may include water, and the persons handling the milk. Nonpathogenic microflora may come directly from the udder and may also enter in the milk from milker's hands, utensils, cow barn, water. Therefore, proper milking, cleaning and sanitizing procedures of equipments and environments are essential tool to ensure quality of milk. Many countries have implemented laws and regulations concerning the composition and hygienic quality of milk and milk products to protect both the consumers and the public health. Unfortunately, these laws and regulations are not often adhered in developing countries included. Some studies show that a big percentage of people in Bangladesh especially in rural areas consume raw which predisposes them to the risk of contracting zoonosis, and other milk-borne diseases.

Regasa *et al.* (2019) investigated that a number of common bacteria including *S. aureus, Escherichia coli*. *Salmonella*, *Klebsiella* and *Shigella* have been recovered from raw

milk and some of these have been determined to be pathogenic and toxicogenic, and implicated in milk borne gastroenteritis. Pathogens bacteria that have been involved in food borne outbreaks include *Salmonella*, *Staphylococcus aureus* and *E. coli*. These pathogens have been originated from environment in the farm, mixing clean milk with mastitis milk, manure, soil, and contaminated water. The prevalence rates of these bacteria were *E. coli* 70(58 %), *Staphylococcus aureus* 29 (24.2 %), *Shigella spp*. 21 (17.5 %), *Klebsiella spp.* 9 (7.5 %) and *Salmonella* spp. 4 (3.3 %) respectively. Therefore, the presence of these bacteria pathogens in raw milk is considered to be an indicator of poor hygiene and sanitation during milking and post milking processes. Also. all these are pathogenic bacteria that pose serious threat to human health and contribute up to 90% of all dairy related diseases. In developing countries like Bangladesh, most of the milk is produced by smallholder farmers dominated by local herds of cattle. Their milking units are widely distributed throughout in rural areas with a poor infrastructure, while most of the vendor shops, markets and customers are in urban areas. Therefore, the need for good hygienic practices and a streamlined collection, handling and transport system is important but has been always a challenge. Accordingly, there are several disease causing microorganisms that are associated with milk and milk products. All the pathogens mentioned above and the diseases are those associated with raw milk are briefly discussed as separate below.

Wanjala *et al.* (2018) study that *Staphylococcus aureus* is among the most significant pathogens causing a wide spectrum of diseases in both humans and animals. It is the leading cause of foodborne illness throughout the world. The safety of raw milk and raw milk products with respect to staphylococcal poisoning is of great concern around the world. Milk can be contaminated by *Staphylococcus aureus* when there is infection of the mammary gland. In addition, it can be contaminated during or after milking by poor hygienic practices, such as improper washing of hands when handling milk storage equipment and coughing or sneezing. In human, *Staphylococcus aureus* is a leading cause of gastroenteritis resulting from the consumption of contaminated food include milk. The most common symptoms are nausea, vomiting, retching, diarrhea, abdominal cramping, and prostration. Pathogenic strains are usually coagulase-positive and cause disease in their hosts throughout the world. *Staphylococcus aureus* is one of the most significant food-borne pathogens. The pathogenicity of *Staphylococcus aureus* has been recognized for many years and it may cause mastitis or skin disease in milk producing

animals or lead to foodborne intoxication in milk and milk products. Human carriers can also contaminate milk. Five serologically distinct enterotoxins (A, B, C, D, and E) are recognized, with enterotoxin A most frequently involved in food poisoning outbreaks. The minimal intoxication dose is 100 nanogram and sometimes less. Subsequently, *Staphylococcus aureus* is also common cause of mastitis in dairy cattle and can enter the milk supply from sores on the teats of cows or from the hands and nasal discharges of dairy farmers and workers. Hereafter, high level of *Staphylococcus aureus* isolation from personnel and equipment besides food samples reveals that the hygiene practice is substandard.

Parseelan *et al.* (2018) Studied that Gram-negative, non-spore forming rods. Some of them are human and animal pathogens producing intestinal infection and food poisoning. The genera of pathogenic importance in milk include *Salmonella, Escherichia, Klebsiella, Shigella,* Yersini a pestis and other disease causing bacteria such as *Proteus* spp., *Serratia* spp., *Enterobacter* spp. and *Citrobacter* spp. The important Coliform and non-coliform bacteria belong to the family *Enterobacter* iaceae, including the genera of *Escherichia, Salmonella, Klebsiella* and *Shigella*.

Annal Selva Malar *et al.* (2018) studied that distribution *Escherichia* coli (*E. coli*) is one of normal inhabitant microorganisms of large intestine in human and warm blooded animal. The main source of *E. coli* in raw milk and milk products is fecal contamination together with poor hygienic practices (Lara *et al.*, 2016). *E. coli* and its pathogenic strains in food is of major concern because of its transmission through contaminated milk and dairy products to human. Beside, *E. coli* in raw milk is an indicator of feacal contamination which implies poor hygienic conditions and unsanitized environment since these bacteria are of faecal origin. The recovery of *E. coli* from food is an indicative of possible presence of entero-pathogenic and/or toxigenic microorganisms, which could constitute a public health hazard. It is transmitted to humans primarily through consumption of contaminated foods, such as raw or undercooked ground meat products, raw milk a contaminated raw vegetables and sprouts.

Kandil, *et al.* (2018) studied that *E. coli* is frequently occurring organisms in milk whenever the methods of production, transportation, handling and sale of milk are unhygienic. The milk sold in raw forms and because of possibilities of contamination with *E. coli* poses a great hazard to public health. Raw milk is a known vehicle and

medium for pathogen like *E. coli*. Most *E. coli* are harmless, but some are known to be pathogenic, causing severe intestinal disease in man and has become serious threat to the dairy industries ranging from mild diarrhea to potentially fatal hemolytic uremic syndrome (HUS), hemorrhagic colitis and thrombotic thrombocytopenic purpura. In recent years, *E. coli* 0157:H7 strain has become a very important milk-borne pathogen and castles are considered as its main reservoir. Though detection of *E. coli* in milk reflects fecal contamination, environmental coli forms have also been detected in milk.

Abdissa *et al.* (2017) investigate that *Salmonella* is a Gram-negative bacterial genus of the *Enterobacter* iaceae family with a strong pathogenicity that can cause cross-infection between humans and animals. *Salmonella* causes fever, diarrhea, gastroenteritis, and sepsis in humans, as well as intestinal damage in both humans and animals (Mughini *et al*. 2018). In addition, that *Salmonella* species are known pathogenic microorganisms that can cause food poisoning through consumption of contaminated milk and milk products. Hence, *Salmonella* food poisoning is one of the most common and widely distributed diseases in the world, estimated to cause 1.3 billion cases of gastroenteritis and three million deaths worldwide. Many factors such as improper hygienic conditions in the farm, food handlers, and consumption of raw milk and milk products are the sources of *Salmonella* infections. Therefore, *Salmonellae* are considered among the most important enteric foodborne pathogens whose presence in the food constitutes a severe health hazard. Many outbreaks of human illness have been associated with the consumption of raw or inadequately heat treated milk or their dairy products. Contamination of raw milk with *Salmonella* spp. is mostly due to infected persons and contamination of the environment, since natural infections of the udder are rare and seldom contribute to human food poisoning. Despite that, *Salmonellae* are considered among the most important enteric foodborne pathogens and many outbreaks of human illness have been associated with the consumption of raw or inadequately heat treated milk or their dairy products.

Abdel -Hameed. (2017) reported that contamination of raw milk with *Salmonella* spp. is mostly due to infected persons and contamination of the environment, since natural infections of the udder are rare and seldom contribute to human food poisoning. As well as *Salmonellae* spp. are also considered as the public health concern since they produce infection ranging from a mild self-limiting form of gastroenteritis to septicemia and typhoid fever. However, awareness of food-borne outbreaks as a result of consumption

of contaminated raw milk in the low and middle income countries remains to be very scanty including Bangladesh. Consequently, for the prevention of *Salmonella* contamination in milk and Milk products, we may apply proper hygienic measures during milking and handling of milk after milking. Efficient cleaning of all utensils and equipment, effective training and education the farmers to improve awareness of milk borne zoonosis and risk factors can be followed. Good Manufacturing Practice (GMP) and (HACCP) system s is suggestive to avoid *Salmonella* contamination.

Chouhan. (2015) stated that the genus *Klebsiella* belongs to a member of the family *Enterobacter* iaceae. *Klebsiella* is a gram negative, rod-shaped, non-motile bacterium. This species can be found everywhere in nature. In recent years, *Klebsiellae* have become important pathogens in multi-resistant infections in hospitals. *Klebsiella pneumoniae* and *Klebsiella oxytoca* are the two members of this genus responsible for most human infections. The incidence of *Klebsiella* spp. (K) in raw milk of different animal species was determined whereby thirty-one strains of *Klebsiella* were isolated from raw milk. On the basis of biochemical characterization, the strains were divided into 4 species as follow *K. pneumonia, K. ozaenae, K. planticola* and *K. rhinoscleromatis.* The gastrointestinal tract and the hands of personnel were reported as principal reservoirs of *Klebsiella*. Bovine mastitis is caused by a variety of bacteria; among them, *Klebsiella* sp. *Klebsiella* sp. is an opportunistic bacterium that can cause primary bacteremia as well as urinary tract infection in human and animal. Thus, the milk and its products might represent important sources of pathogenic *Klebsiella*, *Klebsiella* pneumonia was isolated from mastitic cows especially from those kept in wood products bedding while *Klebsiella pneumoniae* and *Klebsiella oxytoca* are the two members of this genus responsible for most human infections. Previous reported indicated that *Klebsiella* sp. Has zoonotic importance. *Klebsiella* sp. is notoriously appeared in dairy food products and it is reported that they are responsible for clinical as well as subclinical bovine mastitis. *Klebsiella* sp. was notoriously and ubiquitously appeared in milk along with their products that have zoonotic importance. Subsequently, it is quite difficult to control bovine mastitis originated from *Klebsiella* sp. infection. In humans, *K. pneumoniae* is an important cause of nosocomial infections like pneumonia, septicaemia, urinary tract infection, and life-threatening septic shock *Klebsiella rhinoscleromatis,* that is unable to utilize citrate, induces tissue destructive infections in

the nose and pharynx besides its effect on the urinary tract soft tissue as a secondary invader.

Ohud *et al.* (2012) studied that Shigellosis, an acute diarrhoeal disease, is caused by Gram-negative bacterium, *Shigella*, belonging to the family *Enterobacter* iaceae, with four species viz *Shigella dysenteriae* (serogroup A), *Shigella flexneri* (serogroup B), *Shigella sonnei* (serogroup C) and *Shigella boydii* (serogroup D). *Shigella is an* important human food-borne zoonosis bacterial pathogen, and can cause clinically severe diarrhea. In humans; they can cause dysentery, an intestinal disorder the clinical manifestation of which depends on the person and also on the *Shigella*e strain. There are about 1.8 million patients died for diarrhea and a majority of these cases 160 million cases have been attributed to *Shigella* in the world for every year. *Shigella* dysenteriae, implicated in epidemics, leads to death. Environmental risk factors of shigellosis include water supply, sanitation, and household environment including fly aggregation. Therefore, milk and dairy products have been associated with few dysenteric outbreaks, hence the importance of detecting for the presence of *Shigella* in these foods. Moreover, the prevalence of these bacteria in milk and dairy products is not negligible and consequently they seem to have moved from time to time into the industrial domain. Detection of *Shigella*e is usually done by culture dependent methods. Shigellosis is endemic in many developing countries and also occurs in epidemics causing considerable morbidity and mortality. It is estimated to cause at least 80 million cases of bloody diarrhea and 700,000 deaths each year.

2.3. Physico- chemical analysis of raw milk

Hasan and Rakib. (2016) were detailed the physico-chemical analysis of raw milk. A total of 100 raw milk samples were collected from different dairy farms, vendor shops and retailer shops. The physico-chemical analysis of milk was carried out. Raw milk was analyzed for, clot on boiling, alcohol tests and Methylene blue reduction test. Results of clot-on- boiling and alcohol tests on the milk samples were fairly good but deteriorated fast when purchased and stored in consumers' containers without cooling. Alcohol and clot-on-boiling tests showed that the milk was of fairy good quality at the selling point. It was concluded that the quality of raw milk distributed to consumers should be improved by observing HACCP concepts including proper sanitation and hygiene and should be processed, cooled to 4-5℃ and packaged before distribution. Out of 100

samples, 60 samples were found yellowish white, 20 were white, 10 samples were light yellowish white and remaining 10 were yellowish white in colour. These variations in colour may be due to the differences in nature of feed consumption or the breed of cow or the fat and solid contents of milk.

Bharti *et al.* (2015) investigated the physico-chemical assessment of raw milk available in Nakla upazila, Sherpur, Bangladesh. Individual raw milk samples were collected from different local markets of Nakla upazila, Sherpur, Bangladesh. Sensory analysis was examined by a panel of experienced judges. The organoleptic properties of milk such as color were evaluated with the help of eyes per standard scored of physico-chemical analysis. The colours of all the milk samples from different local markets were golden yellowish, yellowish white and whitish. These differences in colour may be due to the differences in nature of feed the cows consumed, the breed, forage consumption, feeding schemes, milking incidence, milking process, seasonal changes, lactation period and adulteration. Also such studied of physico-chemical quality of raw milk in central part of Cote 'D' Ivoire. Pysico-chemical properties of each milk sample from cow species and physicochemical properties of milks mixed from various farms were evaluated. The quality and composition of raw milk depends on its physiochemical parameters that vary from one area to another. Moreover, they are affected by several factors such as type of breeds, forage consumption, feeding schemes, milking incidence, milking process, seasonal changes, lactation period and adulteration.

Soomro *et al.* (2014) examined that physico-chemical analysis included were clot on boiling, alcohol tests and Methylene blue reduction test. Studied physicochemical assessment of raw and raw milk. A total of 150 samples of milk consisting of 90 raw and 60 raw were collected from supermarkets. Raw milks were purchased from different vendors and the brand milks were brought from different shops. Whereas, the physical examination revealed 13% and 0% samples had light yellow while 7% and 0% showed bloody colour from various canteens of educational institutes and public places, respectively. was carried out to assess the physical, chemical quality and detection of adulteration in raw milk collected from dairy farms of five different places of Mymensingh sadar upazila (BAU Sheshmore, BAU KR market, train going vendor, sweetmeat shop and Dhudmohol) in Bangladesh. Results shows that milk from sweet meat shop had 100% yellowish white colour, normal (milky) flavor and free flowing liquid whereas other sources milk varies with their percentage in terms of physical

parameters. The previous studied report the normal milk has a yellowish white color due to the presence of fat, casein and the presence of small amount of colouring matter. These differences in colour may be due to the differences in nature of feed consumption or the breed of cow or the fat and solid contents of the milk.

2.4. Bacterial load determination

Talukder *et al.* (2019) Microbial load is a major factor in determining milk quality. It indicates the hygienic level exercise during milking, cleanliness of the milk utensils, condition of storage, manner of transport as well as the cleanliness of the udder of the individual animals. The enumeration of total bacterial count (TVBC), isolation of bacterial isolates and identification of pathogenic bacteria, that bacterial load of milk is a significant factor in determining its quality and safety. Such previous studied were carried out for the detection of total bacterial count (TBC), coliforms, *Staphylococcus* count and *Salmonella-Shigella count*. In raw milk sample one highest number of viable bacterial count, coliform count and *Staphylococcus* count were found. The higher microbial population (> 10^5cfu/ml) in aseptically drawn milk or detection of pathogenic microorganisms in raw milk is an indicative of unhygienic milk production. Fresh milk from healthy animal contains relatively few bacteria (10^2-10^3 cfu/ml) which increase up to 100 fold or double in less than three hours during its storage at normal temperature, depending on the initial microbial population and the temperature of storage. Whereas the lower microbial load was noted in individual cow milk (10-10^4 cfu/ml), which increase to 10^6 cfu/ml at vendor shops milk and to 10^7 cfu/ml in collection center respectively, in which at dairy processing units indicate contamination of milk after milking. The high bacterial load could also be associated with the original heavy load of bacteria in raw milk before pasteurization.

Korma and Negera. (2018) determination of sanitary, total viable bacterial count (TBC), total staphylococcal count (TSC), Total coliform count (TCC) and Total *Salmonella-Shigella* count (TSSC) was performed. The highest TVBC, TSC, TCC and TSSC were 6.8 x105 cfu/ml, 85cfu/ml, 4.5x105 cfu/ml and 3.4x10^4cfu/ml, respectively. According to guidelines elaborated by the International Commission on Microbiological Specifications for Foods. Total bacterial count in raw milk below 10^4 CFU/g is indicative of their acceptable quality, the counts of 10^4 to10^5CFU/g indicates their permissible quality, whereas bacterial count exceeding 10^5CFU/g is unacceptable. The

studied that total bacterial count was used as an important indicator of the microbial quality of the raw milk. The reported showed that high total bacterial load in raw milk indicates contamination possibly from lactating cows, milking equipments, storage containers, unsatisfactory hygiene/sanitation practiced at farm level, unsuitable storage condition, unclean udder and/or teats, poor quality of water used for cleanliness and dirty hands of milkers. Consequently, the total bacterial, total staphylococcal count, counts, total coliform counts, total *Salmonella* counts and decolonization time of the samples were determined. The total bacterial counts ranged from $7.5x10^4$ to $8.4X10^4$ CFU/ml, total *Staphylococcus* ranged from $8.3x10^4$ to $8.6x10^4$cfu/ml, total coliform counts ranged from $1.0X10^4$ CFU/ml to 2.0x104 CFU/ml, while the total *Salmonella* counts were between $1.0x10^4$ and $1.6x10^4$cfu/ml. The bacteria were characterized and identified as *Staphylococcus* sp, *Escherichia*, and *Salmonella* sp.

Oumer *et al.* (2017) analyzed that the lack of knowledge about clean milk production, use of unclean milking equipment and lack of potable water for cleaning purposes were some of the factors which contributed to the poor hygienic quality of raw milk at farms and milk collected centers. All the raw milks had high bacterial load which ranged from 1.75×106 to 1.22×108 cfu/ml. The most frequent cause of high bacterial load is poor cleaning of the milking system. Therefore, bacterial count high was due to milking dirty udders, maintaining an unclean milking and housing environment, and failing to rapidly cool milk to less than 40°F. Bacterial load in milk indicates the degree level of hygiene practiced in the whole milk production process. A total bacterial count is an indicator for prolonged storage of milk especially when stored at room temperature. Microbiological quality of milk samples was analyzed using Total Viable Bacterial Count (TVBC), Total Coliform Count (TCC), Total *Staphylococcus* Count (TSC) and Total *Salmonella-Shigella* Count (TSSC) techniques. Critical hygienic indicator for food and food stuffs is total microbial load. Compare to four techniques four shows the high contamination value in milk samples (TVBC 12.48×10^5 cfu/ml, TCC 6.4×10^5cfu/ml, TSC, TSS 3.48×10^2 cfu/ml and 4.85×10^2 cfu/ml). The average total viable bacterial counts of can rinse were $3.11x10^6$. Fresh milk drawn from a healthy cow normally have a low microbial load, but the loads may increase up to 100 fold or more once it is stored. Contamination of mastitis milk with fresh clean milk may be one of the reasons for the high microbial load of bulk milk.

Yacine Titouche *et al.* (2016) studied that the source of Coliform bacteria in bulk tank storage milk is the udders of cows or unsanitary milking practices. the Coliform count is an indication of the effectiveness of cow preparation procedures during milking and the cleanliness of the cows' environment. Coliforms can also incubate on residual films of milking equipment. The Coliform count should be less than10 cfu/ml. A Coliform count between 100 and 1000 usually indicates poor milking hygiene and a Coliform count >1000 suggests that bacterial growth is occurring on milk handling equipment. Different study done in Ethiopia show that, coliforms were recovered from raw milks and the count range between 4.03 log cfu/ml and 6.57 log cfu/ml. The mean Coliform Plate Count (CPC) was found to be 4.3 (log10 cfu/ml) with more counts recorded in vendors which ranged from 3.3 to 5.4 (log10 cfu/ml). Coliforms are other bacterial group which causes milk deterioration which is associated with the level of hygiene during and subsequent handling.

Chimuti *et al.* (2016) according to coliform count of milk directly collected from udder, from storage containers at farm level and distribution containers upon arrival at selling point is different and the count was 2.47, 4.93 and 6.52 log10 cfu ml-1 respectively. That show the coliform count progressively increased by 2.46 log10 cfu ml-1 (99.6%) for milk samples taken from production to arrival at selling point and by 1.59 log10 cfu ml-1 (32.3%) between sampling from milk storage containers at the farm level to sampling from distribution containers upon arrival at selling points. The difference between these results may be due to a difference in awareness of farmers to control hygiene, transport, and storage conditions in Morocco, loads of 1.7×10^4 CFU/ml for total coliforms and 6.8×10^3 CFU/ml for *E. coli* have been reported in raw milk. Likewise, were reported in Nigeria, it was reported that 88.43% milk had a mean total coliform counts of 20×100–3×10^7 CFU/ml. The national standard for milk quality is 100 CFU/ml. In Cameroon, it has been announced that 87.1% milk had coliforms levels below 3 log10 CFU/ml with a mean load of 3.83±0.86 log10 CFU/ml while contamination by *E. coli* was 79.5% with a mean load of 2.25±1.44 log10 CFU/ml. While in Bangladesh, the mean coliform counts were reported as 2.66–5.94 log10 CFU/ml by 1.84 log10 CFU/ml and 3.48–7.38 log10 CFU/ml.

Belbachir *et al.* (2015) investigated that the bacteriological quality for most raw milk sample collected from vendor shops was poor with a total plate count of 7.54 log10 cfu/ml).The overall mean of total plate count of 7.25 log10 cfu/ml in this study was

higher than those found in the study of 6.46 log10 cfu/ml who mentioned that, improper hygienic practices during milking process, poor storage temperature, health and hygiene of cows and procedures used in cleaning and sanitizing the milking and storage equipment to affect the microbiological quality of raw milk. In addition to this ineffective sanitizing routine, the cow's environment that leaves manure in contact with cows' udder also contributed to the high bacterial load in raw milk. The microorganisms present can originate from interior of the udder, its exterior and/ or milking equipment. High initial microbial count in milk of $>10^5$ cfu/ml in evidence of serious faults in milk production hygiene, whereas production of milk having counts consistently below 10^5 cfu/ml reflects good hygiene practices. Some countries have adopted different standards suited to local conditions. For example, the standard plate count for America is no more than $3x10^5$ cfu/ml, while the standard for Kenya is no more than $2x10^6$ cfu/ml. In Sweden the accepted limit for the total number of bacteria and somatic cell count in raw milk is $1x10^5$cfu/ml and $4.99x10^3$ somatic cells/ml respectively. The standard plate count for raw milk should be equal or less than 30,000 cfu/ml.

Asaminew *et al.* (2015) stated in North Africa, in Egypt, total coliforms and total *Staphylococcus* count were detected in 89.5 and 65.8% examined raw milk samples with mean counts of 1.65×106 and 3.69×105 MPN/ml, respectively. *E. coli* was isolated from 52.6% raw milk samples. Similar work reported mean total coliform counts of 3.28×102-1.4×103 CFU/ml in Egypt with the dominant isolated coliforms of 8% *E. coli*, 14% *Salmonella*e spp., and 15% *Staphylococcus* stated that the average counts of the total coliforms and coliforms in milk samples of Morocco were as high as 2.6×103 and 1.9×102 CFU/ml, respectively; they also showed that 52% milk samples showed an unsatisfactory quality since the samples exceeded the maximum acceptable counts of fecal coliforms (102 CF.

CHAPTER III

MATERIALS AND METHODS

3.1. Description of the study area

The study was conducted in dairy farms located at Dinajpur Town, under the Rangpur division which located at a distance of 420 km along North of Dhaka. It is situated between 88°10 and 92°41 East longitudes and between 20°34 and 26°38 North longitudes. The total area is about 147,570 km2. Annual minimum temperature varies from 8°C to 13.4°C and maximum temperature vary from 25.5°C to 36.8°C. Annual rainfall is over 2000 mm with seasonal and regional variations from 5500 mm in the Northeast to 1500 mm in the West. The highest rainfall in the monsoon season (June to September) varies from 750 mm per month in the Northeast to about 500 mm in the West. The humidity is the highest (95%) in July and the lowest (36%) in December (Hamid *et al.*, 2016).

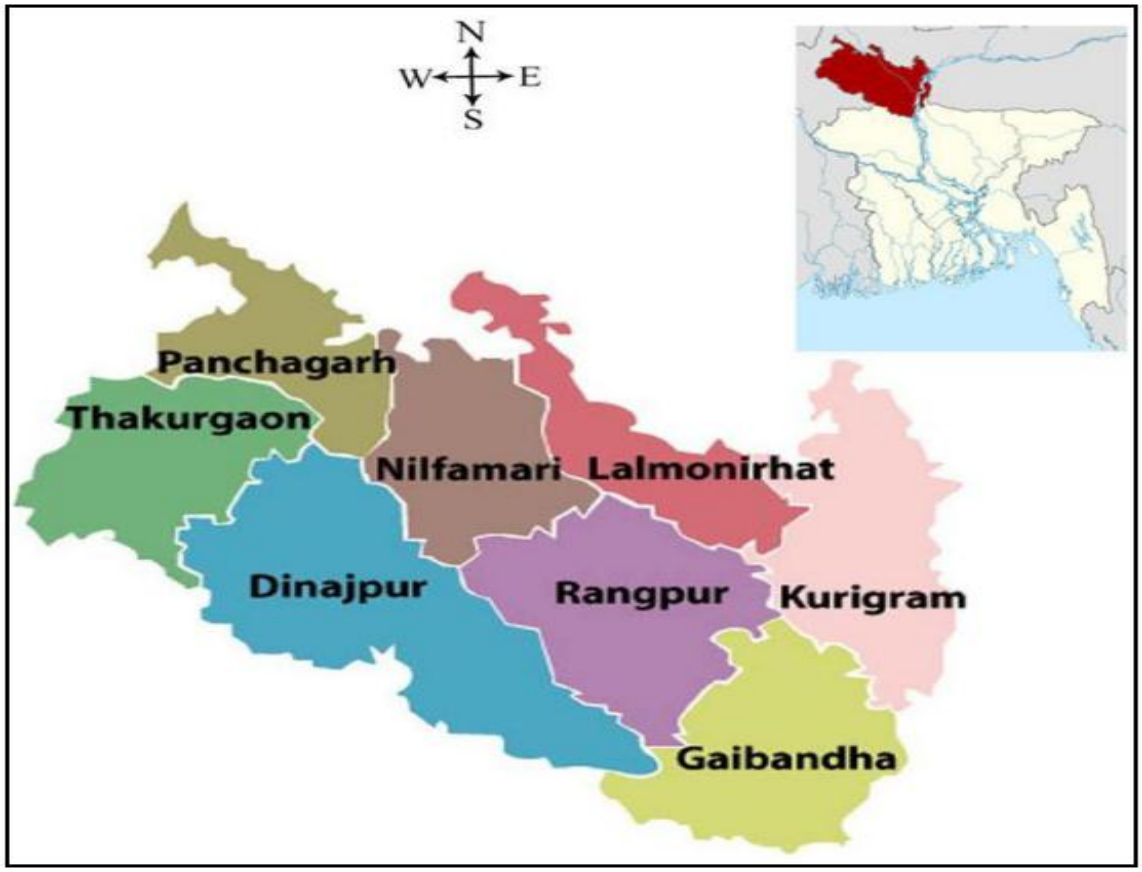

Fig. 1: Study area map

3.2. Experimental site and duration

A cross sectional study design was employed. The study involved different actors and nodes along the dairy value chain who were farmers and milk vendors used milk. The inclusion criteria of the study participants included, availability of milk during the time of sample collection and willingness to participate in the research. Therefore, this present research work was performed in the Bacteriology Laboratory of the Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, Bangladesh and the duration of the experiment was 6 months (January to May/2020).

3.3. Collection of Samples and Handling Procedures

All dairy farms and vendor shops fulfilling the inclusion criteria were considered for sampling. A total of 45 (25 dairy farms, 20 milk vending shops) samples were collected from selected dairy farms and vendor shops based on availability, voluntary basis to provide milk, transportation and accessibility of time for sampling and laboratory work. Accordingly, dairy farms having less than or equal to 5 dairy cattle were categorized as small scale, 5 to 10 dairy cattle as medium scale and greater than 10 dairy cattle as large scale dairy farms (ILRI, 2007). Hence, out of the 15 dairy farms included in this study, 4, 6 and10 was small, medium and large scale dairy farms, respectively. 15-20 mL of milk samples were collected from each dairy farms using sterile test tubes. Similarly, vendor shops 20 samples were collected designated by each vendor (V1, V2, V3 and V4). Thereafter a total of 45 pooled samples was collected. Each milk sample was labeled and placed in the icebox and transported to the HSTU Microbiology Laboratory. The samples were kept in the refrigerator at 4°C and culture was conducted within 24 hours (Kebede, 2005). The samples were prepared according to the technique recommended by the International Organization for Standardization (ISO 8261:2001).

3.4. Administration Questionnaire and Observation Survey

Before the formal survey, preliminary visits were made to get the consent of the farmers, locate the farms and to give a brief description to each respondent on research objectives and potential benefit of involving in the study. The revised version of the questionnaire that was used in the pilot study was translated into 'Bangla', the National language that was clearly understood by the majority of Bangla. Then Semi-structured questionnaires

were used to assess the hygienic and handling practices of milk in dairy farms and vendor shops. The farm owner, milking personnel and farm attendants were interviewed. Also, the questionnaire was used to collect information on possible risk factors for bacterial contaminations in milk. Risk factors considered in the current study were sanitary conditions of the barn/milking environment, hygiene of milking cows' udder and milk handlers, hygiene of milking equipment with special emphasis to hygiene of milking procedures and milk handling practices, utensils used for milking, milk storage and uses of milk (for selling or domestic purposes). Furthermore, milk consumption behaviors and their awareness on the risk of zoonotic diseases that are associated with the consumption of raw milk was also assessed. While administering questionnaires, direct observation on general cleanliness, hygienic conditions and practices concerning milk was also done and noted at the same time. Upon finishing of the administration of questionnaires, milk samples were collected for laboratory analyses. Sometimes milk was sampled first before administering questionnaires because some farmers wanted to transport and sell to the milk vendors.

3.5. Bacteriological Analysis

3.5.1. Laboratory equipment's

All items of glass wares including were used during whole period of experiments were including; Petri dishes, test tubes, conical flask, , slides, racks, water bath , hot air oven, cotton, hand gloves, plastic syringe (5 ml) Pipette, micro-pipette (1 ml, 1000µl, 100 µl), glass slides, magnifying glass, marker pen, ice-box , cover slips, inoculating loop and rack, autoclave, refrigerator, pipettes, cylinder, glass plate, slides, colony count machine, digital weight balance and stirring machine, phase contrast microscope and Trinocular Microscope.

3.5.2. Preparation of Different Bacteriological Culture Media

The samples were analyzed within 2-6 hours of collection. The different types of media were prepared for both differential and selective growth, Gram's staining and morphological characteristics, biochemical test, sanitary quality milk tests (Methylene blue test and Alcohol test, Standard Plate Count), Antibiotic sensitivity test and finally characterization of PCR for further identification of specific properties and characteristics of different microorganisms. All media used in the present study were

prepared respectively and according to the instructions provided by the manufacturing firms and checked for sterility and protocol. The above media were prepared separately by the following method:

3.5.3. Liquid Media Preparation

3.5.3.1. Nutrient Broth Media (NB)

Nutrient broth the medium was prepared by adding 13 g of nutrient broth powder to one litter of distilled water and well mixed. The pH was adjusted to 7.4. The mixture was distributed in 5 m1 volumes into clean bottles, and then sterilized by autoclaving at 121 °C (15 lb/inch2) for 15 minutes. Peptone water This medium was prepared by dissolving 10 g of peptone water and 5g sodium chloride in 1littre of distilled water. The mixture was distributed in 5 ml volumes into clean bottles, and sterilized by autoclaving at 121°C (15lb/inch2) for 15 minutes (Carter, 1979).

3.5.3.2. Buffered Peptone Water

To obtain Buffered peptone water (BPW), 20 g of the BPW powder was dissolved in 1000 of distilled water according to the manufacturer's instructions (OXOID® Ltd., Basingstoke, Hampshire, England). Original BPW powder is a mixture of 10 g/l peptone, 5 g/l sodium chloride, 3.5 g/l disodium phosphate and 1.5 g/l potassium di-hydrogen phosphate. Each 6 ml of the mixture was dispensed in new sterile test tube, sterilized by autoclaving at 121°C for 15 minutes and cooled to 25°C for serial dilutions. This media preparation was follow in many published reference methods, including the current International Organization for Standardization (ISO) method for the detection of many organisms in foods (Roesch *et al.,* 2004).

3.5.4. Solid Media Preparation

3.5.4.1. Nutrient Agar Media (NA)

Twenty-eight grams of Nutrient agar (NA) was prepared and dissolved in 1000 ml of cold distilled water in a flask and then autoclaved at 121◦C, 15 psi. The media was then dispensed into sterile petridis while liquid and left for a while get solidify. Using sterile technique, a NA agar plate was streaked by picking a loop full of colony of 24-hour fresh pure culture with an inoculating loop by means of three quadrant streak plate method to obtain isolated discrete colonies. The plates were then incubated at 37◦C for 24 hours.

After the incubation period the 18 growth patterns of the bacteria were evaluated for size, pigmentation, form, margin, elevation and texture (Cappuccino and Sherman, 2005).

3.5.4.2. MacConkey agar

The media were prepared by suspending 51.50 grams. Mac Conkey Agar in 1000ml distilled

water. The media were heated to boiling with gentle swirling to dissolve completely. The media were sterilized by autoclaving at 121^{o}C for 20 minutes at 15lbs pressure. Overheating was avoided. Then media were cooled to 45-50^{o}C and poured into sterile Petridis. The surface of the medium was dried when inoculated (Carter, 1979).

3.5.4.3. Mannitol Salt Agar (MSA)

111 grams Mannitol Salt Agar base powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely (necessary calculation was done for required number of plates). The medium was then sterilized by autoclaving at 1.2 kg/cm2 pressure and 121° C for 15 minutes. After autoclaving the medium was put into water bath at 450- 500C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass Petri dishes (medium sized) and in 15 ml quantities in sterile glass Petri dishes (large sized) to form thick layer there. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the Petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. Petri dishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.5.4.4. *Staphylococcus* Agar No.110

Staphylococcus Agar No.110 is used as a selective medium of *Staphylococcus aureus*. Suspend 149.5 grams in 1000 ml of distilled water. Mix thoroughly. Heat, to boiling, to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Resuspend the precipitate by gentle agitation to avoid bubbles and pour the plates while the medium is hot. After autoclaving the medium was put into water bath at 450- 500C to decrease the temperature *(*Lehrfeld and Morris, 1992).

3.5.4.5. Plate Count Agar (PCA)

Add 17.5 grams to 1 liter of distilled water, dissolve by bringing to the boil with frequent stirring, mix and distribute into final containers, sterilize by autoclaving at 121°C for 15 minutes. After autoclaving, the medium was poured into each sterile petri dish and allowed to solidify. After solidification of the medium in the petri dishes, these were incubated at 37°C for overnight to check their sterility and used for cultural characterization or stored at 4°C refrigerator for future use (Cater, 1979).

3.5.4.6. Eosin Methylene Blue (EMB) agar

This test was done to select and isolate Gram negative organisms, and coliforms, and to differentiate among the family of *Enterobacter* iaceae. The main use of this test was to isolate fecal coliforms and to detect for fecal contamination. Thirty-six grams of EMB agar base was added to 1000 ml of water in a flask and boiled to dissolve the medium completely. The medium was sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121°C for 15 minutes and to 50°C and shake the medium in order to oxidize the methylene blue (i.e. to restore its blue colour). Then 10 ml of medium was poured into each sterile Petri dish sized and allowed to solidify (Goodridge *et al.*, 2004).

3.5.4.7. *Salmonella-Shigella* Agar (SSA)

63.0 grams SS agar powder was dissolved in 1000 ml of distilled water. It was mixed well until a homogeneous suspension is obtained. It was heated with frequent agitation and boiled for one minute. It did not sterilize by autoclaved. It was cooled to 45ºC and 50°C and distributed in Petri plates and allow the medium to solidify partially uncovered (Leifson *et al.*, 1935).

3.5.4.8. Xylose Lysine Deoxycholate (XLD)

Xylose Lysine Deoxycholate agar is a selective growth medium used for the isolation of *Salmonella* and *Shigella* species from clinical samples and from food. Suspend 56.68 grams of dehydrated medium in 1000 ml purified or distilled water. Heat with frequent agitation until the medium boils. Note: this media does not autoclave. And then transfer immediately to a water bath at 50°c. After cooling, pour into sterile Petri plates (ISO-6579, 2002).

3.6. Microscopic Observation of the bacteria

All the potential bacteria were observed under microscope in order to study their visual properties. Gram staining was done to differentiate between two principle groups of bacteria: Gram positive and Gram negative.

3.6.1. Morphological characterization by Gram's staining method

Smears were prepared from the culture by emulsifying a part of a colony in a drop of normal saline on a glass slide, dried and fixed by gentle heating. Crystal violet was then applied on each smear to stain for one minutes and then washed with running water. Few drops of Gram's iodine were then added to act as mordent for one minute and then again washed with running tap water. Acetone alcohol was then added (acts as decolorizer) for few seconds. After washing with water, safranin was added as counter stain and allowed to stain for 2 minutes. Then the slides were washed with water, blotted and dried in air and then examined under light microscope with high power objective (100X) and Trinocular microscope using immersion oil. Gram-positive bacterial cells appeared violet in colour while that of Gram-negative bacteria appeared red (Buxton and Fraser, 1977).

3.6.2. Preparation of Biochemical Media

Several biochemical tests were carried out in order to have a presumptive identification of the potential bacteria chosen before. Most of the methods were done according to the microbiology laboratory manual (Cappuccino and Sherman, 2005). The biochemical tests performed were Triple sugar iron agar test, IMViC test (Indole production test, Methyl red test, Voges- Proskauer test, Citrate utilization test), MIU test (Motility test, Indole test and Urease test) Catalase test, Oxidase test, and Cetrimide agar respectively test as described by (Ali *et al.*,2008).

3.6.3. Triple sugar iron (TSI) agar slant (Hi-media, India)

65 grams TSI agar base powder was mixed in 1000 ml of cold distilled water in a flask and mixed thoroughly, then heated to boiling for dissolving the medium completely. The medium was then sterilized by autoclaving for 15 minutes at 121°c maintaining a pressure of 1.2 kg/. Then 20/10 ml of medium was poured into each sterilized test tubes and allowed to cool and to solidify (kept in horizontal position). The test organisms were

culture into TSI agar slant by stab streak methods. After solidification test tube were used for biochemical characterization and incubated at 37°c for 24 hours.

3.6.4. Motility, Indole, Urease (MIU) medium (Hi-media, India)

18.0 grams of MIU agar (Difco) was suspended in 950 ml of cold distilled water taken in a conical flask and heated up to boiling to dissolve the medium completely. Dispense in 95 ml. amounts into flask and sterilize by autoclaving at 15 lbs pressure (1210C) for 15 minutes. Cool to about 50-550C and aseptically add 5ml. sterile 40 % basal medium. Mix well and dispense into sterile test tubes. Allow to cool in an upright position. The sterility of the medium was jugged and used for cultural characterization or stored at 4^0C in refrigerator for future use (Carter, 1979).

3.6.5. Indole Production test

Distilled water solution was prepared by diluting 15.23 grams' powder into 1000ml of the distilled water and then 5mls of this solution was added in test tubes by using a sterile plastic disposable pippete. A fresh sterile plastic loop was used to inoculate the colonies to bijou tubes before incubation at 37°C for 48 hours. After incubation was added the prepared below Kovac 's reagent. The tubes were gently shaken and examined for red coloured ring formation on the surface of the tube. Formation of this red ring is an indication of positive Indole reaction.

3.6.5.1. Kovac's reagent

This solution was prepared by mixing 25 ml of concentrated Hydrochloric acid in 75 ml of amyl alcohol and to this mixture 5 grams of paradimethyl-aminohenzyldehide crystals were added. This was then kept in a flask equipped with rubber cork for future use (Merchant and Packer, 1969). This reagent was prepared to observed for indole production by added 5 drops of Kovac 's reagent directly into the tubes.

3.6.6. Methyl Red-Voges Proskaure broth

Methyl Red-Voges Proskaure broth, it is very useful in separating members of the family *Enterobacter* iaceae and some other organism including *Staphylococcus* . This medium was prepared by adding 15gram of powder to 1 liter of distilled water, mixed well, distributed into test tubes in 5ml amount and sterilized by autoclaving at 121ºC for 15 minutes. Subsequently MR-VP were inoculated separately test tubes by using sterile

technique, small amount of the experimental bacteria from 24-hours old pure culture and then were incubated MR and VP for 48°C and 72°C hours at 37°C respectively. After that were added two below prepared solutions of Methyl Red test and Alpha- naphthol solution correspondingly (Cheesbrough, 1985).

3.6.6.1. Methyl red test

The indicator methyl red (MR) solution was prepared by dissolving 0.1 gm of Bacto methyl red (Difco) in 300 ml of 95% alcohol and diluting this to 500 ml with the addition of 200 ml of distilled water. This Methyl red test was done to determine the ability of the bacteria to oxidize glucose with the production and stabilization of high concentration of acid end products. After 48-hour of incubation (MR) media were added 5 drops of methyl red reagent were added. A positive reaction was indicated by appearance of a red colour while yellow coloration shows negative (Cappuccino and Sherman, 2005).

3.6.6.2. Alpha- naphthol solution

Alpha- naphthol solution was prepared by dissolving 5 grams of Alpha- naphthol in 100 ml of 95% ethyl alcohol. This indicator and reagent was used after incubation of VP for 72°C hours at 37°C. The addition of 5% alpha naphthol was added followed by 0.2 ml (4 drops) of 40% KOH (reagent B) with Voges Proskauer (VP). A positive reaction was indicated by development of bright pink colour within 30 minutes.

3.6.7. 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 121°C. the media was cooled to about 50-55°C and 100ml 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. (Cappuccino and Sherman, 2005).

3.6.8. Citrate utilization test (Hi-media, India)

Citrate utilization test was done to differentiate among enteric organisms on the basis of their ability to ferment citrate as a sole source of carbon by the enzyme citrate permease. Suspend 24.28 grams of powder were suspended in 1000 ml purified/ distilled water and then boiled to dissolve completely, then sterilized by autoclaving at 121ºC for 15 minutes. Simmons citrate agar slants of 5 ml were poured in each test tube and formerly allowed to set in the slope position until solidified. Afterward using by g sterile technique, small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the test tube by means of a streak inoculation method with an inoculating needle and the test tube were incubated for 24 hours at 37°C. (Cappuccino and Sherman, 2005).

3.6.9. Catalase Test

Catalase test was done to determine the ability of the bacteria to degrade hydrogen peroxide by producing the enzyme catalase. Catalase-positive bacteria include strict aerobes as well as facultative anaerobes while, catalase-negative bacteria may be anaerobes, or they may be facultative anaerobes that only ferment and do not respire using oxygen as a terminal electron acceptor. A microscopic slide was placed inside a petri dish. Using a sterile inoculating loop, a small amount of bacteria from 24-hour pure culture was placed onto the microscopic slide. 1 drop of 3% H2O2 was placed onto the organism on the microscopic slide using a dropper and observed for immediate bubble formation. (Cappuccino and Sherman, 2005).

3.6.10. Oxidase Test

Oxidase test was done to determine the presence of the enzyme cytochrome oxidase in the bacteria. A small piece of filter paper was soaked in Gaby and Hadley oxidase test reagent and let dry. Using an inoculating loop, a well isolated colony from pure 24-hour culture was picked and rubbed onto filter paper and observed for colour change (Marbach *et al*., 2010).

3.6.11. Antibiotic Sensitivity

To determine the drug Sensitivity and resistance patterns of isolated bacteria organisms used different types of commercially available antimicrobial discs, (Mast diagnostics

Mersey side, UK.) which were showed in (Table 1). Approximately ten antimicrobials such as Ciprofloxacin (CIP), Chloramphenicol (C), Erythromycin (E), Levofloxacin (LE), Amoxicillin (AMX), Azithromycin, Gentamycin (GEN), Cefixime (CFM), Ampicillin (AMP), Tetracycline (TE) were selected from main class of antimicrobials and that were commonly used by the veterinary and human clinician found in the Department Microbiology laboratory at HSTU, and investigated for sensitivity testing. The antibiotic discs were placed on the surface of Muller Hinton agar plate media previously seeded with appropriate amount of the organism to be tested. Each disk was pressed down to ensure complete contact with the agar surface. The plates were incubated at 37^{o}C for 18-24 hours. Subsequently, the plates were examined for the development of zone of inhibition around the discs. After measuring the zone of inhibition, it was classified as sensitive, intermediate and resistant according to National Committee for Clinical Laboratory Standard (NCCLS) break point to interpret the inhibition zone (Quinn *et al.*, 2002).

3.6.12. Mueller Hinton Agar

Mueller Hinton Agar (HI-MEDIA, India) is used in antimicrobial susceptibility testing by the disk diffusion method. 38 grams of Mueller Hinton agar powder was suspended in 1000 ml of distilled water. It was mixed well. It was heated agitating frequently and boiled for about one minute. It was dispensed and sterilized in autoclave at 116 - 121°C (15 lbs. sp) for 15 minutes. It was cooled to 45° or 50°C (Carter, 1979).

Table 1: Antibacterial used in antibiotic sensitivity test

Sl. No.	Name of the antibiotics	Disc concentration (µg/disc)
1	Levofloxacin (LE)	5 µg
2	Ciprofloxacin (CIP)	5 µg
3	Cefixime(CFM)	5 µg
4	Ampicillin(AMP)	30 µg
5	Chloramphenicol (C)	30 µg
6	Amoxicillin(AMX)	30 µg
7	Azithromycin(AZM)	30 µg
8	Gentamycin (GEN)	10 µg
9	Tetracycline (TE)	30 µg
10	Erythromycin (E)	15 ug

Source: (CLSI, 2015)

3.6.13. Maintenance of stock culture (Glycerol solution)

Glycerol solution was used for preservation and long term storage of the isolated colonies. This stock culture was used to maintained microorganism and for the purpose of keeping the microorganism in a viable condition and also used for further identification of the organisms**.** This was prepared by mixing nutrient broth with glycerol solution. Thereafter, the nutrient broth was mixed with equal volume of 20% Glycerol solution and bacterial isolated colonies and then was dispensed into the cryovials for the inoculation of isolated colonies. For long term storage of the isolates, inoculated vials were stored at –20°C. Moreover, the isolated organisms were given code name and ID for convenience identification and indication.

3.7. Methods

The following methods were used for the isolation and identification of bacteria.

3.7.1. Experimental Layout

The experimental work was divided into following steps: First step physio- chemical analysis. Secondly, bacteriological analysis comprised enumeration of total viable count (TVC), total colifrom count (TCC), total staphylococcal count (TSC) and total *Salmonella-Shigella* (TSS) for the determination of sanitary quality. Thirdly cultural properties were performed for the isolation and identification of the bacteria of the collected sample using cultural, staining and biochemical characteristics.

EXPERIMENTAL LAYOUT

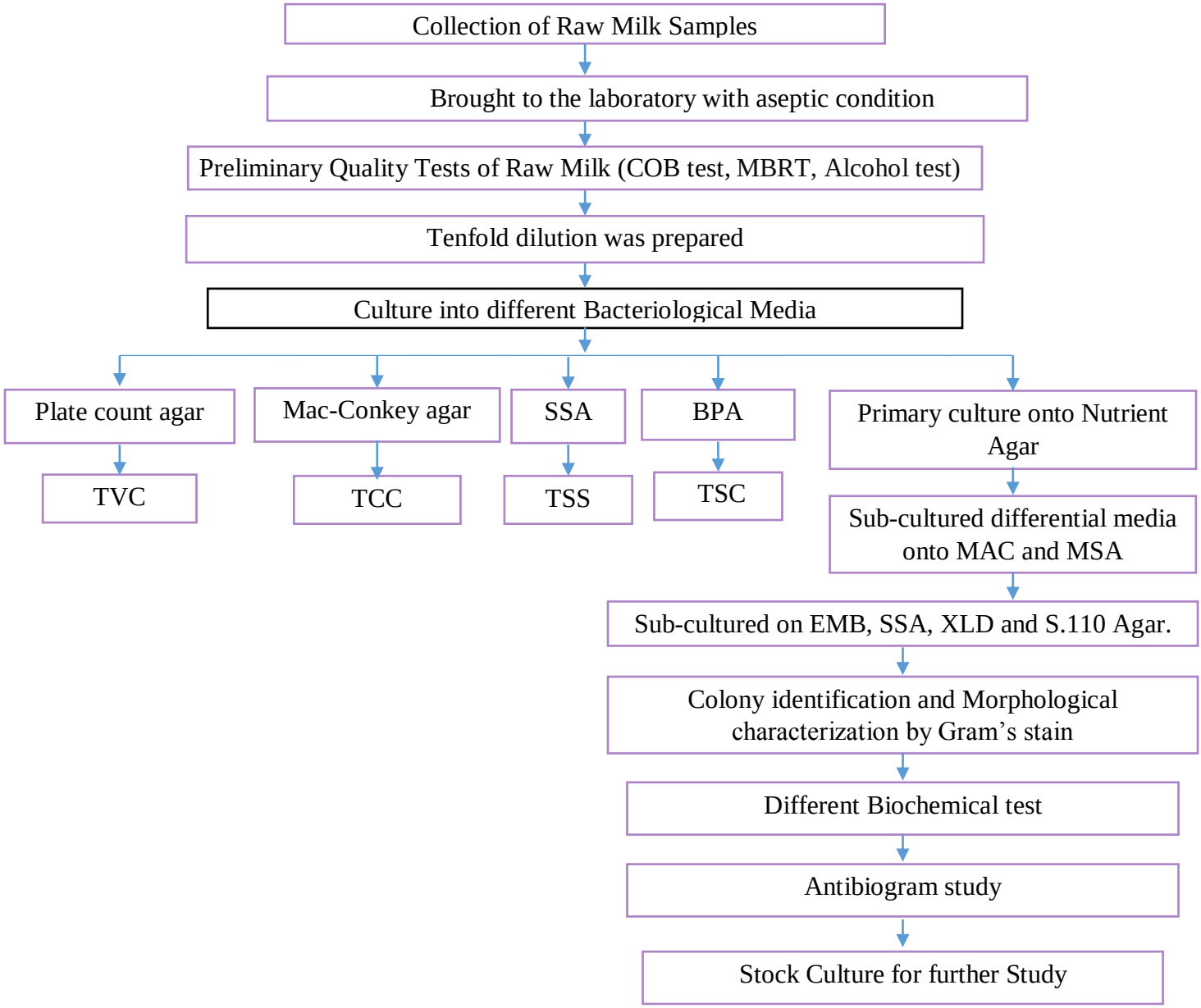

Fig. 2: The schematically illustration of layout of the experiment

3.7.2. Laboratory Analysis and Milk Samples Procure

Laboratory analyses were carried out in the Hajee Mohammad Danesh Science and Technology University (HSTU), at Dinajpur using conventional microbiological testing of different methods. First was analysis and checking preliminary the quality test included organoleptic of physical examination, Clot-On-Boiling Test**,** Alcohol test, Methylene blue reduction test and Standard plate count (SPC) were analyzed. Along with bacteriological investigation comprised enumeration of total bacterial count (TBC), total coliform count (TCC), *Salmonella-Shigella* count (TSS) and total staphylococcal count (TSC) for the determination of sanitary quality and of milk contamination from dairy producers and vendors and test sample for the presence of pathogenic bacteria. In this test of bacterial load were considered, a few selected pathogens including *E. coli*, *Salmonella* spp., S. aureus and were targeted. Bacterial isolates were then identified according to the Bergey's manual of determinative bacteriology (Buchanan and Gibbon, 1984), and manual for the identification of medical bacteria (Cowan and Steel, 1996).

3.7.3. Sampling and handling of milk samples

Milk samples were collected from all the actors along the dairy value chain. In that aspect, milk samples were collected from farmers, vendor shops/milk selling points and cafeteria, milk at farm level, milk samples were obtained directly from the containers used during milking, distribution and storage. About 15 ml of milk sample was collected and put in a sterile falcon tubes and placed in a cool box with ice packs. Thereafter within two to six hours' samples were transported to the Hajee Mohammad Danesh Science and Technology University (HSTU)and stored at -80ºC until analysis. Samples were prepared according to the technique recommended by (ISO8261: 2001). Types of milk samples collected are summarized in Table (2).

Table 2: Types of milk samples collected for laboratory analysis

Type of milk	Source	No. of sample
Raw milk	Dairy farmers	25
	Vendor shops	20

3.7.4. Evaluation of Bacteriological Quality of Milk Tests

Sanitary methods of handling milk must be strictly adhered to rigidly in order to provide safe milk for human consumption. Furthermore, since milk is a good growth medium, even a small number of non-pathogens can multiply considerably if the milk is not kept refrigerated. Because the consumer has no way of knowing whether or not the milk delivered to the home or purchased in the store is contaminated, a number of standard tests are carried out periodically on milk in that area. From the results of these tests, milk is classified into grades designated as Excellent, Good, Fair and poor while decolorization were marked as A, B, C and D (Volk and Wheeler, 1980). Tests commonly employed to determine the quality of milk include plate count, dye-reduction (Methylene blue reduction, Alcohol test, and clot on boiling (COB).

3.7.4.1. Preliminary Quality Milk Test

3.7.4.1.1. Physical Examination (Organoleptic tests)

This test is performed first and involves assessing the milk with regard to its smell, appearance and colour. This test is quick and cheap to carry out, allowing for segregation of poor quality milk. No equipment is required, but the tester should have a good sense of sight and smell. Milk that cannot be adequately judged in this way is subjected to tests that are more objective (Lore *et al*., 2006). The organoleptic test should be the first test to be carried out on all milk received at the dairy producers and vendor shops. The poor quality milk should be immediately noted and obviating the need to proceed with other quality control tests (Kurwijila, 2006). The rapid segregation of low quality milk samples at milk receiving platform was based on (Shirai *et al*.,1992). Milk grade should have good colour, flavour and texture (sight, smell and taste) based on the procedure described by (Lore *et al*., 2006).

3.7.4.1.2. Alcohol test

This test was also performed according to method described by (Tassew and Seifu, 2011). The alcohol test was done by using a 68% ethanol solution. Tests were done immediately after the samples were delivered to the laboratory. A quantity of 5 ml of milk sample and 5 ml of 68 percent ethanol were placed in test tube. The test tube was inverted several times with the thumb held tightly over the open end of the tube and then

the tubes were examined for formation of curd particles denotes positive alcohol test. Such samples were rejected as negative for the summarized result.

3.7.4.1.3. Clot-On-Boiling Test

This test was performed according to the method described by Bari (2001). A quantity of 5 ml of milk was placed in a test tube and it was placed in the boiling water for five minutes. Finally; the test-tube was carefully removed from the water bath and examined the sample showing precipitated formation and recorded as positive C.O.B. test while sample milk that did not showed floccules or precipitated particles were recorded as negative. The milk also assessed for the presence acid milk or abnormal milk (e.g. colostral or mastitis milk) to assess milk acidity**.**

3.7.4.1.4. Methylene blue reduction test

Methylene blue is a blue-colored reagent which is used to estimate the bacterial population of a given milk sample. A known dilution of the methylene blue solution is added to the milk sample and observation is made at fixed intervals until the blue color disappears. The number and species of organisms present in the milk determines the time required for the disappearance of the blue color in the milk (Kurwijilla *et al.*, 2006). This test is usually used for grading the quality of raw milk before pasteurization. On the basis of this test, raw milk is graded as follows; Very good: not decolorizing in 5 hours, Good: decolorized in less than 4 hours, but not less than 3 hours. Fair: decolorized in less than in 2 hours, but not less than 1 hour and poor: decolorized in less than ½ hour as described (Yirsaw, 2004). Therefore, dye reduction time is inversely proportional to the presence of total number of bacteria in sample; hence the greater the bacterial population, the shorter is the dye reduction time (Teka, 1997).

3.7.4.1.5. Standard plate count (SPC)

The standard plate count of raw milk gives an indication of the total number of bacteria present in the milk at the time of pick up. Obviously, very clean milk will have lower bacterial counts than milk collected or handled under unsanitary conditions. The standard plate count is a basis for grading milk (Volk and Wheeler, 1980). Milk samples are plated on standard plate count agar media and then incubated for 48 hrs at 37^{o}c to encourage bacterial growth. Single bacteria or clusters grow to become visible colonies that are then counted. All plate counts are expressed as the number of colony forming

units (CFU) per milliliter (Murphy,1996). This method is used mainly to estimate the bacterial population of raw milk prior to heat treatment. The standard plate counts generally accepted as the most accurate and informative method of testing bacteriological quality of milk (Godefay and Molla 2000). It is sensitive but also labour intensive and is inaccurate for high count milks (Slaughuis *et al*., 1996). Plate count standards have been developed to ensure satisfactory production hygiene and that the product is safe (Table 3). The plate count method has been conducted as a valuable adjunct to guide sanitarians in correcting sanitation failures and improving milk quality (IDF, 1990).

Table 3: Grade of raw milk based on SPC

Bacterial count/ml	**Grade**
Not exceeding 200,000	Very good
200,000 – 1,000,000	Good
1,000,000-5,000,000	Fair
>5,000,000	Poor

Source: Kurwijilla *(*2006) and Yirsaw, (2004).

3.7.5. Bacteriological Quality of Raw Milk Sample

3.7.5.1. Preparation and Examination of milk samples for determination of sanitary quality

All steps of preparation of milk samples and determination of their sanitary quality are carried out using sterile sampling equipment and procedure as per recommendation of International Commission for the Microbiological Specification of Foods (1987). The bacteriological tests considered for determination of the bacterial load in raw milk samples were total viable count (TVC), total staphylococcal count (TSC) total coliform count (TCC) and total *Salmonella-Shigella* count (TSS). For these four procedures of standard plate count agar (PCA), Baird-Parker (BP), Eosin methylene blue (EMB), Deoxycholate citrate agar (DCA) agar were plating on selective media respectively. These bacterial load was determined by standard method as described by ICMSF (1998). The obtained results of the above tests were then compared with the limit of Gulf standard, recommended microbiological standards as described by (Rehman *et al.*, 2011). All the bacterial counts were the average of at least three independent

experiments. Peptone water and buffer peptone water and were used for serial seven-fold dilutions (10^{-7}).

3.7.5.2. Preparation of serial dilution, inoculation and incubation

Buffer peptone water (BPW) was used as the diluent. This was prepared by diluting 15.23 grams of peptone water powder into 1000ml of the distilled water. A total of 10 tubes were dispensed with 9 ml of sterile (BPW). Tenfold serial dilution of the samples was made up to 10^{-7} in sterile normal saline. Then, 1 ml of the milk sample was added into the 9 ml peptone water (10^{-1} dilution). After complete solidification, all the Petri dishes were inverted and placed in the incubator at 37 ^{o}C for 24 hours to allow for bacterial growth. After the incubation period, bacterial colonies on the culture plates were countered manually. Two consecutive plates with countable colonies were considered for record (ISO4833-1: 2013).

3.7.5.3. Bacteriological Count

After the incubation period, bacterial colonies on the culture plates were countered manually. Two critical dilutions per each sample were counted. A plate was divided into quarters using a marker-pen and colony forming units were counted on at least two critical dilution plates by the without of colony counter. Two consecutive plates with less than 300colonies were considered for record (ISO 4833-1:2013).The countable bacterial colonies from two consecutive plates of each sample were converted into colony forming units per millilitre (cfu/ml) using the formula of Number of CFU/ g = Number of CFU/ (Volume plated in ml × total dilution used).

3.7.5.4. Total Viable Count (TVC)

Standard procedures described by (Rodrigues *et al.,* 2017) was used for total viable count of the collected milk samples. Tenfold dilution was prepared by transferring 1 ml of milk sample to 9 ml of Buffer peptone water (BPW. For the enumeration of total bacterial count, 1 ml of each tenfold dilution was transferred and spread on seven plate count agars (PCA) using a fresh pipette for each dilution. The plates were then kept in an incubator at 30°C for 24-48 hours. Following incubation plates exhibiting 30-300 colonies were counted. The total bacterial count was calculated according to (ISO 4833: 2003). The results of the total bacterial count were expressed as the number of organism or colony forming units per gram (CFU/gm) of milk sample (Richardson, 1985).

3.7.5.5. Total Staphylococcal Count (TSC)

For the determination of TSC the procedures of sampling, dilution and streaking were similar to those followed in total bacterial count. Only in case of staphylococcal count, Baird-Parker (BP) agar medium was used. Media were inoculated and after incubation at 37°C for 24 hours. Thereafter typical convex shaped and surrounded by a clear zone colonies were counted. The total staphylococcal count was calculated according to (ISO 6888-3: 2003). The results of the total Staphylococcal count were expressed as the number of organism or colony forming units per gram (CFU/gm) of ice cream sample (Richardson, 1985).

3.7.5.6. Total Coliform Count (TCC)

Total coliform was determined by the same method used in the enumeration of total viable bacteria. The medium used for coliform was MacConkey agar. Inoculated plates were incubated at 37°C for 24 hours. The colonies that were considered for total Coliform Count were only those that were dark red in colour with metal sheet. The total coliform count was calculated according to (ISO 6888-3: 2006). The results were expressed as the number of organism or colony forming units per gram (CFU/gm) of milk sample (Richardson, 1985).

3.7.5.7. Total *Salmonella* and *Shigella* count (TSSC)

Salmonella Shigella agar which is a selective and differential medium for the isolation of enteric pathogens was used for the isolation and enumeration of *Salmonella* and *Shigella* by means of direct plating method. For the determination of total *Salmonella-Shigella* count the procedures of sampling, dilution was detected as per the procedure outlined by FDA (2001) which were similar to those followed in total Coliform Count (TCC). Media were inoculated and after incubation at 37°C for 24 hours. Typical colonies were counted and total *Salmonella-Shigella* count was calculated according to (ISO, 1995). The results of the TSSC were expressed as the number of organism or colony forming units per gram (CFU/gm) of milk sample (Richardson, 1985).

3.8. Isolation and Identification of Major Pathogenic Bacteria from Raw Cow Milk Samples

3.8.1. Isolation and Identification of *Staphylococcus aureus*

Isolation and identification of *Staphylococcus* spp were performed as per procedures described by (Carter, 1979). After the serial dilution of peptone water and primary culture was performed in Nutrient agar. Then were sub-culturing was performed on Mannitol (MS) agar, Bair pairk agar media and pure culture was obtained by used *Staphylococcus* agar no.110 media. The representative Staphylococci isolates colonies was performed by Gram staining according to the methods described by (Merchant and Packer, 1967) to determine the size, shape, and arrangement of bacteria. Stained slides were examined under light microscope at 100X magnification and Trinocular microscope with immersion oil to enhance visible of colony morphology. Subsequently, Isolated organisms were subjected to biochemical tests included catalase test, oxidase test, triple sugar iron (TSI) agar slant reaction, methyl red-Voges Proskauer (MR-VP) test, indole reaction, and motility indole urease (MIU) test as procedure mentioned by (Cheesbrough, 1985 and Jahan *et al.*,2015).

3.8.2. Isolation and Identification of *Escherichia* coli

The methods used in isolation and identification *Escherichia* coli were determined according to the method previously described by (Addo *et al.*,2011). For each sample, dilutions were made by aseptically withdrawing 1 mL of each sample into 9 mL of 0.1% sterilized buffered peptone water, then serial dilutions were prepared. A 10 µL was drawn from appropriate dilutions and plated on EMB Agar. The sterile glass beads were used to spread the sample on agar, and plates were incubated at 37 ºC for 24 h. The positive colonies which showed circular colonies with greenish-black colonies with metallic sheen pink colour were subculture to obtain pure colonies and transferred on MaCconkey agar and incubated at 37°C for 24 Hrs. Five to six pink to red colour of lactose fermented were randomly picked, and subsequently sub cultured on fresh EMB agar plate showed circular colonies with greenish-black colonies with metallic sheet. The presumptive identified colony was performed by Gram's staining to determine *E. coli* as microscopically characteristics. Bacterial isolates were further biochemically characterized by Indole, Methyl red, Voges Proskauer, Citrate utilization test (IMViC test) and Triple Sugar Iron Agar (TSI) test. Appropriate positive and negative controls

were used to make a distinction between positive and "false-positive" reactions as per the procedure described by (Butland, *et al.,* 2008).

3.8.3. Isolation and Identification of *Klebsiella*

Isolation and identification of *Klebsiella* were performed according to the method described by Carter (1986). Initially samples were enriched in nutrient agar(NA) at 37º C for 24 hours. The overnight cultures were streaked on MacConkey agar and then the likely suspected colonies of sample showed bright pink or red colonies were identified as lactose fermented was transferred and sub-cultured onto Eosin Methylene Blue (EMB) agar the growth was indicated, large mucoid, pink to purple colonies with no metallic green sheen on EMB agar. The obtained bacterial colonies were examined macroscopically for colony morphology (shape, color, and arrangement) and microscopically by Gram's staining. Single isolated colony was picked for the preparation of smear and stained for the examination of morphological characters of the isolates as per procedures described by (Cheesbrough, 1985). The isolated strains were subjected to a series of different biochemical tests using the procedure of (ISO, 2003) to confirm *Klebsiella*. Catalase test, indole production test, Methyl red test, Voges-Proskauer test, MR-VP medium, motility indole urease (MIU) and Simon citrate agar performed on all suspected isolates to confirm the *Klebsiella* as described (Chandrasekaran *et al.*,2014).

3.8.4. Isolation and Identification *Salmonella* spp and *Shigella*

Total *Salmonella- Shigella* count were carried out using tenfold dilution of samples up to 10^{-7} dilution by buffer peptone water. Before performing the test, a pure culture of the organism was allowed to grow in Nutrient agar (NA). The colonies showing the desired morphology and colour were again streaked on MacConkey agar plates showing the presence of colourless and transparent colonies were considered for further identification. Sample of non-lactose fermenter were taken and sub-culture were streaked on the Hekton enteric agars (HEA), Xylose- lysine decarboxylate (XLD), Brilliant Green agar (BGA) and *Salmonella-Shigella* agar (SSA) and incubated at 37°C for 24 hrs. The Gram staining was performed by taken a small colony from the representative *Salmonella-Shigella* colonies were picked up from MC, SS, HEA, XLD, and BGA plates to determine the size, shape, and arrangement. The colonies suspected to be of *Salmonella* and *Shigella* species were subjected to biochemical tests which consisted of

triple sugar iron (TSI) agar, simmon's citrate agar, motility indole urease (MIU), Indole reaction and MRVP broth. Then tubes were kept in an incubator for 24,48 or 72 hours at 37 ^{0}C respectively. An alkaline slant with acid (yellow colour) butt on TSI with hydrogen sulphide production, positive for lysine (purple colour), negative for urea hydrolysis (red colour), negative for tryptophan utilization (indole test), negative for Voges proskauer (yellow-brown ring), and positive for citrate utilization (blue colour) were considered as *Salmonella* positive. The isolate *Salmonella* were identified according to the previously as described (Amagliani *et al.*, 2012).

3.8.5. Antibiotic sensitivity of isolates

The bacterial isolates from raw milk samples were tested against five species isolated were included: Ciprofloxacin (CIP); Chloramphenicol (C); Erythromycin (E), Levofloxacin (LE), Amoxicillin (AMX), Azithromycin, Gentamycin (GEN), Cefixime (CFM), Ampicillin (AMP), Tetracycline (TE) to know the sensitivity, resistance and intermediate pattern of isolates bacteria using Kirby Bauer Method (Bauer *et al,* 1996).

3.9. Statistical Analysis

Data obtained from a questionnaire survey and observational studies of bacteriological quality and the results of the laboratory investigations were entered into MS-excel spread sheets. Data was analyzed using Statistical Package for Social Sciences (SPSS) software, version 25.0 with descriptive statistics used to summarize the results and chi-square was used to determine to compare the between above and below the accepted limit of CFU/mL count within and between variables. For statistical inference, the level of significance was taken as 0.05 was considering as a significant association at 95% level of confidence.

CHAPTER IV

RESULTS

4.1. Questionnaire Survey and Observation

In this study, from total 45 (25 farmers, 20 vendors) of the respondents were interviewed and the result from questionnaire showed that majority of the participants were males in both dairy farms and vendors (83%) in overall mean compared to females (17%).The highest age proportion of the respondents age were ranged 18-40 years which accounts about (67%)while the rest of the respondents were above 40 years which holds (33%) in the study sites (Table 4). Regarding in this study, males constituted a large part of respondents in all categories. About their level of education, the majority of (62%) had completed primary education whereas (25%, 13%) of owners had completed secondary and college/university respectively (Table 4).

Table 4: Demographic characteristics and distribution of the respondents (N=45).

Variables	Categories	Dairy farms (%)n =25	Vendors (%)n=20	Overall mean (%)
Gender	Male	19 (76%)	18(90%)	83%
	Female	6(24%)	2(10%)	17%
Age	15-40	16(64%)	14(70%)	67%
	Above 40	9(36%)	6(30%)	33%
Educational level	Primary education	15(60%)	13(65%)	62%
	Secondary education	6(24%)	5(25%)	25%
	College/university	4(16%)	2(10%)	13%

4.2. Practices related to the animal management and hygienic condition of dairy farms

All farms included in this study had (84%) cross-breed (Holstein-Friesian with indigenous) lactating cows but (16%) of them of were keeping local breed. The overall average amount of milk produced by local breed cows was 1.4 litter /day for 180 days of lactation. The improved cows produced 11 litter /day for 263 days of lactation length (Table 5). The study farm types were categorized into three groups based on the number of dairy cattle owning as small scale (52%), medium scale (36%) and large scale (12%) dairy farms. Likewise, management systems were also grouped as the semi-intensive and

intensive system (72% and 28%) accordingly. According to the response of the interviewers and observations (44%, 20%) stated that cattle were reared in open and house confirmed respectively. However, (36%) showed that cattle were kept in both housing systems. Similarly, the response from respondents indicates that the studied farms were mostly used feedstuffs like natural grass and Stover, roughage and concentrate follow by mixed both as shown by (10%, 44%, 20% and 28%) of the respondents respectively (Table 5). A mix of Roughages with concentrates was main feed compared to the other feed stuff but they were not feed concentrated only due to heath aspect. The majority of the respondents were indicated that were clean the barn daily basis (88%), twice a day (8%) and (10%) were reported that they clean twice a day and ones two days respectively. General information on type of houses, management, types of animals owned and animal health aspect were summarized in (Table 5).

Table 5: Summary the types of livestock kept, management cleaning practiced of dairy farmers with the respective proportion of the respondents (n= 45).

Variables	**Categories**	**Dairy farms(%) =25**
Cattle breed	Local breed	4(16%)
	Cross breed	21(84%)
Farm type/herd size	Small scale	13(52%)
	Medium scale	9(36%)
	Large scale	3(12%)
Management system	Intensive	7(28%)
	Semi-intensive	18(72%)
Type of Housing	Confirmed/ Closed	5(20%)
	Open air house	11(44%)
	Both	9(36%)
Feeding source	Natural grass only	2 (10%)
	Roughages and Concentrates	11(44%)
	Minerals	5(20%)
	A mix of both	7(28%)
Barn/house cleaning interval	On daily	22(88%)
	Twice a day	2(8%)
	Ones two days	1(4%)

4.3. Hygienic practices during milking, storage and distribution of milk dairy farms

Generally, it was observed that (100%) of all interviewed farmers were done milking manually in entirely dairy producers (Table 6). About, (72%) of the interviewed farmers indicated that they were washing hands before milking and only (28%) did not clean their hands rather they massage the udder with bare hands. Similarly, most of the dairy milk producers cleaned the udder and teats of cows before milking. Moreover, (88%) of respondents said that they wash the udder of milking cows before milking and (12%) of them did not wash the udder or clean cow teats before milking. However, it was observed that most of them did not use detergents for cleaning of udder and teats rather they cleaned only by tap water. During observation, it was observed that (64%) of the dairy producers was use the common towels for udder drying, (32%) of them used individual towels in each cow to dry udder and (8%) reported they practice bare hand and never used towel to dry the udder their hand during milking (Table 6). Those farms that had towels, they reuse it for cleaning and sanitizing of other material. This may result in recontamination of the udder. Besides the hygiene milking environments and the person involving milk was looked unhygienic and there was opportunity increase of microbial contamination of milk and bacteria can access to the milk through colonization of the teat canal or spread mastitis.

Accordingly, (80%, 20% and 4%) of the respondents had access to wells/bore holes' water, tap water and river water respectively for sanitary including washing hands, udder milk utensils and/or equipment washing. Among the interviewed of (96%) were milking their animals twice a day while (4%) were milking one a day. All respondents understood that the quality of milk was mostly related to cleanliness of containers and milking practice at farm level. More than (72%) of the interviewed dairy producers reported to use aluminum container (buckets) during milking whereas (28%) used plastic containers. As observed the utensils cleaning was moderate but not efficient and not well dried and there were the possibilities indicated for microbial contaminations of milk. The water used for cleaning/washing purpose in (76%) was used cold tap water with soap while about (24%) had used warm water with soap. The most common means of milk transportation to final destinations was mainly done using public transport, motorcycle /bicycles, private cars (56%, 32%, 8%), respectively and remaining (4%) was used on foot. The process between collection and delivery was taking 2 -5 hours during transactions. However, these vehicles were not appropriate for raw milk transportation because

its lacks cooling facilities and stored at room temperature until completion of selling and there were the possibilities indicated for microbial contaminations of milk.

Table 6: Hygienic practices of milk during milking, storage and distribution of milk dairy farms

Variables	**Categories**	**Dairy farms(%) =25**
Milking method	Hands	25(100%)
	Machine	0(0.0%)
Washing hands before milking	Yes	18(72%)
	No	7(28%)
Wash udder and teats before milking	Yes	22(88%)
	No	3(12%)
Udderand hand drying	Common towel	16(64%)
	Individual towel	8(32%)
	Bare hand	3(8%)
Water sources used for cleaning	Tap	5(20%)
	Wells/bore holes	20(80%)
Milking frequency per day	Once a day	1(4%)
	Two times a day	24(96%)
	Three times a day	1(4%)
Utensils for milking	Plastic container	18(72%)
	Metal/ Aluminum	7(28%)
Containers Washed with	Cold tap water with soap	19(76%)
	Warm tap water with soap	6(24%)
Means of delivery of transport	On foot	1(4%)
	Public	14(56%)
	Private car	2(8%)
	By motorcycle / bicycles	8(32%)

4.4. Hygienic condition and practices towards respondents on dairy farms and vendor shops and their public health awareness.

The results of this study showed that all dairy producers and vendor shops interviewed were cleaned their storage milk containers used different cleaning methods (Table 7). It was observed that the microbiological quality of water using during cleaning was uncertain, contributing to contaminate the milk. Overall, about (35%, 44% and 21%) used water with supplemented soap and detergents, tape water/normal and hot water respectively to wash milking equipments, milk storage and transportation containers of milk. Thus, to use water only with no detergent and tape water for cleaning can contribute to the poor quality of milk and there was a possible source of milk contamination. As observed in the present study (52%) of the vendor shops usually keeping milk used refrigerator to maintain a low temperature and prevent a high microbial contamination and to increase the shelf of milk while (48%) of all dairy farmer's respondents and some of vendor shops were store milk at room temperature milk after milking and delivering. Also vehicles were not appropriate for raw milk delivery because its lacks cooling facilities. since there was no refrigeration facility and other cool system the milk after milking and delivering (Table 7).

The survey results showed that milking cleaning utensils method and equipment were common among most of the interviewees of dairy farmers and vendor shops. Overall, about (35%, 44% and 21%) were water with detergent/disinfected, tape water/normal and hot water respectively to wash milking equipments, milk storage and transportation containers of milk (Table 3). Thus, to use water only with no detergent and tape water for cleaning can contribute to the poor quality of milk and there was a possible source of milk contamination. With regarding the respondents and direct observation, it was found that the dairy producers and vendor shops were commonly used storage plastic containers, plastic bags and soda/ water bottles (47%, 18% and 35%) respectively (Table 7).

Regarding the producers or milk suppliers' delivery milk to the retailers and the sellers were stored the storage plastic containers and filled- fitted with plastic bag, bottles included soda and water bottles as to sell their consumers (Table 7). However, fitting of milk storage containers with plastic bags and bottles was not the safe procedure as it contaminates the milk and making it unsafe for consumption. Findings of this survey

have shown that (27% and 31%) of milk retailers were reported to sold milk to the household and individual customer respectively whereas the rest of (43%) were sold milk and both customers of individual and household customers. As observed in the present, about (72%) of the interviewed producers and vendor shops consume boiling milk while the remaining (28%) raw milk before consumption. The survey data showed that, (98%) of the milk producers and vendor shops did not testing the quality of milk, while only (2%) of such dairy producers were employed to testing milk quality by using lactometer (Table 7).

Despite the fact, about (72%) respondents were aware about the risk knowledge of public health hazards associated with consumption of unboiling cow milk however, (28%) did not aware risk of milk consumption and there is potential risk of contamination by zoonotic pathogens. Likewise, as indicated in (Table 6), about (78%) different dairy farms and vendor shops consumers had no experience or unware of zoonotic potential and milk borne pathogens which concerned with milk safety and most of the respondents reported they were suffered from food borne infections of unknown origin. At the same time, the consumers were not conscious that *Staphylococcus* , *Salmonella*, and *E. coli* and other diseases can be transmitted from animals to humans through drinking raw milk or not well boiling. In the study areas prioritized milk quality related constraints by the respondents during questionnaire and observation. About, (33%) of dairy producer and (67%) of vendor shops respectively were reported that they were limited awareness on hygienic handling of milk (Table 7).

Table 7: Hygienic condition related to quality milk production among dairy farmers and vendors and their public health awareness (N=45)

Variables	Categories	Dairy farms (%)n=25	Vendors (%)n=20	Overall mean
Type of Cleaning of Storage	Water with detergent	16(56%)	3(15%)	35%
	Hot water	3(12%)	6(30%)	21%
	Normal /tap water	8(32%)	11(55%)	44%
Storage method before selling milk	Use of refrigerator	2(8%)	19(95%)	52%
	At room temperature	23(92%)	1(5%)	48%
Type of milk sold on dairy farms and vendor shops	Raw milk	25(100%)	19(95%)	98%
	Boiled milk	0(0%)	1(5%)	2%
Who were Customers?	Household	7(28%)	5(25%)	27%
	Individual customers	8(32%)	6(30%)	31%
	Both	10(40%)	9(45%)	43%
Containers used to stored milk	Storage plastic container	21(84%)	2(10%)	47%
	Plastic bag	0(0.0%)	7(35%)	18%
	Soda/water bottles	4(16%)	11(55%)	35%
Habit of milk consumption	Raw milk	2(8%)	4(20%)	14%
	Boiling Milk	23(92%)	16(80%)	86%
Practice of testing quality	Yes	1(4%)	(0%)	2%
	No	24(96%)	20(100%)	98%
Awareness on risk of getting diseases	Yes	21(84%)	12(60%)	72%
	No	4(16%)	8(40%)	28%
Experience of suffering from food borne infection	Yes	6(64%)	4(20%)	42%
	No	19(76%)	16(80%)	78%
Limited awareness the hygienic quality of milk and training	Yes	9(36%)	6(30%)	33%
	No	16(64%)	14(70%)	67%

4.5. Bacteriological Quality of Raw Milk Sample

A total of 45 liquid milk samples, 25 raw milk samples from dairy farm designated as small scale, medium scale and large scale respectively and while the rest of 20 sample were collected from different vendor shops. The required were (denoted as V1, V2, V3 and V4). The raw milk samples were subjected to bacteriology laboratory of Hajee Mohammad Danesh Science and Technology University (HSTU) to determine the microbial load and microbial quality test of raw milk.

4.5.1. Preliminary Quality Tests of Raw Milk

The different preliminary quality tests of milk were performed included to Organoleptic properties, alcohol test, clot-on-boiling test and Methylene blue reduction test. In addition, standard plate count (SPC) was used to determine bacteriological quality of raw milk. Milk quality test was an important aspect of milk quality for both health and processing into different milk products. This also is used as criteria when processors were developed a quality scheme payment to farms. A Total of 45 milk samples were collected from small holder farms and Vendor shops to ensure and measure the milk quality analysis based on the above method. The result of milk quality was summarized (Table 6, 7, 8).

4.5.2. Physical analysis milk test (Organoleptic evaluation)

Sensory analysis was examined by a panel of experts with the help appearance score to assess consumer's acceptance of milk quality. The organoleptic properties of milk such as color, flavor and texture were evaluated with the help of eyes, nose and tongue respectively as per standard score card procedure described by (ISO, 1995). Current study the panelist staffs from Laboratory Microbiology at (HSTU) were selected to assess the sensory acceptability of milk that collect from the study sites. Each sample was observed for general appearance of colour by observed (normal colour of milk as yellowish white, slight white), texture (free flowing water or thin or watery) and flavour (normal flavour of milk) and with the help of a panel of expert according to (Khan *et al.*, 2005). The obtained results about the colour, flavour, texture of organoleptic characteristics test were presented in the (Table 8). The physical parameters were studied after collection and sampling of milk from different dairy farms and vendor shops. The physical parameters were mainly organoleptic colour, flavour, texture were presented in the (Table 8).

The Physical analysis of raw milk samples were done in relation to color score of milk samples were presented in (Table 8), the all milk sample from dairy farms (MS and LS) showed (100%) yellowish white while (SS) dairy farms revealed (90%) Yellowish white while the remaining of (10%) was indicate white only (Table 8). In the same way, the colour of all milk samples from different vendor shops were also showed yellowish white and light yellowish white according to (V1, V2, V3 and V4). The only V2 was indicate (100%) yellowish white. Whereby the milk sample collected from (V1, V3 and V4) of vendor shops were yellowish white of (75%, 86% and 70%) and (15%,14% and 30%) were light yellowish white respectively (Table 8). Changes in milk colorway be due to the differences in nature of feeding habit, animal breed fat, casein might change the color of milk because colour of milk depends upon these factors. The animal which eats more consolidated feed has more yellowish or pale color of milk compared to pasture feeding animals. Also to adulteration of milk is another factors that might alter the color of milk like water.

The flavors score of milk were presented in (Table 8), according to flavour all twenty-five (25) samples dairy farms had normal flavour (100%). in Comparatively, all vendor shops had different flavour. Among the vendor shops only V2 had (100%) flavour butV1, V3 and V4 had flavour of (85%, 80% and 95%) while the rest of (15%, 20% and 5%) of milk had no odd flavour at the time of the experiment respectively. This indicated that all milk sample vendor shops were not clean and fresh. (Table 8). This flavour (pleasant aromatic) differences among vendor shops in milk sources are due to addition of long term storage, milk process and lack of storage facilities, lactation period of cow adulterated milk and also due feeding scheme of animal.

Similarly, the texture of raw milk sample was examined before starting the experiment. All the milk samples collected from different dairy farm had normal texture (100%) of free flowing liquid). However, the vendor shops milk sample were varying from them regarding to texture and V1 and V3 (100%) normal texture milk samples but, the V2 and V4 showed (95% and 90%) free flowing liquid while (5% and 10%) shown watery texture respectively (Table 8). The texture difference in milk sample might be due to breed quality of the milking cows or percentage of water in milk. This might also be due to the fact that the farmers take hygienic measures during milking and not to allow the cows to feeding some sorts flavoured feed prior to or during milking their cows. In addition, high level of microbial loads brought about by unhygienic distributions of raw milk also reduces the standard of milk texture.

Table 8: Physical parameters of milk samples collected from two different of dairy farms and vendor shops.

Physical parameter	SS	MS	LS	V1	V2	V3	V4
Colour	90%YW 10% W	100% YW	100% YW	75% YW, 25% LYW	100% YW	86% YW 14% LYW	70% YW 30% LYW
Flavour	100% Normal flavour	100% Normal flavour	100% Normal flavour	85% flavour $15% no flavour	100% flavour	80% flavour 20% no flavour	95% flavour 5% no flavour
Texture	100% Free flowing liquid	100% Free flowing liquid	100% Free flowing liquid	100% Free flowing liquid	95% Free flowing liquid 5% water flowing	100% Free flowing liquid	90% 10% Free flowing liquid

Legend: SS= Small scale, MS=Medium scale, LS= large scale, V1, V2, V3 and V4=Vendor shops, YW= Yellowish white and LYW= Light Yellowish White.

4.5.3. Alcohol and clot-on-boiling tests

The results pertaining to the clot on boiling test and alcohol test are presented in (Table 9). The procedure was performed as per recommendation of American Public Health Association (1960). The both of tests are important in milk processing for identification of abnormal milk and developed acidity. The entire milk samples collected from dairy producers of small scale, medium scale and large, scale were showed negative results for both COB test and Alcohol test which means there was no developed acidity and precipitated formation in milk sample and were a symbol of good quality (Table 9). At the same time, COB tests and Alcohol test of vendor shops of (V1, V2 and V4) were showed positive which indicates that milk was developed acidity and precipitated formation which may be milk adulteration, poor handling practices, keeping and transportation while only V3 was showed a negative which means there was no developed acidity level in milk sample which presented a better quality. The result of the COB test and alcohol test among dairy producers and vendor shops showed that deterioration of milk quality increases as the steps increase towards marketing (vendor shops). The result also shows high level of acid concentration in the milk samples from vendor shops were indicate low quality milk (Table 9)**.**

Table 9: Values for clot-on-boiling and alcohol test of different milk samples from different dairy farms and vendor shops

Dairy farms and Vendor shops (V)			
Milk sources	**No. of milk samp test**	**No. of Alcohol test positi**	**No. of Clot-on-Boiling Test positive**
SS	5	Negative	Negative
MS	8	Negative	Negative
LS	12	Negative	Negative
V1	5	Positive	Positive
V2	5	Positive	Positive
V3	5	Negative	Negative
V4	5	Positive	Positive

V= Represented Vendor shops of different vendors selling milk, **SS**=Small scale, **MS**= Medium scale and **LS**=Large scale dairy farms

4.5.4. Methylene blue reduction test (MBRT)

The decolorization time and grading of the samples on the basis of the methylene blue reduction test were shown in (Table 10). Analyses were performed according to the methods described by (AOAC, 2000) to determine the Methylene blue test (MBRT). To identify the decolonization time of milk quality and to specified grading of each sample from varied dairy farms and vendor shops were selected respectively to check milk quality those representative the entirely milk samples of different site collections. From the result of these test value of milk decolorization time were designated as A, B, C and D. Likewise the results of grades were classified into grades nominated as Excellent, Good, fair, and Poor, which matched to decolorization time. Equally, increase in microbes and decreases the quality of milk graded. The data regarding to methylene blue reduction test(MBRT) was given in (Table 10).

Table 10: Decolonization time and grading of the milk samples on the basis of the (MBRT) in different milk sources.

Dairy farms and Vendor shops (V)			
Site collection	**No. of sample**	**Decolorization time (Hrs)**	**Grade**
SS	3	D	Poor
MS	2	B	Good
LS	2	A	Excellent
V1	3	D	Poor
V2	3	D	Poor
V3	3	B	Good
V4	3	C	Fair

Legends'= Vendor shops, **Excellent(A)=** Decolorization time of more than 8 hours, **Good (B)=**Decolorization time of 6-8hours, **Fair(C)=**Decolorization time of 2-6hours and **Poor(D) =**Decolorisation time of less of 2 hours.

4.6. Bacterial Load and Quality of Milk Samples

According to guidelines elaborated by the International Commission on Microbiological Specifications for Foods (ICMSF, 2005). The level of bacterial contamination was determined using TBC, TCC, TSC and TSS using in colony forming unit (CFU/mL), the findings, where categorized as below and above the accepted limit of standard ($1x10^5$ CFU/mL). The bacterial counting raw milk below 10^5 CFU/g is indicative of their acceptable quality, the bacterial counts of 10^4 to10^5CFU indicates their permissible quality, whereas bacterial count exceeding 10^5CFU/mL is unacceptable. In view of these guidelines, present results showed marginally acceptable quality of the analyzed milk samples (Table 10). The bacterial load had grown within the range that can be counted as recommended by the ISO protocol were examined. The current study indicated the presence of bacterial contaminants in milk samples collected from dairy farms and milk vending shops (Table 10). The enumerations of bacterial counts shown in (Table 11) were from two main sources of dairy farms (SS, MS and LS) and vendor shops (V1, V2, V3, and V4).

4.6.1. Total Viable count (TVC)

Total Viable Count (TVC) of bacteria was carried out on plate count agar media using pour plate techniques. The results, presented in (Table 11), showed that the average TVC (cfu/ml) were 2.6×10^6(log6.4), 1.9×10^3(log3.3) and 1.8×10^3 (log 3.2) for raw milks collected from different sources of SS, MS and LS dairy farms respectively. The TBC for vendor shops of milk samples, (V1, V2, V3 and V4 were4.5×107 (log 7.6), 2.3×10^5 (log 5.6), $1.3x10^5$(log 5.1) and $2.9x10^8$(log 8.4) correspondingly (Table 11).

4.6.2. Total staphylococcal count (TSC)

Staphylococcus Baird-Parker (BP) medium was used for the enumeration of total staphylococcal count (TSC) in the milk samples. The average values of TBC (cfu/ml) were 7.3×10^5 (log 5.8), 2.1×10^4 (log 4.3) and 1.6×10^2 (log 2.2) for SS, MS and LS, respectively. Similarly, the average TBC (cfu/ml) of V1, V2, V3 and V4 were $8.2x10^4$ (log 4.9), $7.6x10^5$ (log 5.8), $8.1x10^9$ (log 9.9) and$7.2x10^5$ (log 5.7) in sequence (Table 11).

4.6.3. Total Colifrom count (TVC)

MacConkey agar medium was used for the enumeration of total colifrom count (TCC) in the milk samples. The average values of TCC (cfu/ml) of milk samples collected from SS, MS and LS were 3.9×10^3 (log 3.5), 4.7×10^3 (log 3.6) and 2.5×10^4 (log 4.3) respectively. The results for vendor shops milks, V1, V2, V3, and V4 were $1.6x10^4$ (log 4.2), 2.3×10^2 (log 2.3), 3.5×10^6 (log 6.5) and $3.7x10^7$ (log 6.7) successively (Table 11).

4.6.4. Total *Salmonella- Shigella* count (TSS)

The average mean measures of TSS (cfu/ml) of milk samples were 2.3×10^2 (log 2.3), 9.9×10^5 (log 5.9), and $2.4x10^3$(log 3.3) for SS, MS and LS respectively. In the raw milks the TSS count (cfu/ml) were The Average raw milks of TSC (cfu/ml) were $2.7x10^2$ (log 2.4), 3.2×10^5 (log 5.2), 1.2×10^2 (log 2 .0) and 3.3×10^3 (log 3.5) for V1, V2, V3 and V4 separately (Table 11).

Remarkably, in comparison within dairy farms, the highest bacterial count was recorded in small scale dairy farms 2.6×10^6cfu/ml (log 6.4). There was strongly statistical significant difference ($p<0.005$) with the mean of bacterial load from all milk samples were greater than permissible limits of $2x10^6$cfu/ml (Table 11). However, the lowest was record medium scale and large scale farms 1.6×10^2 cfu/ml (log 2.2) and1.9×10^3cfu/ml

(log3.3) respectively. Conversely, there was no statistically significant variance (P< 0. 05) among medium scale and large scale dairy farms while analysis and observed bacterial load in milk samples collected from the two dairy farms. Therefore, the bacterial count evaluation and quality of milk samples were observed that the medium scale and large scale dairy farm was superior good quality of milk due to presence of less numbers of bacterial count/load in milk sample that indicates two dairy farms were practice with good hygienic practices like clean udder and teats of cow's, soap using for milking vessels, containers and equipments, hand washing and soap using for milking vessels and comply with good general sanitation (Table,7 and 8).

Similarly, when comparing the bacterial count enumerated of vendor shops, V3 and V4 had shown highest bacterial count of $8.1x10^9$ (log 9.9) and $2.9x10^8$ (log 8.4) in collected milk sample. Whereas the V3 showed lowest bacterial count of 1.2×10^2 (log 2.0). In generally, the all the milk samples brought from on vendor shops were found higher bacterial load than the acceptable level 1 x 10^5 to $2x10^6$cfu/ml according to Kivaria, *et al.* (2006). According to the vendor shops, the overall mean value of bacterial load from milk sample demonstrated in the laboratory and data analysis showed that (V1, V2, V3 and V4) had strong significantly association difference of (P > 0.05). In addition, among all four vendor shops of bacterial count was between the critical points of (p< 0.000). Meanwhile, the mean value of bacterial load demonstrated a limited increase (P > 0.05) from dairy farms to vendor shops level (Table 11). The Highly bacterial count indicated the contamination in raw milk samples which directed by insufficient hygiene at milking or infection occurred from the skin of animals, milkers hands, animals shed and milking utensils. This also might be due to the mean delivery steps of milk transportation from dairy farms to the vendor shops without refrigerator which might give for the high level of contamination. On the other hand, narrow mouthed bottles included soda, water bottles, plastic bags and proper cleaning of milk containers can could contribute for high level contamination. (Table 7 and 8). Therefore, the results of this study showed clearly disregard and lack of interest accorded by small scale dairy farms and vendor shops to the hygienic practices as shown (Table 9).

In the present study, the milk samples with a bacterial load ranging from $1x10^6$ to $5x10^6$ CFU/mL were considered as poor quality as suggested by (Sherikar *et al.*,2004). but samples with bacterial load of less than $2x10^5$CFU/mL were graded as good quality. According the to present study, the total milk collected (48%) from the different farm

settings and (60%) from milk vending shops were graded as poor quality respectively while (52% and 40%) from diary and vendor shops were graded and considered as good quality (Table 12).

Table 11: Summary of bacterial count from milk samples presented as CFU/ml and Log form

Sample source	No. of sample	TVC (cfu/ml)	Log	TSC (cfu/ml)	Log	TCC (cfu/ml)	Log	TSS (cfu/ml)	Log	P-value
SS	5	2.6×10^{6}	6.4	7.3×10^{5}	5.8	3.9×10^{3}	3.5	2.3×10^{2}	2.3	0.001
MS	8	1.9×10^{3}	3.3	2.1×10^{4}	4.3	4.7×10^{3}	3.6	9.9×10^{5}	5.9	0.074
SL	12	1.8×10^{3}	3.2	1.6×10^{2}	2.2	2.5×10^{4}	4.3	$2.4x10^{3}$	3.3	
Vendor shops										
V1	5	4.5×10^{7}	7.6	$8.2x10^{4}$	4.9	$1.6x10^{4}$	4.2	$2.7x10^{2}$	2.4	0.000
V2	5	2.3×10^{5}	5.6	$7.6x10^{5}$	5.8	2.3×10^{2}	2.3	3.2×10^{5}	5.2	
V3	5	$1.3x10^{5}$	5.1	$8.1x10^{9}$	9.9	3.5×10^{6}	6.5	1.2×10^{2}	2.0	
V4	5	$2.9x10^{8}$	8.4	$7.2x10^{5}$	5.7	$3.7x10^{7}$	7.6	3.3×10^{3}	3.5	

Table 12: Quality of milk samples tested on the basis of bacterial load

Milk Quality grade (Dairy farms and Vendor shops)			
Site collection	**Sample number**	**Good Quality**	**Poor quality**
SS	5	2(40%)	3(60%)
MS	8	3(37.5%)	5(62.5%)
LS	12	8(67%)	4(33%)
Total	**25**	**13(52%)**	**12(48%)**
V1	5	2(40%)	3(60%)
V2	5	1(10%)	4(90%)
V3	5	3(60%)	2(40%
V4	5	2(20%)	3(60%)
Total	**20**	**8(40%)**	**12(60%)**

4.7. The Bacteria Isolated from Raw Milk Sample

The milk samples with high bacterial load and graded as poor quality were further processed for bacteriological examination used differential and selective media and for further biochemical tests. In the course of the study, out of 45 samples 30 were found to be positive among bacteria belong to the five genera were isolated. Accordingly, different bacterial species with their respective prevalence rate were recorded (Table 13). The type of bacteria isolated from raw milk were both Gram negative and Gram positive

organisms. Mostly, Gram-negative bacteria were isolated in raw milk sample including *E. coli*, *Klebsiella* spp, *Salmonella* spp *and Shigella* spp. While Gram positive bacteria was isolated only *Staphylococcus* spp (Table 11). The results showed isolation rate of *Staphylococcus aureus* (33.3%), *E. coli* (23.3%), *Salmonella* spp. (20%), *Klebsiella* spp. (14.2%) and *Shigella* (10.7%). According to the prevalence of isolated bacteria *Staphylococcus aureus* (33.3%) was showed the highest prevalence of bacteria isolated follow by *E. coli* (23.3%). Subsequently, different bacterial species with their respective prevalence rate were recorded (Table 13).

Table 13: Frequency of bacterial species isolated from raw milk samples

Bacterial species Isolated	**Dairy farm**	**Milk vending shops**	**Total Samples Positive no. (%)**
Staphylococcus	4	6	10 (33.3%)
E. coli	3	4	7(23.3%)
Salmonella	3	3	6(21%)
Shigella	1	2	3(10.7%)
Klebsiella	3	1	4(14.2%)
Total	**14**	**16**	**30**

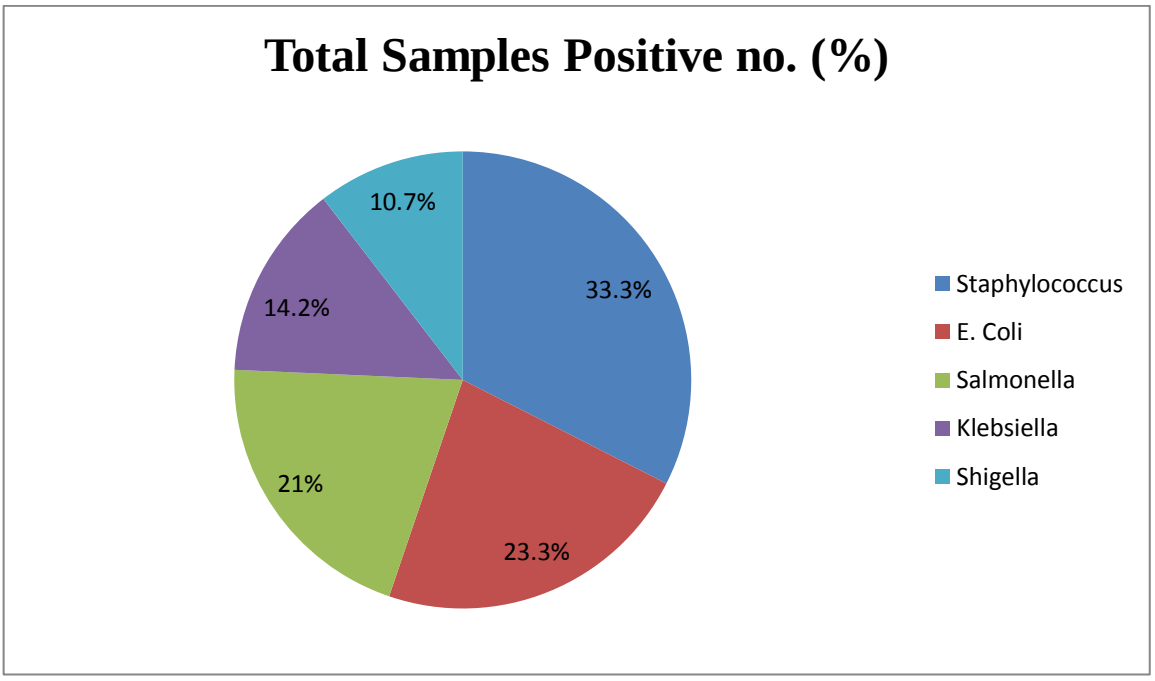

Fig 3: Histogram presenting the difference type of bacteria isolated from raw milk

4.7.1. Results of Isolation and Identification of bacteria with their cultural characteristics

Bacterial isolation and identification was done according to Quinn *et al.* (200). In this study most predominant bacteria isolated and cultured the raw milk sample were *Staphylococcus* spp, E. coil *Klebsiella*, spp *Salmonella* spp and *Shigella* spp. The findings of this study share more likely evidence on the milk quality test and bacterial load in the raw milk sample revealed (Table7, 8 and 9). These bacterial isolates exhibited on the media were presented in (Table 12 and Plate 7, 8, 9, 10, 11, 12, 13,15 and 15) respectively.

Table 14: Cultural characteristics of the bacterial isolates

S/No	Suspected case of Bacteria	Name of Media	Colony characteristics
01	*Staphylococcus* spp	Nutrient agar	Small, regular, circular, entire, smooth, convex, opaque, golden yellow colonies
		Mannitol Salt Agar	whitish colony or yellowish colony
		S. Agar no. 110	Golden yellowish colony
02	*Escherichia*. (*E. coli*)	Nutrient agar	Small, regular, circular, translucent colonies
		MacConkey agar	Lactose fermented colon with Rose pink color
		EMB agar	circular colonies with greenish-black colonies with metallic sheen
03	*Klebsiella* spp	Nutrient agar	Large, regular, convex, opaque, mucoid colonies
		MacConkey agar	Large, regular, convex, opaque, mucoid, lactose fermenting colonies with bright pink
		EMB agar	Large, mucoid ,bright pink lactose fermented colony but no metallic green sheet colony
04	*Salmonella* spp	Nutrient agar	Small, white, transparent dew drop like colony
		SS agar	Small colony with non-lactose fermented with dark black center colony
		XLD agar	Red colonies with black center
05	*Shigella* spp	Nutrient agar	3-4 mm in diameter circular, grayish and smooth and colonies
		SS agar	Translucent colorless colony
		XLD agar	bright pink or red appearance color

Legends'. Agar no. 110= *Staphylococcus* Agar No.110, EMB = Eosin Methylene Blue, SSA=*Salmonella Shigella* Agar

4.7.2. Morphological characterization of bacteria by Gram staining technique

Gram's Method of staining was performed as per the procedures recommendation of Cowan (1985) to study the morphology and staining character of each isolated bacterium. The microscopic examination of Gram's staining smears from plate count agar, Mannitol salt agar, MacConkey agar, *Salmonella* and *Shigella* agar and *Staphylococcus* agar no-110, EMB Medium were examined morphologically and staining characteristics. Gram's negative; pink color, mostly rood shape organisms were shown (Plate 16, 17, 18, 19, 20, 21 and 22). For Mannitol salt agar and *Staphylococcus* agar no-110, Gram's positive; violet color, short cocci were found within bundles and singly arranged. (Plate 15).

Table 15: Identification of isolated bacteria pathogens by Gram's staining technique

Sl. No.	Color	Shape	Arrangement	Grams staining	Identification bacteria
01	Violet color	Cocci arranged	Grape like cluster	Gram positive	*Staphylococcus*
02	Pinkish color	Short plumps rods	Single paired or short chain	Gram negative	*E. coli*
03	Pinkish color	Small rod shaped	Single paired or short chain	Gram negative	*Klebsiella spp*
04	Pinkish color	Very short plump rods	Single paired or short chain	Gram negative	*Salmonella* spp
05	Pinkish color	Small rod shaped	Single paired or short chain	Gram negative	*Shigella* spp

4.7.3. Biochemical characterization of bacteria isolated

The bacteria isolated onto conventional media and characterized Gram's staining showed (Table 11 and 10) was performed for biochemical test as for further confirmation and identification of bacteria isolated. In the present study, five species of bacteria isolate and identify were *Staphylococcus* , *E. coli*, *Klebsiella*, *Salmonella* and *Shigella* spp. The isolate bacteria were characterized by biochemical test used various biochemical test included; catalase test, Oxidase test, indole test, Motility indole utilization test, Methyl red, voges-prokaeu (MR and VP) test and Triple Sugar Iron (Table 14). In addition, results of biochemical tests of different isolates revealed that out of five isolates species, four were gram negative while the rest one was gram positive. All the isolates bacteria

were indicating positive reaction in catalase test except *Shigella* which was showed negative with No bubble formation (Table 14 and Plate 23, 24,25). Likewise, all bacteria isolated in oxidase test were revealed negative but only *Staphylococcus* was showed oxidase positive (Table 14 and Plate 23).

Table 16: Biochemical Properties of the Isolated Organisms

Biochemical test	***Staphylococcus***	***E. coli***	***Klebsiella***	***Salmonella***	***Shigella***	**Plate no.**
Catalase test	+	+	+	+	+	23
Oxidase test	+	–	–	–	–	24,25
Indole test	–	+	–	–	–	26
Methyl red (MR) test	+	+	–	+	+	27
Voges Proskaure (VP) test	+	–	+	–	–	28
Simmons Citrate utilization test	–	–	+	+	–	29
Motility Indole Urease	–	–	+	+	+	30
Triple Sugar Iron (TSI) test	R/Y(Alk/A)	R/Y(Alk/A)	R/Gas(+)	R/Y/H_2S(+)	R/Y/Gas(+)	31

Legends: R/Y=Red slant/yellow butt, Alk/A= Alkaline/Acid, += Positive, - = Negative

4.7.4. Observation of Antimicrobial Susceptibility test of Isolated Raw Milk Sample

Antibacterial susceptibility test was performed on Muller-Hinton (MH) agar by agar disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI, 2007) guidelines. A total of five bacterial species were isolates from milk culture samples, Gram staining and biochemical that were confirmed a positive were subjected to antimicrobial susceptibility test against ten antibiotics from different antibiotic classes that are used for veterinary and human health practices. From the results of this study were recorded and classified the zone of inhibition antibiotics as resistant (R), intermediate (I) and sensitive (S) according to the general guidelines prepared by (CLSI, 2007). The results survey this study was isolated bacteria of *Staphylococcus* , *E. coli*, *Klebsiella*, *Salmonella* and *Shigella* spp as showed (Table 15, 16, 17, 18 and 19) respectively.

4.7.5. Antibiotic Sensitivity Test Against *Staphylococcus*

The Antibiotic sensitivity test revealed that the isolated *Staphylococcus* spp. were sensitive to Ciprofloxacin (CIP) and Chloramphenicol (C) while it was resistant to Erythromycin, Amoxicillin (AMX). and Tetracycline (TE) as presented (plate 32).

Table 17: Results of Antibiotic Sensitivity test of *Staphylococcus*

Antibacterial Agents	Disc Concentration	Zone of inhibition (mm)	Interpretation
Amoxicillin (AMX)	30 µg	4	Resistant
Tetracycline (TE)	30 µg	11	Resistant
Ciprofloxacin (CIP)	5 µg	23	Sensitive
Chloramphenicol (C)	30 µg	32	Sensitive
Erythromycin(E)	15 ug	9	Resistant

4.7.6. Antibiotic Sensitivity Test Against *Klebsiella* spp.

From the results (Table 16), it was observed that the most antibiotics isolated *Klebsiella* spp were resistant which include to Amoxicillin (AMX), Azithromycin(AZM) and Ampicillin (AMP) but it was sensitive to Ciprofloxacin (CIP) (C)and Levofloxacin (LE), as point to (Plate 33).

Table 18: Results of Antibiotic Sensitivity test of *Klebsiella*

Antibacterial Agents	Disc Concentration	Zone of inhibition (mm)	Interpretation
Amoxicillin (AMX)	30 µg	0	Resistant
Levofloxacin (LE)	5 µg	29	Sensitive
Ciprofloxacin (CIP)	5 µg	33	Sensitive
Azithromycin(AZM)	30 µg	8	Resistant
Ampicillin (AMP)	25 ug	0	Resistant

4.7.7. Antibiotic Sensitivity Test Against *Escherichia* coli spp.

The Antibiotic sensitivity test shown that isolated *E. coli.* spp. was sensitive to Chloramphenicol (C) but it was resistance to included Ampicillin(AMP), Tetracycline(TE), Ciprofloxacin (CIP), Erythromycin (E) as indicated (Plate 34).

Table 19: Results of Antibiotic Sensitivity test of *Escherichia* coli

Antibacterial Agents	Disc Concentration	Zone of inhibition (mm)	Interpretation
Ampicillin (AMP)	25µg	0	Resistant
Tetracycline (TE)	30 µg	0	Resistant
Ciprofloxacin (CIP)	5 µg	14	Resistant
Chloramphenicol (C)	30 µg	26	Sensitive
Erythromycin(E)	15 ug	12	Resistant

4.7.8. Antibiotic Sensitivity Test Against *Salmonella* spp.

The results showed that antibiotics sensitive of isolated *Salmonella* spp were resistant to Tetracycline (TE) Ampicillin (AMP) followed by Erythromycin (E). In addition, it was observed that *Salmonella* were sensitive to Cefixime (CFM) and Ciprofloxacin (CIP) as designated (Plate 35).

Table 20: Results of Antibiotic Sensitivity test of *Salmonella*

Antibacterial Agents	Disc Concentration	Zone of inhibition (mm)	Interpretation
Cefixime (CFM	30 µg	25	Sensitive
Tetracycline (TE)	30 µg	7	Resistant
Ampicillin (AMP)	25 µg	0	Resistance
Ciprofloxacin (CIP)	30 µg	24	Sensitive
Erythromycin (E)	15 ug	5	Resistant

4.7.9. Antibiotic Sensitivity Test Against *Shigella* spp.

The antibiotic sensitivity test of *Shigella* showed resistance to Amoxicillin (AMX), Levofloxacin (LE), Azithromycin (AZM) while it was to intermediate to Gentamycin (GEN). Also, *Shigella* was sensitive to Chloramphenicol (C) as revealed (Plate 36).

Table 21: Results of Antibiotic Sensitivity test of *Shigella*

Antibacterial Agents	Disc Concentration	Zone of inhibition (mm)	Interpretation
Amoxicillin (AMX)	30 µg	3	Resistant
Levofloxacin (LE)	5 µg	12	Resistant
Chloramphenicol (C)	30 µg	36	Sensitive
Azithromycin(AZM)	30 µg	13	Resistant
Gentamycin (GEN)	10 ug	18	Intermediate

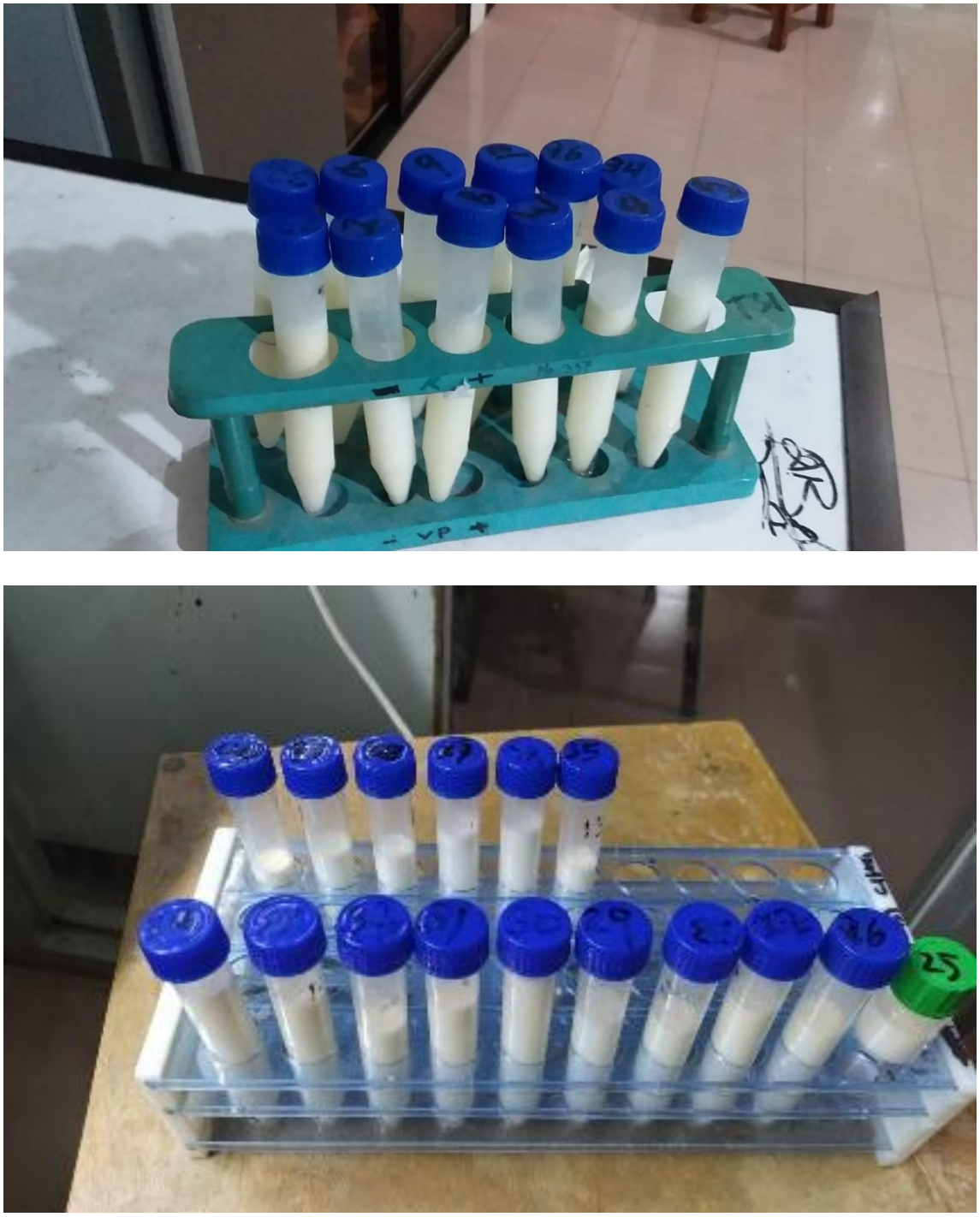

Plate 1: Collection of sample from dairy farms and vendor shops

Plate 2: During collection of questionnaires from dairy farms

Plate 3: Vendor shop interviewed

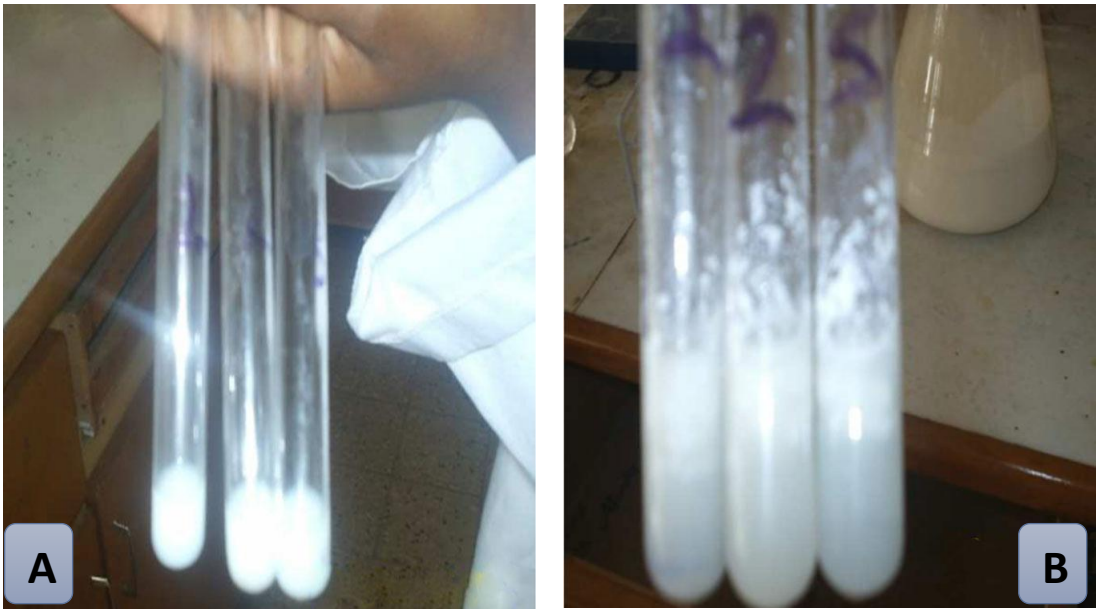

Plate 4: Clot on boiling test developed precipitated formation (A) and Alcohol test observed coagulation formation (B)

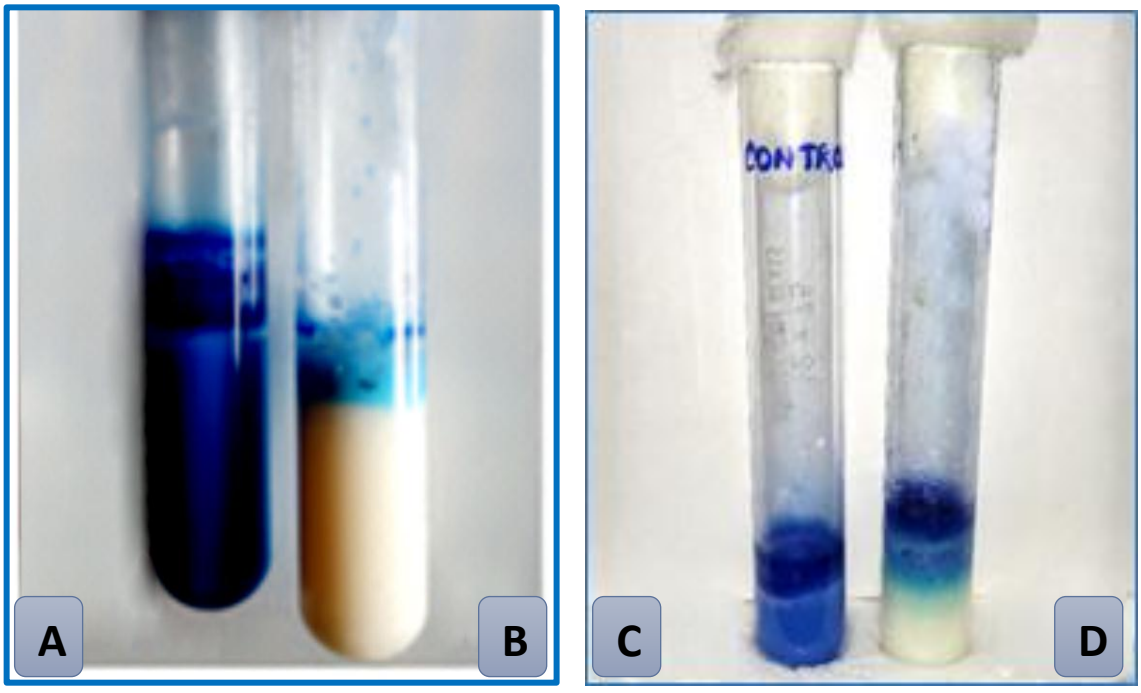

Plate 5: A and C control and B and D Positive Raw milk with Methylene blue reduction test

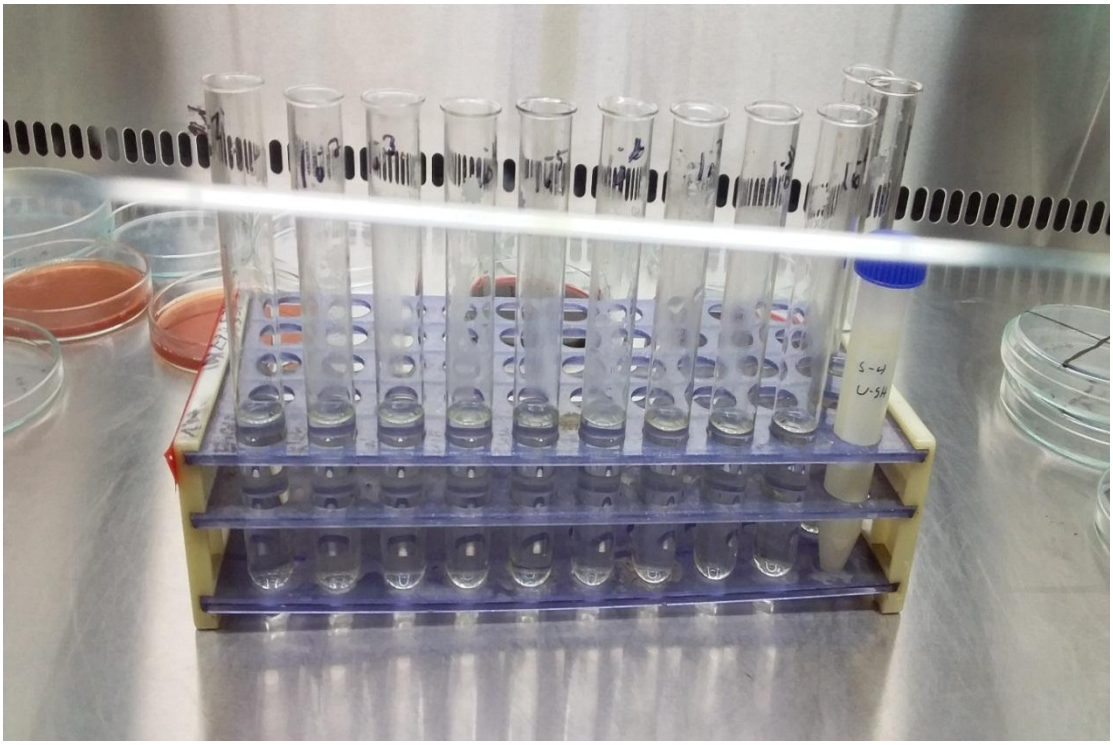

Plate 6: Tenfold serial dilution

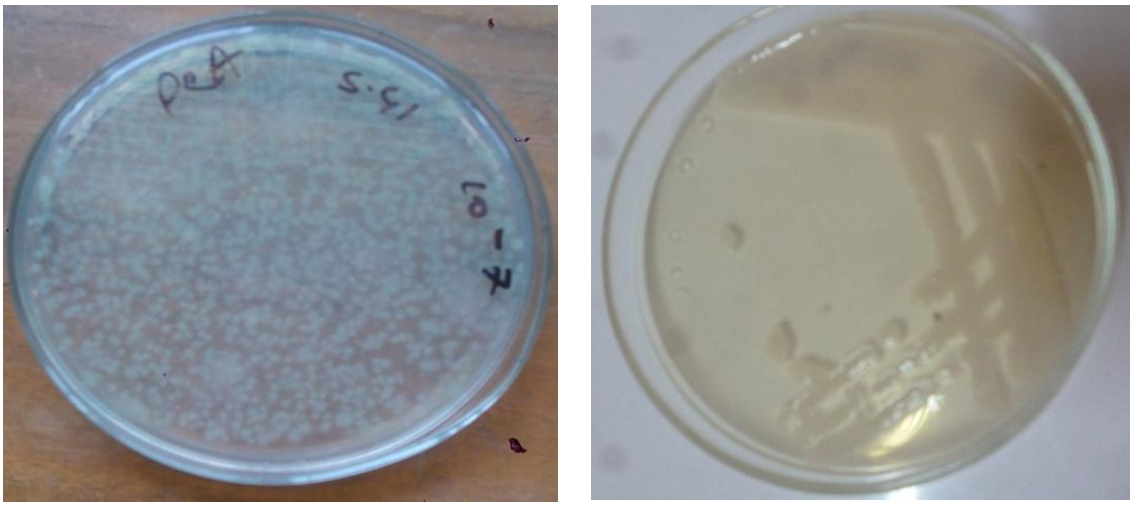

Plate 7*:* (Right) Nutrient agar grown media and (Left) Plate count Agar

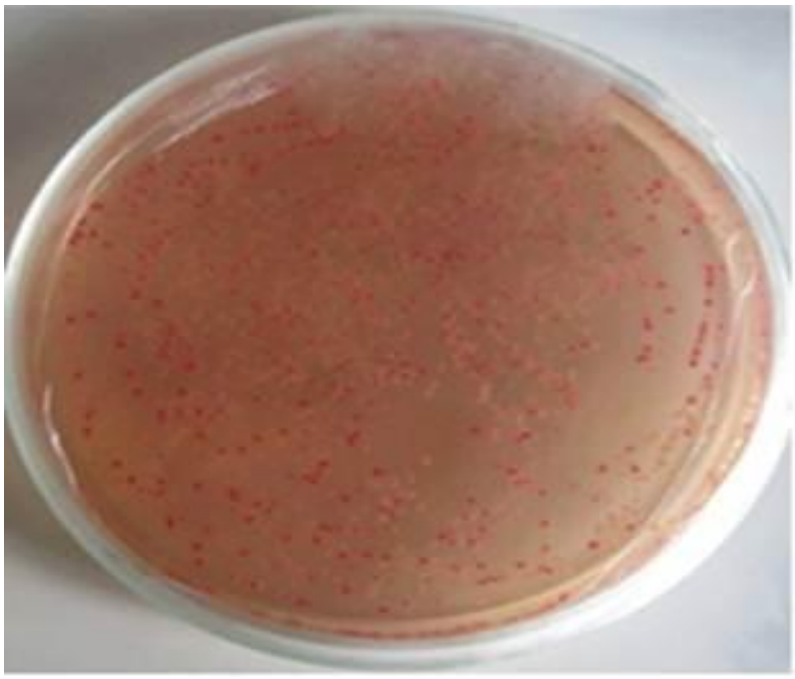

Plate 8: Colony of bacteria in MacConkey agar for Total coliform count (TCC).

Plate 9: Colony of bacteria in Baird-Parker agar for Total *Staphylococcus* count (TSC).

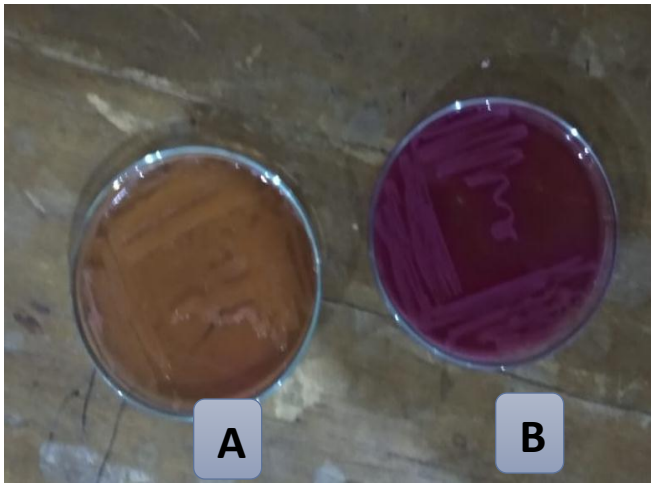

Plate 10: MacConkey agar grow(A) Non lactose fermented, (B) Lactose fermented and (C) uninoculated control media.

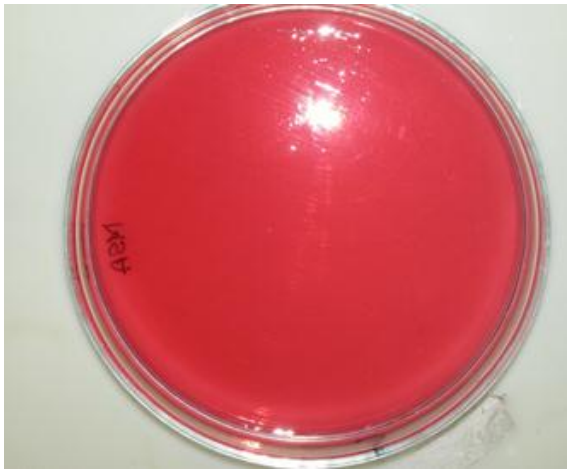


Plate 11: *Staphylococcus spp* on Mannitol salt agar (Right) grown media and (Left) uninoculated control


Plate 12: *Staphylococcus* Agar no. 110 (Right) grown media and (Left) uninoculated control

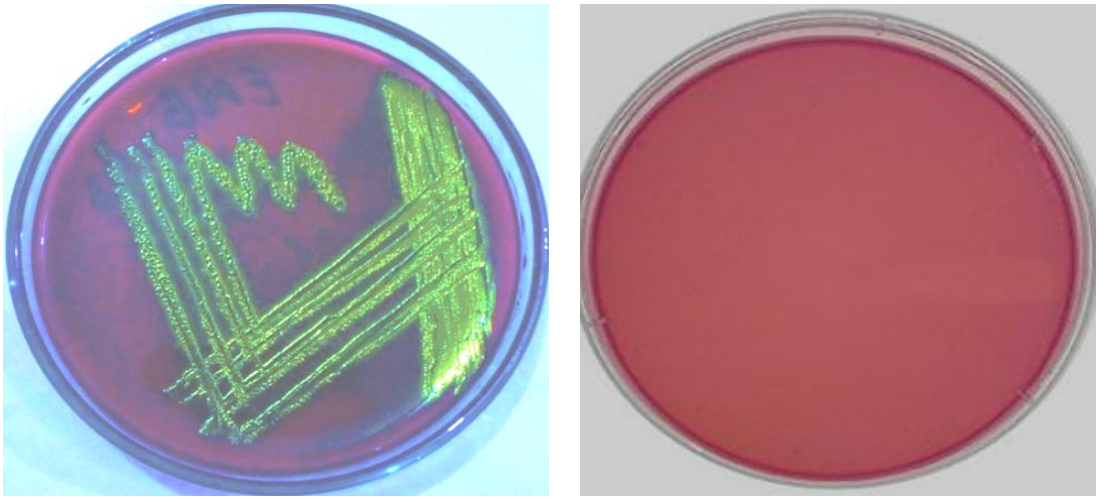

Plate 13: *E. coli spp* on Eosin Methylene Blue (Left) grown media and (Right) uninoculated control media.

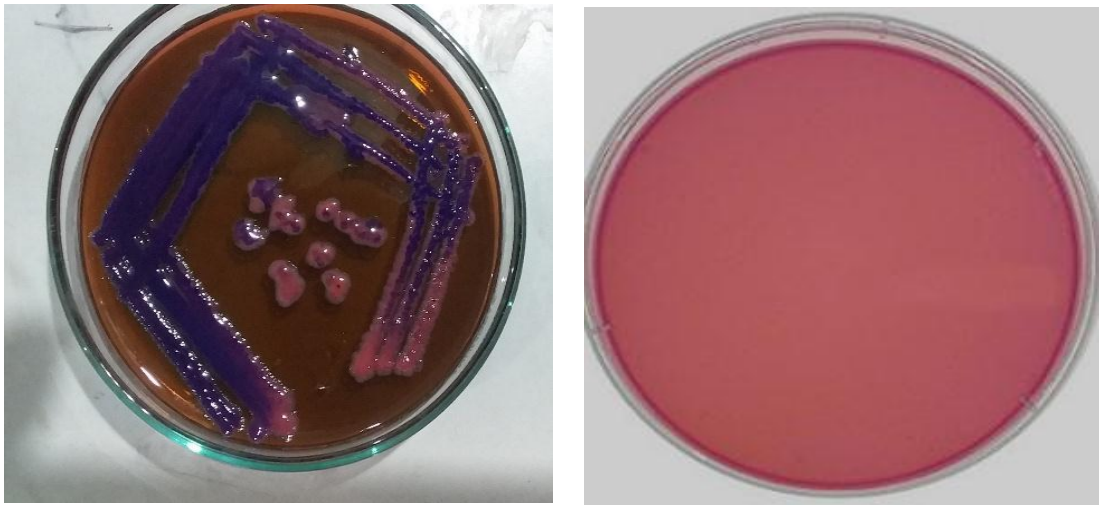

Plate 14: *Klebsiella spp* on Eosin Methylene Blue (Left) grown media and (Right) uninoculated control media

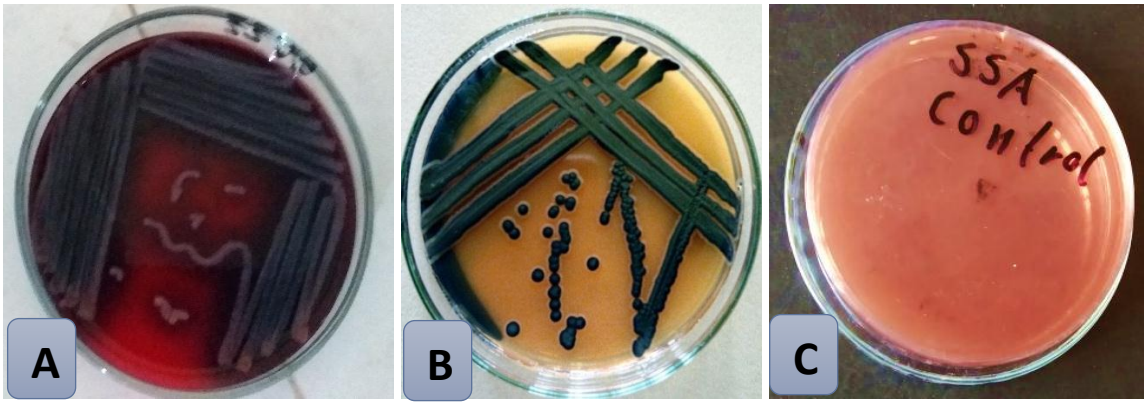

Plate 15: *Salmonella spp* on *Salmonella Shigella* Agar Plate (A and B) and uninoculated control (C) media

Plate 16: *Shigella* spp on *Salmonella Shigella* Agar Plate (Right) grown media and uninoculated control (Left) media

Plate 17: *Shigella* spp on *Salmonella Shigella* Agar Plate (Left) grown media and uninoculated control (Right) media

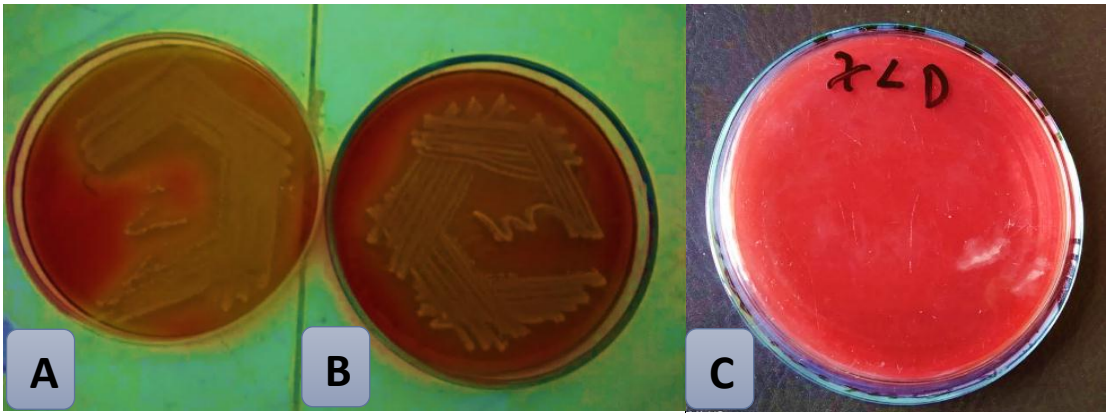

Plate 18: *Salmonella*(A) *Shigella*, (B) Lactose fermented and (C) uninoculated control media on XLD Plate.

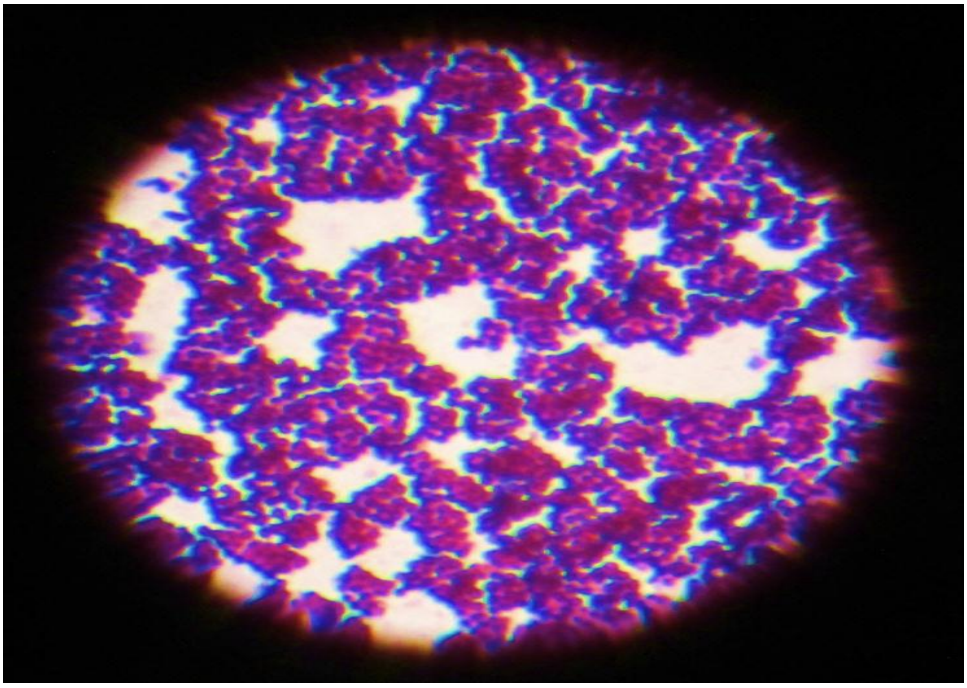

Plate 19: Gram positive *Staphylococcus* spp, violet and cluster shaped was seen under microscope Trinocular magnification

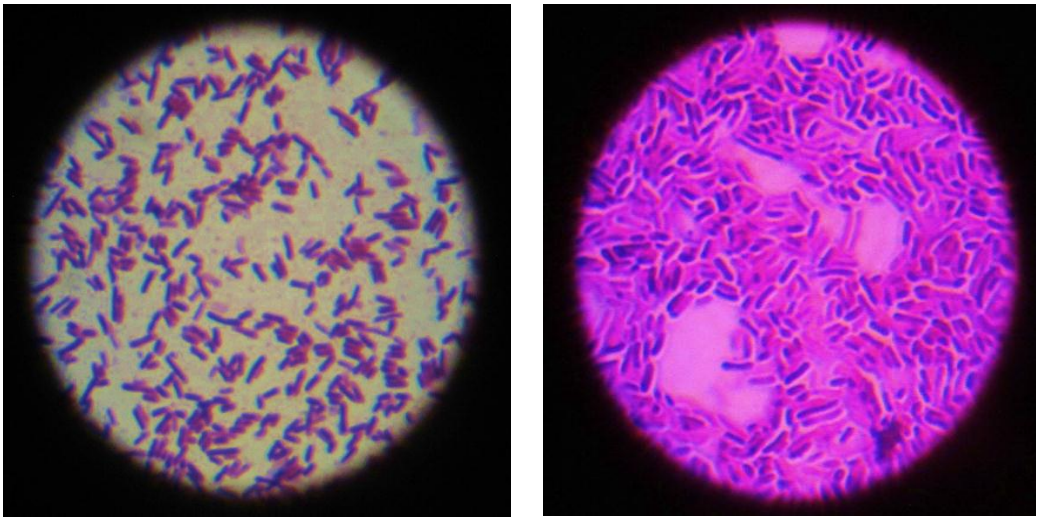

Plate 20: *E. coli* spp. showed Gram positive pink colored, short rods, single or paired seen under at 100x magnification of microscope.

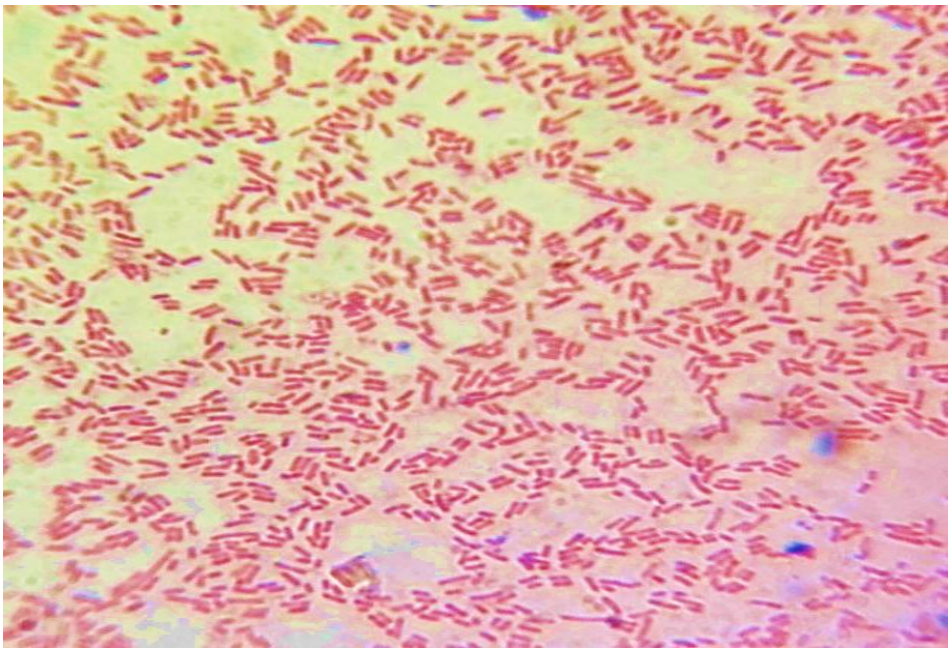

Plate 21: *E. coli* spp. showed Gram positive pink colored, short rods, single or paired seen under Trinocular magnification

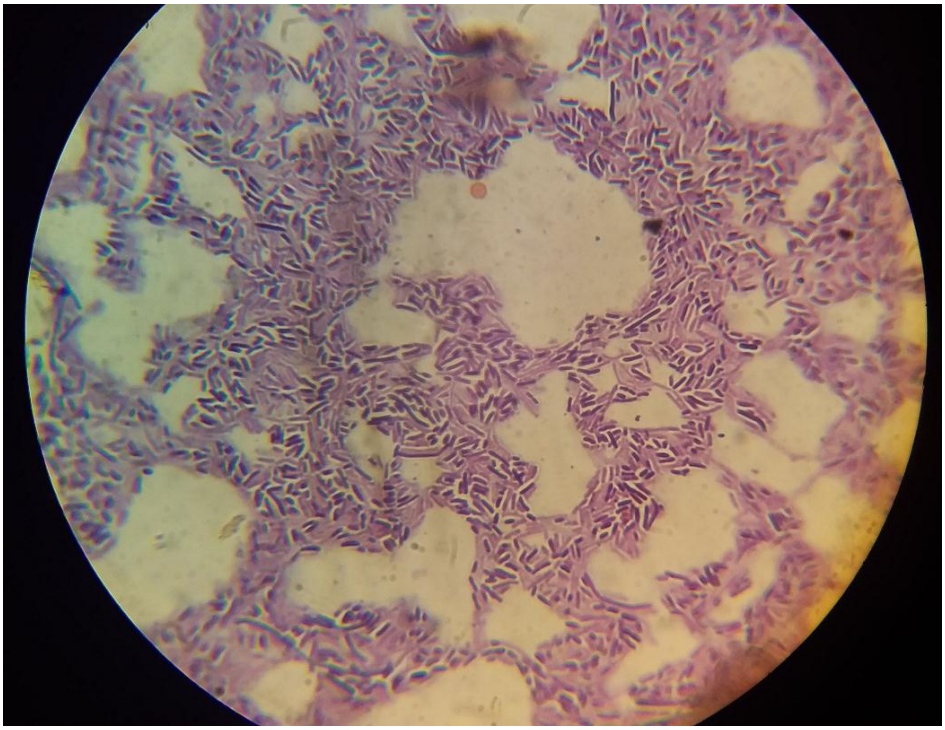

Plate 22: Gram negative *Klebsiella* spp, pink colored, short rods, single or paired seen under Trinocular magnification

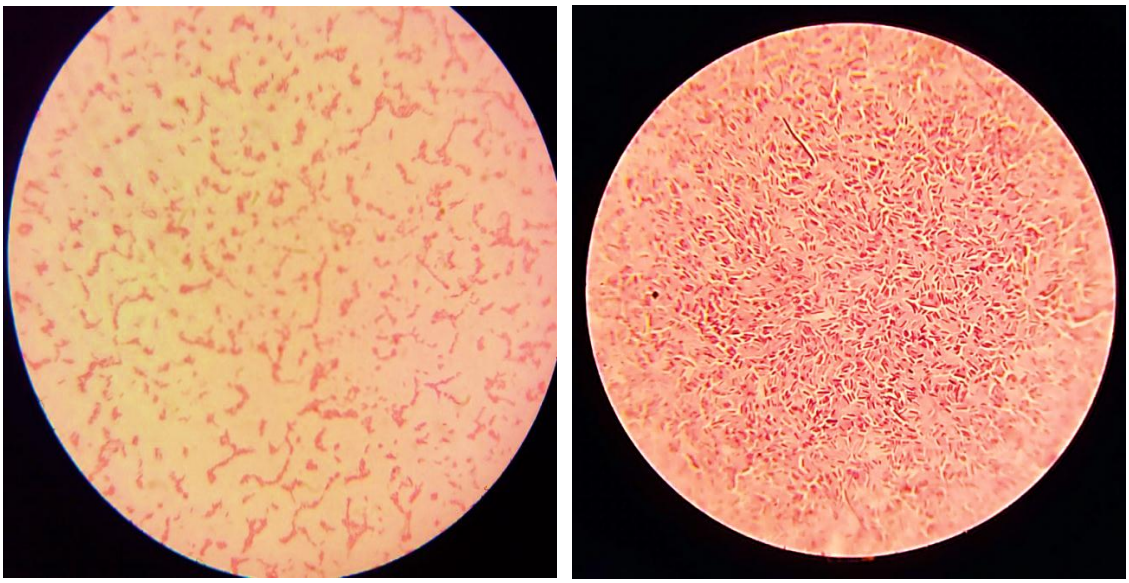

Plate 23: Gram negative *Salmonella*s, pink colour, small rod-shape, arranged in single or pair at 100x magnification of microscope.

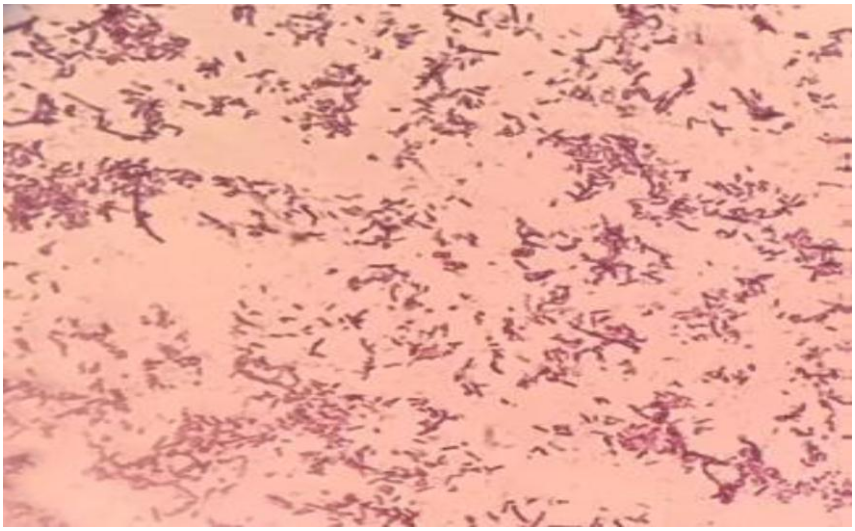

Plate 24: Gram negative *Shigella* spp, pink colored, very short plump, rods, single or paired seen under Trinocular magnification.

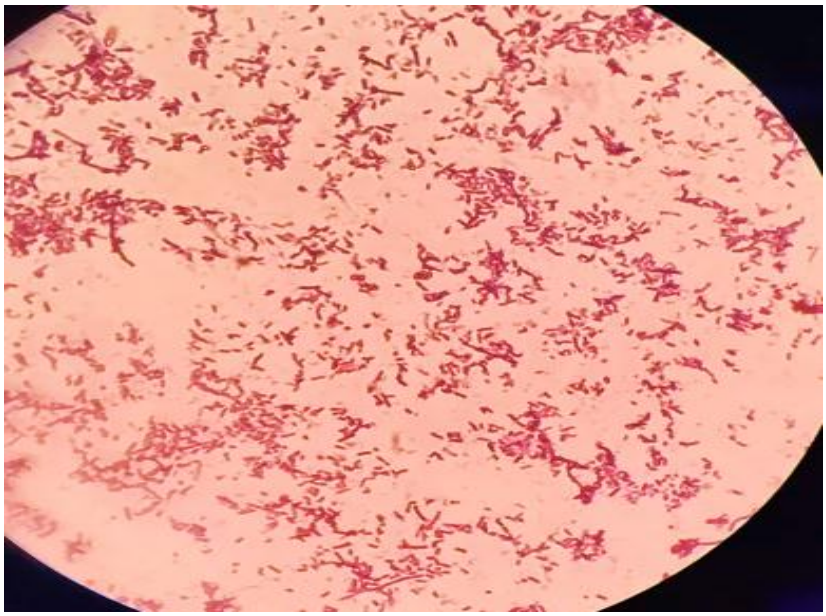

Plate 25: Gram negative *Shigella* spp, pink colored, very short plump, rods, single or paired seen under at 100x magnification of microscope.

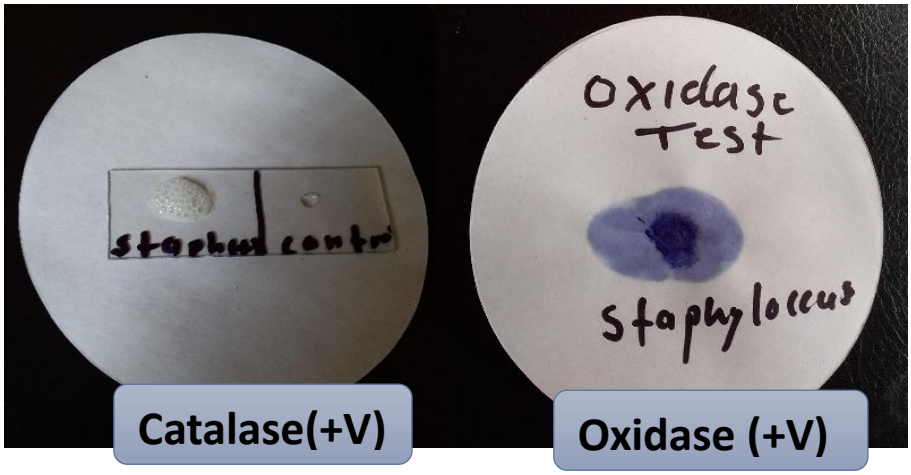

Plate 26: *Staphylococcus* were positive in both Catalase and Oxidase test

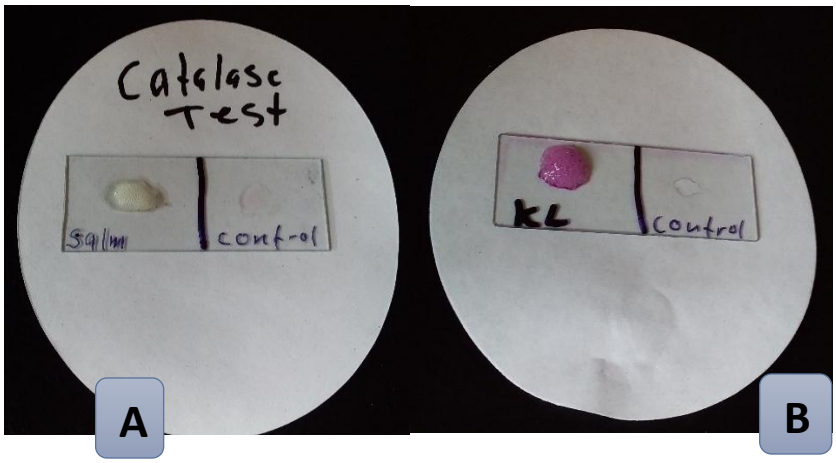

Plate 27: Catalase test result (Left) A= *Salmonella* (Positive) and Left B= *Klebsiella* (Positive)

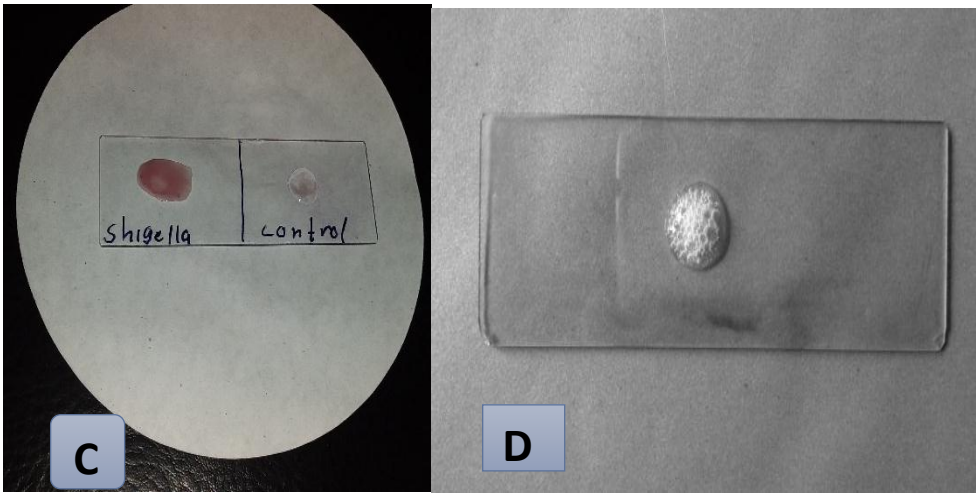

Plate 28: Catalase test result (Left) C= *Shigella* (Negative) and Left D= *E. coli* (Positive)

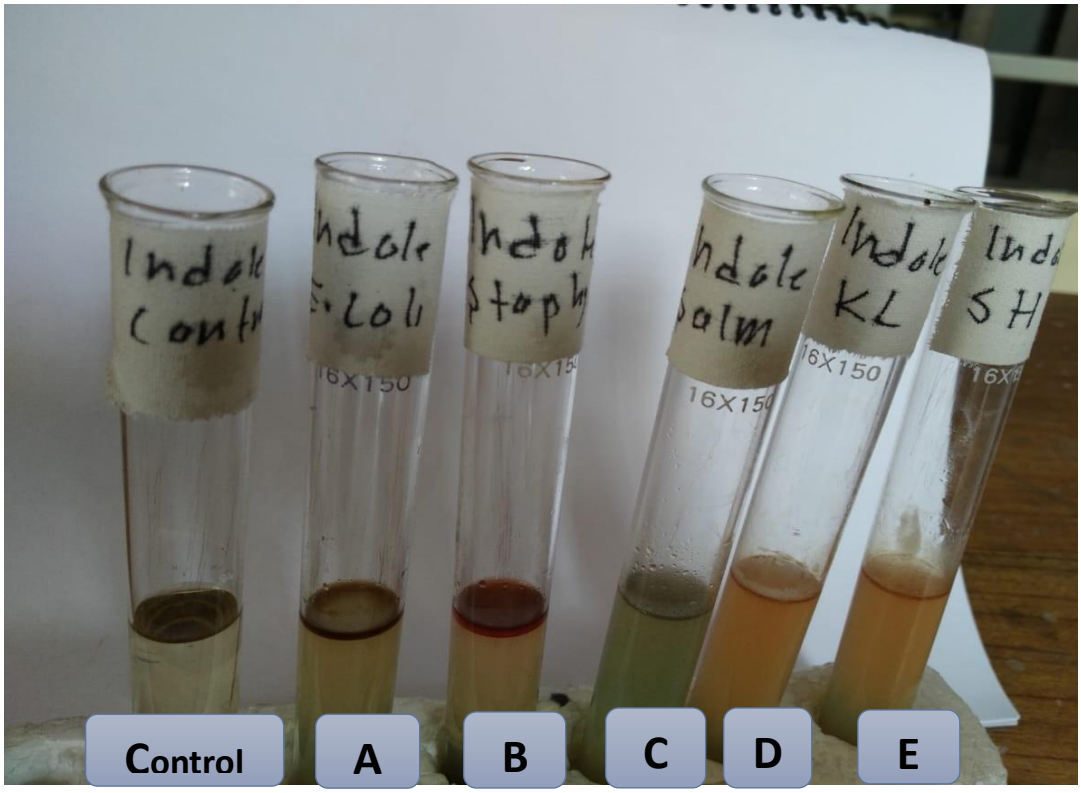

Plate 29: Indole test results (Right) A= *Escherichia* coli (Positive), B=*Staphylococcus* (Positive), C= *Salmonella* (Negative), D= *Klebsiella* (Negative) and E=*Shigella*(Negative) and Uninoculated control (Left).

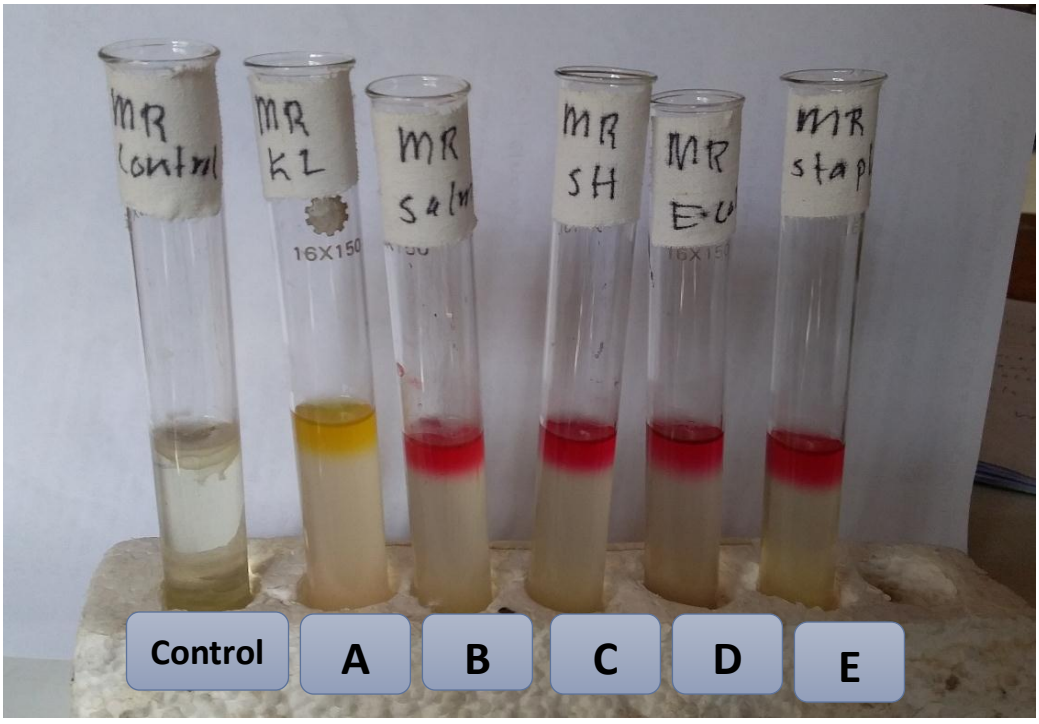

Plate 30: MR results (Right) A= *Klebsiella* (Negative), B= *Salmonella* (Positive), C=*Shigella*(Positive), D= *Escherichia* coli(Positive) and E=*Staphylococcus* (Positive) and uninoculated control (Left).

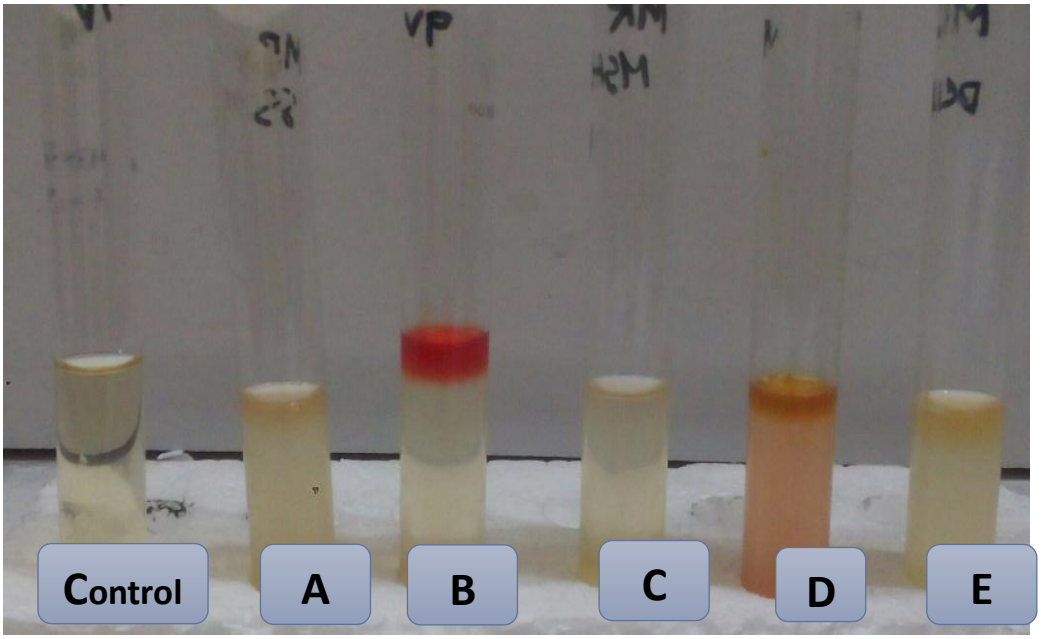

Plate 31: VP results (Right) A= *E. coli (*negative), B= *Klebsiella* (positive), C= *Shigella* (negative*)*, D= *Staphylococcus* (positive) and E= *Salmonella* (negative) and uninoculated control (left).

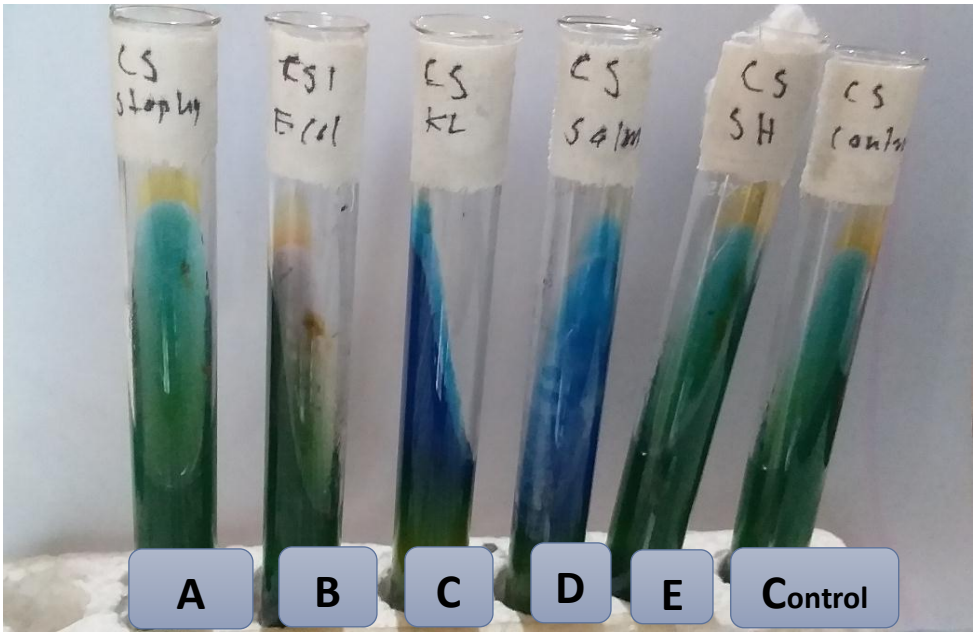

Plate 32: Simmon Citrate Utilization test results (Left) A=*Staphylococcus* (Negative), E= *Escherichia* coli (Negative), C= *Klebsiella* (Positive), D= *Salmonella* (Positive), SH=*Shigella* (Negative) and Uninoculated Control. (Right).

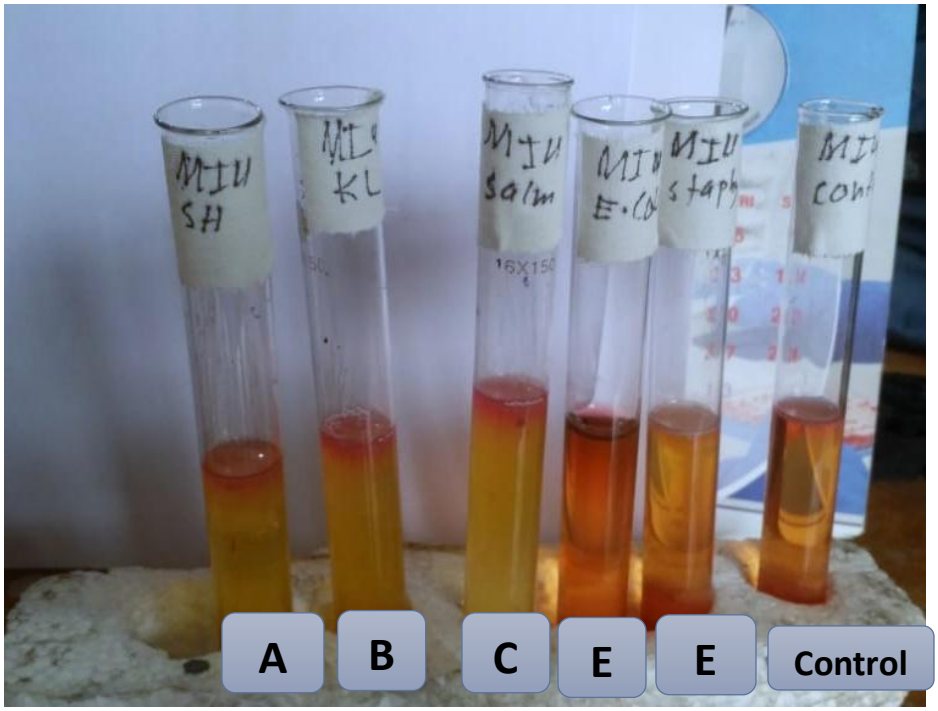

Plate 33: MIU test results (Right), A=*Shigella* spp (positive), B= *Klebsiella* spp. (positive), C= *Salmonella* spp. (positive), D. *Escherichia* coli (negative), E= *Staphylococcus (negative)* spp and C= Control (Right).

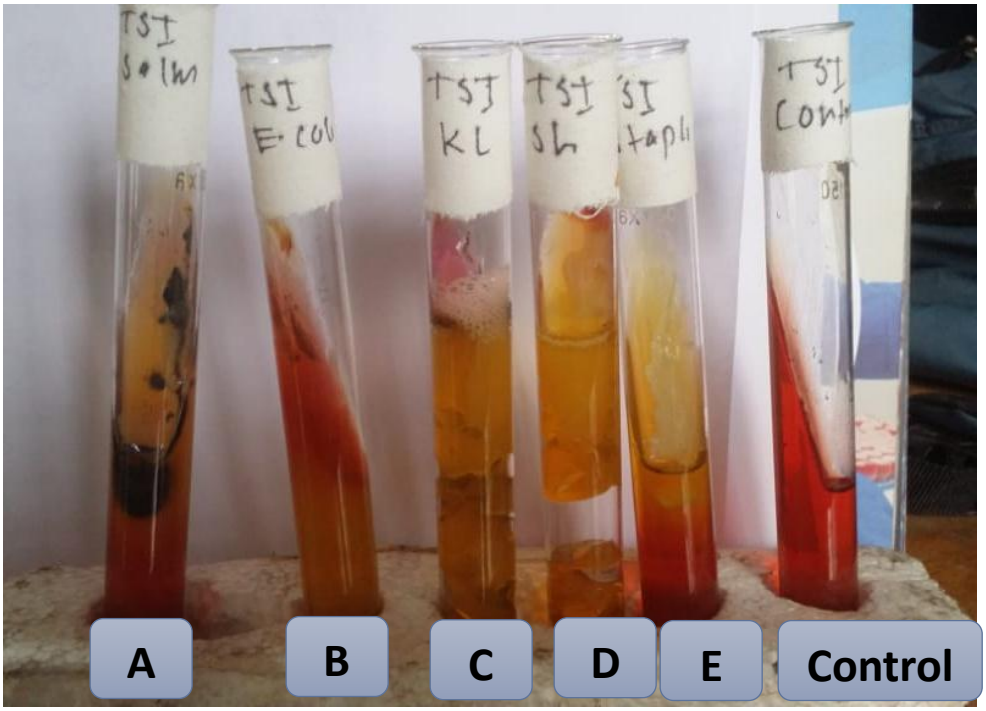

Plate 34: TSI test results (Right), A= E=*Salmonella* spp., B=*Escherichia* coli, C= *Klebsiella*. D= *Staphylococcus* spp. E= *Shigella* spp., and Uninoculated control (left).

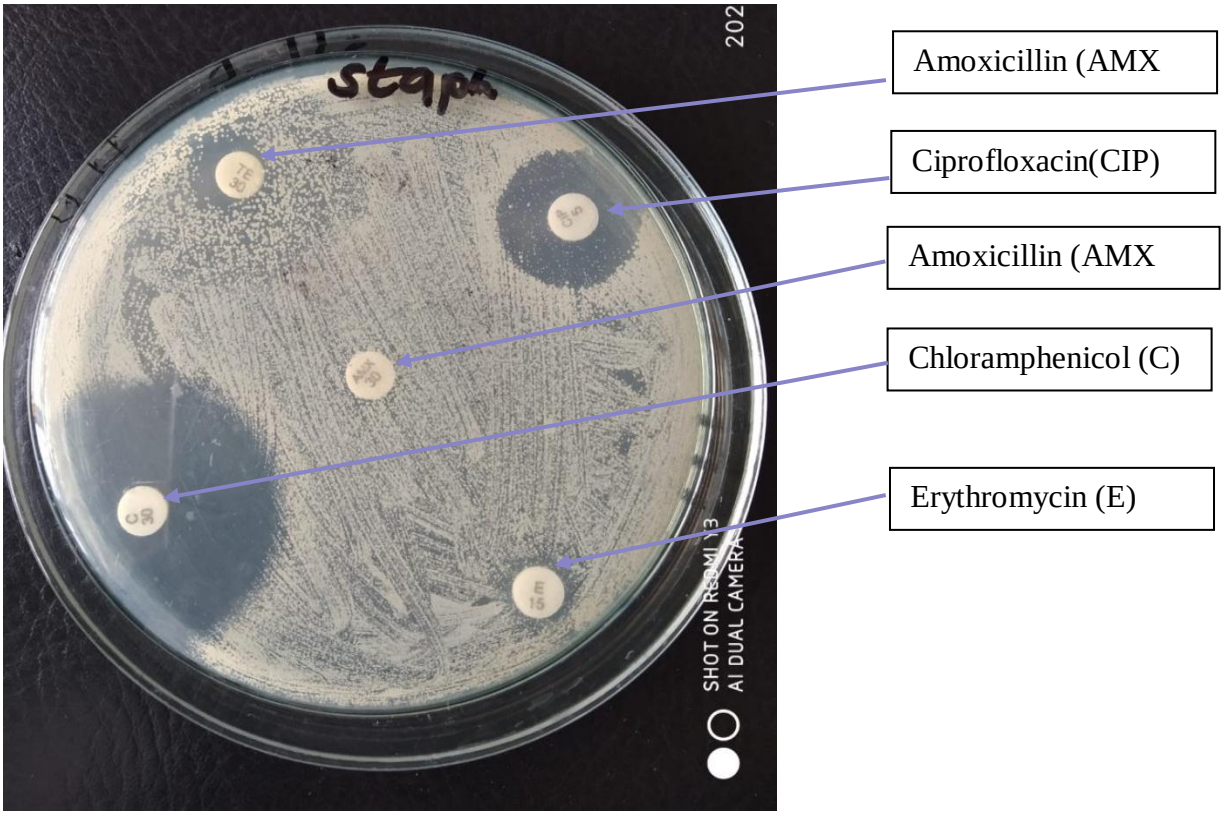

Plate 35: Antibiotics against *Staphylococcus spp.*

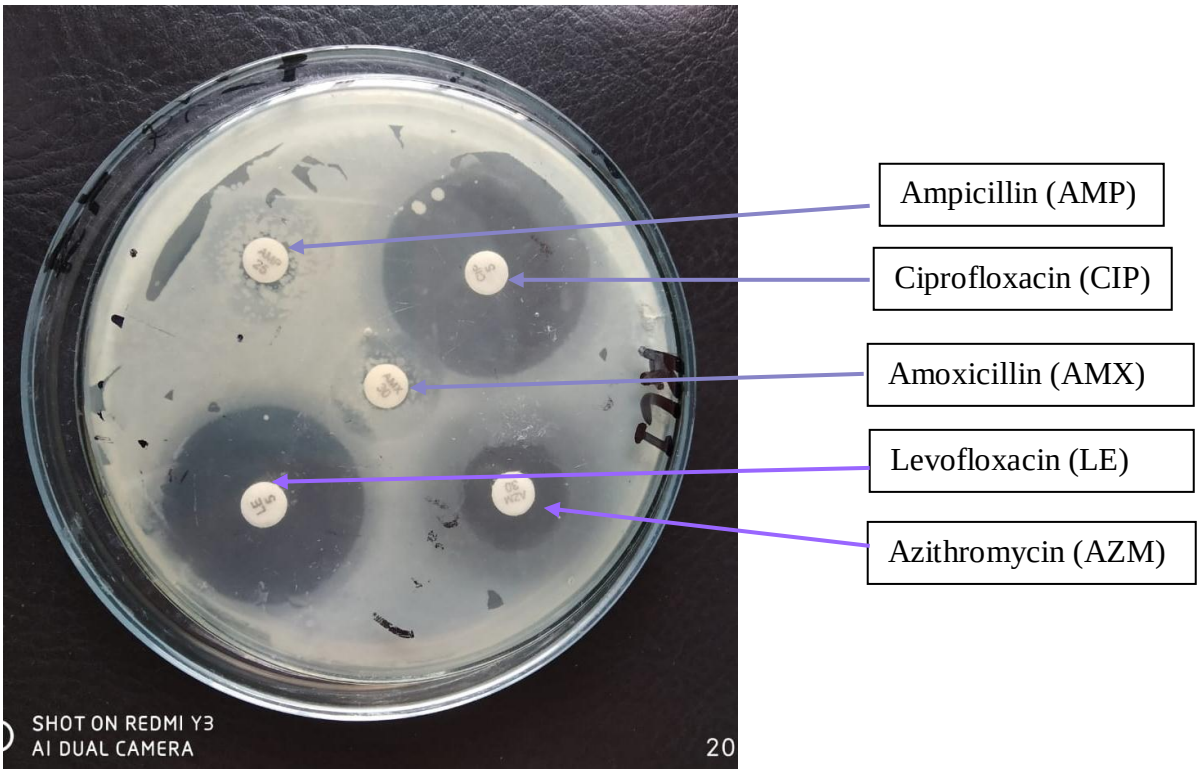

Plate 36: Antibiotics against *Klebsiella spp*

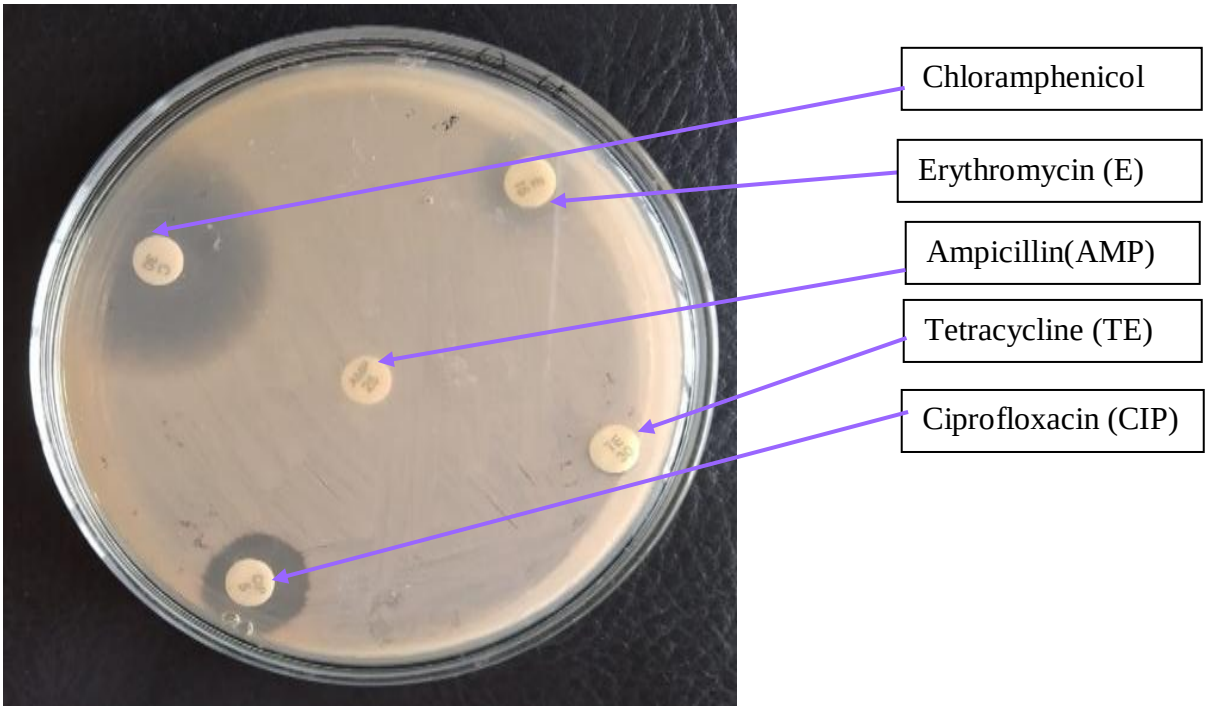

Plate 37: Antibiotics against *E. coli spp*

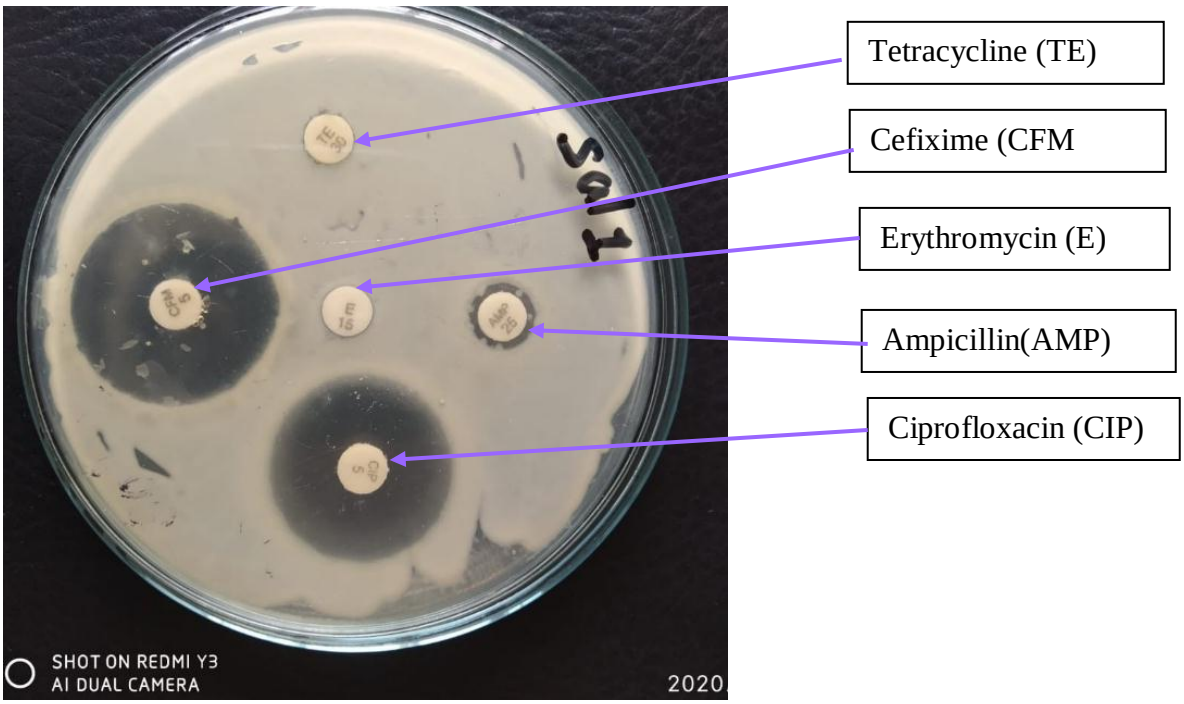

Plate 38: Antibiotics against *Salmonella spp.*

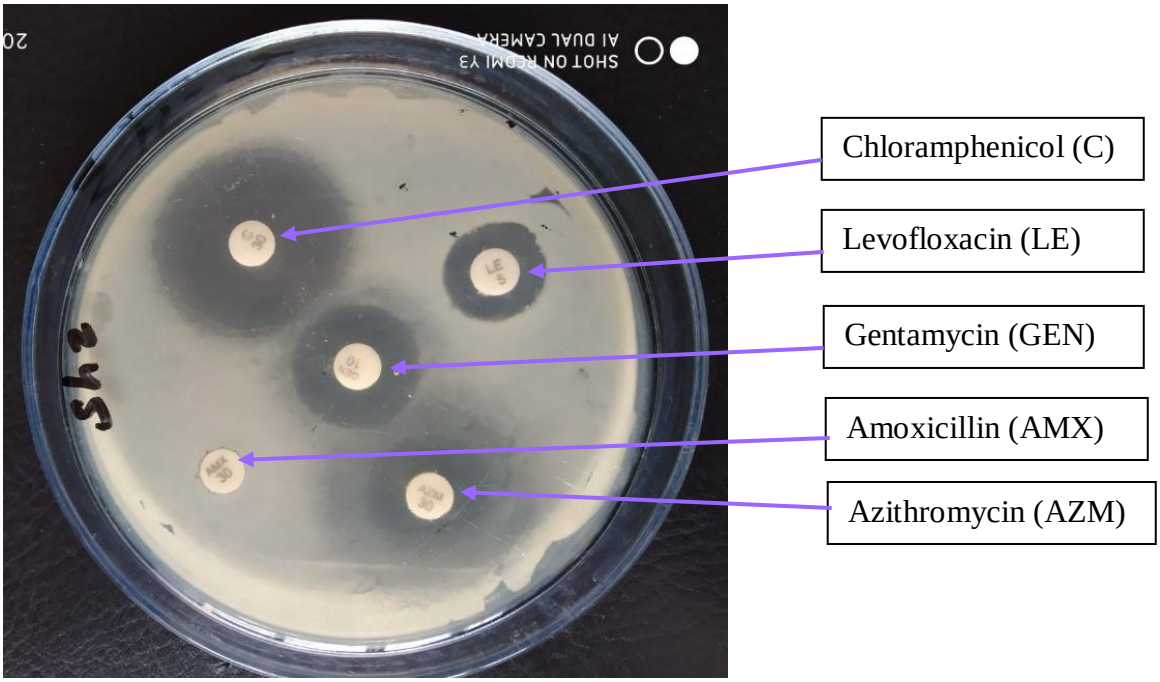

Plate 39: Antibiotics against *Shigella spp.*

CHAPTER V

DISCUSSION

Dairy cattle farming and milk traders are becoming an emerging business sector in most of the developing countries, including Bangladesh, in supporting the economy of the wider community. However, a lot remains to be done in improving major hygienic practice and milk quality has become a main problems of milk producers as well as milk collectors and sellers that primarily related to the worker's practical skills and knowledge on the milk hygienic and handling practices. Therefore, the overall purpose of this study was to assess the bacteriological quality and hygienic practices of raw milk quality in dairy farms and vendor shops in Dinajpur town.

Generally, findings showed that, there are several practices undertaken at farm level and vendor shops such as type of animal house floor, not washing hands and udder/teats before milking, milking sick animals and those with udder problems, water used for cleanliness (hands and milk equipments), type of storage containers used and milk storage duration under room temperature predispose raw milk to microbial contaminations. The current study presented preliminary quality test and bacteriological tests included organoleptic of physical examination, included Clot-On-Boiling Test**,** Alcohol test, Methylene blue reduction test. Also presented was considered the bacteriological tests for determination of the bacterial load in raw milk samples were total bacterial count (TBC), total staphylococcal count (TSC) total coliform count (TCC) and total *Salmonella Shigella* count (TSS).

This study shown that, out of the total respondents of dairy farms and vendor shops (N=45), (83%) of the respondents were males and (17%) were female, that indicated the majority of dairy farms and vendor shops were operationally covered by male. For example, in animal house floor cleaning, milk handling, transporting of dairy producers were entirely undertaken by male. Similarly, almost all collectors and transporter, sellers of vendors who involved in milk collection, transportation and vending were also male. The finding agrees with Amentie *et al.* (2016) and Farah. (2018) those reported that about (94.2% and 65%) of milk handling and marketing were undertaken by male. Regarding the age, almost all of respondents were ranged between 15-40 years comprised (67%) and the remaining proportion of 33% were aged above 40 years,

(35.2%). This result was similarly to the result of Jahan *et al.* (2015) who reported *(*76%) in all the farm categories had completed primary level of education.

Moreover, the poor knowledge and low level of education could be predisposing factor related to the practices of milk handling and quality. The results of the current study showed that the educational background of the most respondents were primary education, about (62%) had completed primary level of education. In agreement with this findings, Daniel. (2008) who documented that (72%) of respondents enrolled in primary school and remaining were attended secondary and graduate level of university.

Similarly, current study established that most of the dairy farms study had (84%) of animal were cross-breed (Holstein-Friesian with indigenous) which means all dairy all farms were established as role of production. Similar observations were reported by Hossain *et al.* (2005), who reported most commercial dairy farms were kept cross breed or exotic rather than local breed. With respect to the farm type/scale, the majority of the farms i.e. (52%) were small-scale dairy farms with less than 5 heads of dairy cows, followed by (36%) medium scale with 5-10 head (12%) having more than 10 head dairy cattle considered as large-scale farms. In line with these findings, it was stated that small and large farms of optimum sizes should contain a maximum and minimum of 5 heads and 10 heads of dairy cows respectively with the report of ILRI. (2007).

With regard to the management system were also grouped as the intensive system as indicated by (80%) and semi-intensive system (16%) according to result (Table 2). Results of this study partially supports according to BBS (2000) those reported by similar findings of (72%) and (20%) of the respondents. In the recent study, the majority of (44% and 36%) dairy farms were kept an air opens and both housing system followed by a confirmed/ closed type housing of (20%). In agreement with these findings, Hossain and his colleagues (2005) have also reported that highest percentage of farmers (80%) provided open house,13% provided both and rest used closed housed.

It was further realized with regard to the feed source of the dairy cattle, the present finding indicated that mostly of dairy farms were used feedstuffs like roughages with concentrates and mixed (44% and 28%) respectively which indicated that most the common feed of intensives farms were stall feeding followed by semi-intensive dairy farms used both stall feeding and grazing system. This agrees with Hossain *et al.* (2004), who report (63%) farmers followed stallfeeding and (37%) farmers followed both stall

and grazing system. At the same time, current finding also showed that most of the respondents (88%) clean the barn on daily basis by removing the feces and other dirty in shade cattle houses except some days of weekends. Similar observation was also reported by Asaminew. (2011) who reported the poor hygienic house will result a negative impact on the quality of milk and milk products produced and processed as well as contamination.

According to the current findings indicated that the entirely respondents of (100%) were used a manual milking. Similar observations have been reported by Das *et al* (2016*)* those stated that most dairy farms in Bangladesh were used manual milking instead of machine milking. Indeed, the hand milking using unwashed hand practiced by famers may indicate that microorganisms on hands could result in contamination of the milk. Meanwhile, about (28%)of the present study showed that the persons involved in milking activities were also not clean with their hands, body and clothes during milking and milking utensils although better than traditional way, these possibilities predisposed milk to microbial contaminations at farm level. Similar observation was reported by Mohamed and & Farah. (2018) in farmers from other parts of Africa.

Cleaning the udder of cows before milking is important since it could have direct contact with the ground, urine, dung and feed refusals while resting. The use of individual towel and following essential cleaning practices during milking is important for the production of quality milk. However, in this study, (64%) of the dairy farmers were used common towel to wash udder with clean water and drying to reduces milk contamination by transient bacteria located on the udder washed their cow's udder (teats) before milking and 8% were not washing rather they were used bare hand. Therefore, the use of common towels in different milking cows during milk can result opportunity access of microbial contamination in milk. and also for cleaning and sanitizing may result in re-contamination of the udder. The present finding is comparable with the result reported by (Zelalem, 2010) who reported (69.4%) most of the dairy producers used common and bare hand.

Moreover, during the study period, (80%) of the dairy farms were used water from wells/bore holes for cleaning of animal house floor, washing hands, udder, milk utensils and/or equipment washing. This is in line with the findings Hossain *et al.* (2005) who reported that 83% of the dairy farms were used water from bore holes/wells. According

to the present study, about (96%) of the most dairy farms were milking their animals twice a day. This is in agreement with Mohamed and Farah. (2018) those reported that about 90% of the respondents milk their animals twice a day.

Production of milk of good hygienic quality for consumers requires good hygienic practices through cleaning of dairy utensils and equipment is essential that anyone handling milk must pay great attention to hygiene contamination from equipment situated between the cow and the storage equipments. During the current study, aluminum and plastic containers were the major utensils for collection and storage of milk were mostly preferred equipments. However, the cleaning is not efficient and utensils are not properly dried were also unhygienic. Stainless steel and aluminum cans are advised in milk storage as they are easily cleaned. The finding of this study is in line with the previous reports by Tafa *et al*. (2015) and Bukuku (2013) those reported aluminum and stainless steel equipment are mostly ideal for milking equipment that can easy being clean.

The common means of milk transportation to final destinations was mainly done using public transport of (56%). The route among the collection and delivery was taking 2 -5 hours during transactions from dairy producer to the vendor shops. However, these vehicles were not appropriate for raw milk transportation because its lacks cooling facilities and stored at room temperature until end of selling and all these gave possibilities for microbial contaminations during stages. Similar finding was also reported Grillet *et al.* (2007) in Africa.

According to the present study, it was observed that the microbiological quality of water using during cleaning was certain, contributing to contaminate the milk. about (44%) of the respondent's tape water/normal without supplement detergents to wash milking equipments, milk storage and transportation containers of milk. This is similar with the report of Nanu *et al.* (2007) who to use water only with no detergent and tape water for cleaning can contribute to the poor quality of milk and there was a possible source of milk contamination.

As observed in the present study (52%) of the vendor shops usually keeping milk used refrigerator to maintain a low temperature and prevent a high microbial contamination. Similarly, Abunna *et al.* (2019) reported that indicated the microbial contamination of raw milk in the market chain, as the milk was typically exposed to high temperature,

road traffic, wind and dusty conditions for prolonged periods of time during the process of milk collection, changing containers and coding contamination.

With regarding the respondents and direct observation, it was found that the dairy producers and vendor shops were commonly used storage plastic containers, plastic bags and soda/ water bottles (47%, 18% and 35%) as presented (Table 7). Moreover, the use of plastic bags in fitting lids of milk buckets, water bottles/soda and scooping were among the causal factors of microbial contaminations in milk. This result is in line with the work of Shirima *et al*. (2003) who reported plastic bags and bottles was not the safe procedure as it contaminates the milk and making it unsafe for consumption.

Likewise, about (44%) of the respondents were used the tape water/normal water and (35%) used the water with supplemented soap and detergents to wash milking equipments, milk storage and transportation containers of milk. Similar findings have been reported in recent studies in Tanzania Mosalagae *et al.* (2011) who reported washing hands with cold water and tape water without detergent lead to insufficient cleaning to remove germs and serves as a major source of microbial contamination of milk.

Concerning of (86%) respondents were aware about the risk knowledge of public health hazards associated with consumption of unboiling or raw cow milk however, (14%) did not aware risk of milk consumption and there is potential risk of contamination by zoonotic pathogens. In addition, the assessment data showed that, (98%) of the milk producers and vendor shops did not testing the quality of milk, while only (2%) of such dairy producers were employed to testing milk quality by using lactometer. This finding is in line with Lumadede *et al.* (2010).

According to the result from (Table 7) showed, about (78%) different dairy farmers and vendor shops consumers had no experience or unware of zoonotic potential from milk borne pathogens which concerned with milk safety and most of the respondents reported they were suffered from food borne infections of unknown origin. The consumers were not conscious that *Staphylococcus* , *Salmonella*, and *E. coli* and other diseases can be transmitted from animals to humans through drinking raw milk or not well boiling. The same findings have been reported Jahan, *et al.* (2012). With regarding to the present study, almost, (33%) of dairy producer and 67%) of vendor shops were reported that they were limited awareness on hygienic handling of milk. This is similar to the finding

reported by *R*eda *et al.* (2014) who reported limited awareness and training may contribute on health risks associated with consumption of milk commercialization of milk.

The results regarding organoleptic characteristics test are presented in (Table 8). There were remarkable differences among the physical parameters like colour, flavour, taste and texture of milk samples obtained from the two different sources. The raw milk collected form dairy producers were showed (100%) for yellowish white and slight yellowish white in colour. This might be due to the fact that the farmers take hygienic measures during milking and not to allow the cows to eat some sorts of flavoured feed prior to or during milking their cows.

At the same time, the milk sample collected from vendor shops were showed various difference colors change. The changes in milk colour may be due to the differences in nature of feed, breed, fat and solids contents of the milk because colour of milk depends upon these factors. Similar type of results found Kivaria, *et al*. (2006) who reported that the color of the most milk samples from Bogra town was yellowish white. Also result showed that the milk from two main source had normal flavour indicated that the milk produced hygienically was normal. Equally, the milk sample collected from dairy farms and vendors had normal texture (free flowing liquid). The result of the present experiment agreed with Rahman *et al.* (2018) and Amin (2005 who showed that the milk, color, flavor and texture were normal (pleasant and aromatic) collected from Bangladesh Agricultural University dairy farm, Mymensingh.

The entire raw milk samples collected from dairy farms were showed negative results of COB test and Alcohol test that indicating that there was no developed acidity in milk which may be due to good practices in handling, keeping, transportation and storage of milk. This finding was supported by Islam *et al.* (2013) and Uddin *et al.* (2016) those found the COB tests and Alcohol test were negative and indicated that there was no developed acidity in milk. also agree with this finding that carried out a research work on comparative study of platform tests on milk in the local vendor shops and fairy farms was found negative result of both test.

The data regarding to methylene blue reduction test (MBRT) were given in Table (10). In all milk sample from vendor shops were grading as poor quality milk. However, dairy farms milk was grading as good milk quality compared the milk collected from vendor

shops. Therefore, the poor quality of milk for vendor shops due are to poor hygienic manner. lower. If the microbial load is low milk is considered of good quality. Furthermore, increase in microbes decreases the quality of milk graded as C and D or poor quality of milk. The result of the test is in agreement with Chatterjees *et al.* (2006).

In the present study, the bacterial count found in dairy producers was ranged from 1.6×10^2 (log 2.2) to 6×10^6 CFU/mL (log 6.4). Compared to the three study targets dairy farms, the highest mean value of microbial load 6×10^6 (log 6.4) was recorded from small scale dairy farms which lower than bacterial count that reported by Reda *et al.* (2014) who found high total bacterial count of (2.34×10^9 (log9.3). Besides, the result showed that there was strongly statistical significant difference ($p<0.005$) with the mean of bacterial load from all milk samples in vendor shops were greater than permissible limits of $2x10^6$cfu/ml (Table11). The present finding is in line with the report of Haile. (2015) on the bacteriological quality of milk from dairy operations. Hence, the result of the current study indicated strong microbial contamination from milk samples in small scale dairy. This reasons might have associated with the higher level of bacterial contamination of the milk in these settings could be due to the lack of knowledge on proper handling of milk, less hygienic conditions in the environment, poor interior quality of material used for milk transportation and storage, lack of proper transportation facilities. Moreover, no statistically significant variation was observed in bacterial load in milk samples collected from medium scale and large scale dairy farms. Results of this study were partially supports of the findings of Khaton *et al.* (2014).

Additionally, the results from vendor shops in bacterial count were ranged from $8.1x10^9$ (log 9.9) to1.2×10^2 (log 2.0). However, this value was much higher than the acceptable value of 1 x 105 bacteria per ml of raw milk (O'Connor, 1994). Also bacteriologically load of high number milk samples from vendor shops than the maximum recommended level of 2.0×10^6 cfu/ml (EAS, 2007) by EAS standards which implied that raw cow milk from different vendor shops had poor microbiological quality. Within vendor shops the bacterial load was found higher in ($8.1x10^9$ (log 9.9) followed by milk vending shops ($2.9x10^8$ (log 8.4) and the lowest bacterial load was found was lowest in the V3 that showed bacterial count of 1.2×10^2 (log 2.0). With regarding to the vendor shops, the overall mean value of bacterial load from milk sample demonstrated in the laboratory and data analysis showed that (V1, V2, V3 and V4) had strong significantly association difference of ($P > 0.05$).This high level of contamination of milk might be due to initial

contamination originating from dairy farms, milk storage, transportation containers of milk, in appropriate vehicles for delivery process between collection and delivery which was taking 2 -5 hours during transactions. These findings agree with Omore *et al.* (2005) who reported that bacterial counts from vendor shops was higher than the dairy producers and subsequently milk quality decreases.

Moreover, the present study indicated the presence of bacterial contamination in milk samples with a bacterial load ranging from $1x10^6$ to $5x10^6$ CFU/mL were considered as poor quality but samples with bacterial load of less than $2x10^5$CFU/mL were graded as good quality according to the procedure Sherikar *et al*. (2004). Among current the milk samples collected, (48%) of the farm settings and (60%) of milk vending shops were graded as poor quality. Likewise, (52% and 40%) from diary and vendor shops were graded and considered as good quality as presented (Table 12). The findings of the present study were in similarly agreement with Reda *et al.* (2014) who found (75%) milk vending shops were graded as poor quality.

Results of antimicrobial susceptibility test showed that most of the isolates of *Staphylococcus* sp., *E. coli*, *Salmonella* sp., *Klebsiella* sp., *Shigella* spwere sensitive to Ciprofloxacin (CIP), Chloramphenicol (C), Levofloxacin (LE) fellow by Cefixime(CFM)but resistant to Ampicillin(AMP) Azithromycin, Ampicillin (AMP), Tetracycline (TE)which were showed very poor efficacies resistance on many isolates bacteria. Only Gentamycin (GEN) was intermediate antibiotic of *Shigella* spp. These findings were closely correlated to Guerin *et al.* (2003) who also observed similar type of findings.

The bacteriological quality and safety of milk is not only affected by the bacterial counts, but also the type and strain of the bacteria are very important. The outcome of this study revealed that 28 % of milk samples were contaminated with at least one bacterium that comprised of *Staphylococcus* sp., *E. coli*, *Salmonella* sp., *Klebsiella* sp., *Shigella* sp., with isolation rates of (33.3%), 23.3%), (20%), 14.2%), (10.7%) respectively. The highest bacterial pathogen was isolates and recovered from milk vending shops (Table 13). The findings of this present study are in agree with other observations regarding the contamination of milk and other ready to eat food stuffs as reported by Daka *et al.* (2012) that reported raw milk may represent an important source of food-borne bacteria and have the effects of level of contamination with each isolated bacteria.

In this study course of bacteria of *Staphylococcus* spp, E. coil *Klebsiella*, spp *Salmonella* spp and *Shigella* spp. were isolated and cultured the raw milk sample collected from dairy farms and vendor shops. Colony characteristics were identified used in differential and selective media. An interestingly, finding of the colony characteristics of the isolates bacteria were both gram positive and gram negative were observed. From the result (Table 12), all media and bacterial morphology were summarized. The present study was comparable with the findings of Quinn *et al.* (200).

In Gram‟s staining, the isolated bacteria exhibited Gram-positive (violet color) cocci arranged in groups or grape like clusters; short coccobacilli or rods arranged in bundles and singly also and Gram-negative (pink color) small rod-shape, arranged single or in paired, motile and non-motile. The study findings were more or less similarly to the findings of Zinnah *et al.* (2007) which were which was supported by the other researchers of Buxton and Fraser. (1977).

In the present study, biochemical tests used for characterization of bacterial pathogens revealed that among the five species of *Staphylococcus* , *E. coli*, *Klebsiella*, *Salmonella* and *Shigella* spp. The isolate bacteria were characterized by biochemical test used various biochemical test included; catalase test, Oxidase test, indole test, Motility indole utilization test, Methyl red, voges-prokaeu (MR and VP) test and Triple Sugar Iron. All the isolates bacteria were indicating positive reaction in catalase test except *Shigella* which was showed negative with No bubble formation (Table 14). The oxidase was only positive reaction in *Staphylococcus* spp. Similar findings have been reported by Ali *et al.* (2008).

CHAPTER VI

CONCLUSION AND RECOMMENDATION

The results obtained in this study showed that milk available to the consumer in Dinajpur town has low quality considering the different stepwise contamination of the milk. The quality of milk produced and channeled starting from the different dairy farm settings in the study area was substandard. The result showed that the microbial quality of raw milk obtained from local dairy farmer was very low. And this was due to the unhygienic condition of milking; unclean milk handling equipment, poor transportation and the use of unhygienic contaminated water were among the important source of milk contamination. High bacterial load, the presence of pathogenic bacteria in several samples not only affects the raw milk quality but definitely pose a safety issue to consumer. According to the bacterial count obtained in this study was generally high compared to the acceptable level of 1×10^5 bacteria per ml of raw milk. The major factor that contributed to poor quality of milk in the study area is due to less hygienic standard and lack of awareness, carelessness of employee and absence of strict sanitation control measures. Therefore, in order to improve the quality and safety of milk produced and distributed to the consumers in the study area awareness creation programs should be initiated to dairy industries, milk distributors, vendor shops and consumers. It was concluded that the microbial quality of raw cow's milk produced and vendor shops in the study area was poor and this suggests the need for improved hygienic practices and handling of milk at all levels of dairy market chain.

Based on the above concluding remarks, the following recommendations are forwarded:

1) Further research could be established for operation standards and strict enforcement of regulatory measures on hygienic standards are of critical importance.
2) Strict hygienic control measures along milking and handling practice to improve hygienic conditions of milk from production to consumption and to enhance quality of milk.
3) Extension of education in all aspects of clean milk production is important in developing awareness among dairy producers and vendors to follow the set standards procedures for milking and proper milk storage.
4) Awareness and training should be given to the public about the danger of consuming the poor hygienic and spoilage milk.

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APPENDIX

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Questionnaire Format for the Assessment of People's Knowledge, Attitude and Practices (KAP) on Assessment of Bacteriological Quality and Hygienic Practices of Milk from Dairy Farms and Vendor shop in and Around Dinajpur district, Northern Bangladesh

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NOTE: This questionnaire Survey is prepared for MSc thesis on " Assessment of people's knowledge, attitude and practices (KAP) on Assessment Bacteriological Quality and Hygienic Practices of Milk from Dairy Farms and vendor shops aspects around Dinajpur town.'' The purpose of this questionnaire Survey is purely academic. Your honesty in responding the right answer is fundamental for the research outcome to be reliable and bring a change in the dairy production sector. Therefore, you are kindly requested to answer the questions in patience and according to the level of your knowledge and I would like to Acknowledge you that your good cooperation by providing true information.

QUESTIONNAIRE FORMA : _____

SECTION I: GENERAL INFORMATION

1). Name of dairy farm or farm owner: _____

2). Address of the farm: _____; phone no. _____

3). Year of establishment of the farm: _____

4). Herd structure: _____

5) Date last reviewed: _____

6) Category of the institution: A. Governmental ☐ B. Private owned ☐

D. Cooperative ☐ E. Other, mention please: _____

8). Occupation: Health Professional Veterinary/animal health attendant

Farmer Sex: Male ☐ Female ☐

9) Respondent´s educational background: A. Formal education ☐ B. Informal education ☐

C. Illiterate ☐

10). What is the number of dairy cows you keep in your farm?

A. Cross breed: ☐ B. Local breed: ☐ C. Both of them: ☐ D. Total: ☐

11). Type of farm: Intensive ☐ Semi-intensive ☐ Extensive ☐

12) Nature/type of the farm: A. Small scale B. Medium scale C. Large scale

13). Feeding regime: a). grazing b). Stall feeding c). Supplemented_____

13). Source of water: a). Pipeline water. b). Well c). River d.) Other_____

14). Housing and cleaning practices: What type of barn do you own?

a). housed b). Fenced c). No barn

15). How frequent do you clean your cows and cow's house/barn?

a**).** Daily b**)**. Two times a week c**)**. Three times a week d**)**. Once a week e**)**. Do not clean

SECTION II: MILK HYGIENE PRACTICES AND EQUIPMENTS OF DAIRY FARMERS

1. Which Milking procedure did you use a). Hand b). Machine c). Both

2). Do you wash your hands before milking? a. Yes_____ b. No._____

3). Are hands washed between milking? a). yes_____. b) No _____

4). Do you wash your cow's udder?

a). Yes_____ If yes, when do you clean it?

b). Wash udder before milking only

c). Wash udder after milking only

d**)**. Wash udder before and after milking e. No_____

5). Frequency of milking per day

a). Twice per day

b). Once a day

c). Three or more times

6). If you clean the udder what materials do you use for drying?

a). Use of towel

b). Collective towel

c). Individual towel

d) Just with bare hands

e). Others (specify

7). What is the source of the water used for washing the udder and milk utensils?

a). Piped/ tap

b). River/ stream

c). Hand dug well

d). Other (specify).

8). How frequently you clean your milking equipment? _____ a). Once per day b). Twice per day c). three times per day d). Others (specify)

9). How often do you wash the container?

a). Before every use

b). After every use

c). Before and after every use.

10). How do you clean milking and milk storage containers?

a). Cold water

b). hot water

c). Cold water and soap

d). hot water and soap

e). Detergent and water

11). What type of milk container do you use for milking purpose?

a). Plastic containers b). Stainless steel c). Nonfood metal container d). Aluminum e). Other

12). What is the means of transportation?

a). On foot b). Van c) auto-rickshaw d). Public transport

e). Private car f.) Other means (specify)

SECTION III: MILK TECHNIQUES, STORAGE AND TRANSPORT TO THE DAIRY FAMERS AND VENDOSHOPS.

1). Where do you transport your products?

a). Own distribution center b) Vendor shops c). Market

2). Type of cleaning of storage used on dairy farms and vendor shops

a). Water with detergent b). Hot water c). Normal /tap water

3). How do you keep the fresh milk before sold or consumed

a) Use of refrigerator b) At room temperature c). Immersing in cool water

d) Traditional cooling system. E). In a hot place f). Where ever no problem.

4). Type of milk sold out on dairy farms and vendors hops

a). Raw milk b). Boiled milk

5). Who were Customers? a). Household b). Individual customers c). Both

6). Type of container used to store and transport milk to market

a). Plastic bags b). Plastic containers. c). Aluminum d). Soda/water bottles

SECTION IV: PUBLIC HEALTH OF PRODUCER AND CONSUMERS AWARENESS LEVEL

1). Habit of milk consumption a). Raw b). boiled c). Fermented milk d). Other_____

2). Do you believe that the raw milk you produce is safe for consumption?

a). Yes_____ b). No_____

3). Do you know any health risk associated with consumption of milk?

a). Yes_____. b). No_____

4). Limited awareness training on hygienic quality of milk from food borne infection

a) Yes _____b) No_____

SECTION V: MILK CATTLE DISEASES AND TREATMENTS

1). Describe major disease you have experienced in your milk cattle during the last year in order of importance.

Local name of diseases	**Symptom**	**Month of occurrence**
1.		
2.		
3.		
4.		
5.		