BACTERIOLOGICAL ANALYSIS OF MILK SOLD IN KATHMANDU AND ANTIBIOTIC SUSCEPTIBILITY PATTERN OF *Staphylococcus species* ISOLATED FROM MILK

A PROJECT WORK SUBMITTED TO THE

DEPARTMENT OF MICROBIOLOGY

AMRIT CAMPUS

INSTITUTE OF SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY

NEPAL

FOR THE AWARD OF

BACHELOR OF SCIENCE IN MICROBIOLOGY

BY

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RECOMMENDATION

This is to recommend that **Soniya Bohara,** Symbol No: 500330012, T.U Registration No: 5-2-33-192-2017 has carried out this project work entitled, **"Bacteriological analysis of milk sold in Kathmandu and Antibiotic Susceptibility pattern of *Staphylococcus species* isolated from milk"** for the requirements to the project work in Bachelor of Science in Microbiology under our supervision in the Department of Microbiology, Amrit Campus, Institute of Science and Technology (IoST), Tribhuvan University (T.U), Nepal,

To our knowledge, this work has not been submitted for any other degree,

She has fulfilled all the requirements laid down by the Institute of Science and Technology (IoST), Tribhuvan University (T.U), Nepal for the submission of the project work for the partial fulfilment of Bachelor of Science (B.Sc.) degree.

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Asst. Prof. Suchitra Thapa
Supervisor
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15th JUNE, 2022

DECLARATION

This project work entitled **"Bacteriological analysis of milk sold in Kathmandu and Antibiotic Susceptibility pattern of *Staphylococcus species* isolated from milk"** is being submitted to the Department of the Microbiology, Amrit Campus, Institute of Science and Technology (IoST), Tribhuvan University (T.U), Nepal for the partial fulfillment of the requirement to the project work in Bachelor of Science (B.Sc.) degree in Microbiology. This project work is carried out by me under the supervision of Asst. prof. Suchitra Thapa in the Department of Microbiology, Amrit Campus, Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal,

This work is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.

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LETTER OF FORWARD

Date: 20th June, 2022

On the recommendation of **Asst. Prof. Suchitra Thapa**, this project work is submitted by **Ms. Soniya Bohara,** Symbol No: 500330012, T.U. Registration No: 5-2-33-192-2017, entitled **"Bacteriological analysis of milk sold in Kathmandu and Antibiotic Susceptibility pattern of *Staphylococcus species* isolated from milk"** is forwarded by the Department of the Microbiology, Amrit Campus, for the approval to the Evaluation Committee, Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal

She has fulfilled the requirements laid down by the Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal for the project work,

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BOARD OF EXAMINATION AND

CERTIFICATE OF APPROVAL

This project work (PRO-406) entitled **"Bacteriological analysis of milk sold in Kathmandu and Antibiotic Susceptibility pattern of *Staphylococcus species* isolated from milk"** by Soniya Bohara, Symbol No: 500330012 and T.U. Registration No: 5-2-33-192-2017 under the supervision of Asst. Prof. Suchitra Thapa in the Department of Microbiology, Amrit Campus, Institute of Science and Technology (IoST), Tribhuvan University (T.U.), is hereby submitted for the partial fulfillment of the Bachelor of Science (B.Sc.) degree in Microbiology. This is report has been accepted and forwarded to the Controller of Examination, Institute of Science and Technology, Tribhvan University, Nepal for the legal procedure.

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ABSTRACT

Milk is a rich source of nutrients. Milk –borne pathogenic bacteria pose a serious threat to human health. *Staphylococcus aureus, Salmonella spp., Listeria monocytogenes with Escherichia coli and Campylobacter* are the main microbial hazards associated with contaminated milk. Therefore, it can cause milk borne diseases like scarlet fever, Brucella, diphtheria typhoid etc. This study was conducted to assess and compare microbial quality of raw milk and pasteurized milk and also determine the antimicrobial susceptibility of *Staphylococcus species* isolated from milk sample consumed in Kathmandu. For this, 30 milk samples (15 raw milk and 15 pasteurized) were collected from different location of Kathmandu district. Total Plate Count and Total *Staphylococcus* Count for each sample were determined by pour plate technique. While for isolation of *Staphylococcus species*, samples were isolated by using selective media (MSA) and characterized by biochemical test. Antibiotic susceptibility testing of isolates was carried out by Kirby Bauer disk diffusion method. Total bacterial count of all raw milk samples were within the range while for TBC of pasteurized milk 93% were within the range. 17 *Staphylococcus species* were isolate from TSC. Among them 15 were identified as *Staphylococcus aureus*. 17 *Staphylococcus species* were 100% sensitive to Cotrimoxazole, Amikacin and Levofloxacin but resistant to Penicillin G (100%),Ceftriaxone (52.92%), Tetracycline (17.68%), Cefoxitin (23.58%), Ampicillin (76.82%) Ciprofloxacin (17.68%) and Chloramphenicol (11.79%). 3(17.68%) of *Staphylococcus aureus* isolated from raw milk samples showed multi-drug resistance and 4(23.58%) MRSA were detected. It is concluded that the milk produced by small scale farm from different places of Kathmandu district are not of quality and can be potential source of milk-borne infection. It is recommended that routine assessment of microbial quality of milk should be done for the safeguard of consumer health.

***Keyword*:** Antibiotic susceptibility testing, *Staphylococcus species*, Total Plate Count, Total Staphylococcus Count, Multi-drug resistant.

शोधसार

दूध पोषक तत्वको धनी स्रोत हो । दूधबाट हुने रोगजनक ब्याक्टेरियाले मानव स्वास्थ्यको लागि गम्भीर खतरा निम्त्याउँछ । *Staphylococcus aureus, Salmonella spp., Listeria monocytogenes with Escherichia coli* र *Campylobacter* दूषित दूधसँग सम्बन्धित मुख्य माइक्रोबियल खतराहरू हुन् । तसर्थ, यसले दूधजन्य रोगहरू जस्तै स्कार्लेट ज्वरो, ब्रुसेला, डिप्थेरिया टाइफाइड आदि निम्त्याउन सक्छ। यो अध्ययन कच्चा दूध र पाश्चराइज्ड दूधको माइक्रोबियल गुणस्तर मूल्याङ्कन गर्न र तुलना गर्न र काठमाडौंमा उपभोग गरिएको दूध नमूनाबाट पृथक स्टेफिलोकोकस प्रजातिहरूको एन्टिमाइक्रोबियल संवेदनशीलता निर्धारण गर्न गरिएको थियो। । यसका लागि काठमाडौं जिल्लाका विभिन्न स्थानबाट ३० दूधको नमूना *(*१५ कच्चा दूध र १५ पाश्चराइज्ड*)* संकलन गरिएको थियो । प्रत्येक नमूनाको लागि कुल प्लेट गणना र कुल स्टेफिलोकोकस गणना pour plate प्रविधिद्वारा निर्धारण गरिएको थियो। सटेफिलोकोकस इसपेसिस को अलगाव को लागी, नमूनाहरु को सेलेकटिभ मिडिया(MSA) को उपयोग गरेर अलग गरिएको थियो र बायोकेमिकल परीक्षण गरियो। आइसोलेटहरूको एन्टिबायोटिक ससेपटिवल टेसट किर्बी बाउर डिस्क प्रसार विधि द्वारा गरिएको थियो। कच्चा दूध नमूनाहरूको कुल ब्याक्टेरियाको गणना दायरा भित्र थियो जबकि पाश्चराइज्ड दूधको TBC दायरा भित्र ९३% थियो । १७ सटेफिलोकोकस इसपेसिस TSC बाट अलग गरिएका थिए। तीमध्ये १५ जनालाई स्टेफिलोकोकस ओरियस भनिएको थियो । १७ वटा सटेफिलोकोकस इसपेसिस, *Cotrimoxazole, Amikacin* र *Levofloxacin* को लागी १००% संवेदनशील थिए तर *penicillin G (*१००%*), Ceftriaxone (*५२.९२%*), Tetracycline (*१७.६८%*), Cefoxitin (23.58%), Ampicillin (*७६.८२%*), Ciprofloxacin (*७६.८२५*), Chloramphenicol (*११.७९%*)* रेसिसटेनट देखियो । ३ *(*१७.६८%*)* काँचो दूध नमूनाहरूबाट अलग गरिएको सटेफिलोकोकस *aureus* ले मलटि ड्ग रेसिसटेनट *(MDR)* देखाए र ४*(*२३.५८%*) MRSA* पत्ता लगाइयो । काठमाडौं जिल्लाका विभिन्न स्थानबाट साना फार्मबाट उत्पादित दुध गुणस्तर नभेटिएको र यसता दुधको उपभोगले दुधजन्य संक्रमणको सम्भावित स्रोत हुन सक्ने निष्कर्ष निकालिएको छ । उपभोक्ताको स्वास्थ्यको सुरक्षाका लागि दूधको माइक्रोबियल गुणस्तरको नियमित मूल्याङ्कन गरिनुपर्छ भनी सिफारिस गरिन्छ ।

कीवर्डः एन्टिबायोटिक ससेपटिविलिटि टेसट, सटेफिलोकोकस इसपेसिस, टोटल प्लेट काउनट टोटल सटेफिलोकोकस काउनट, मलटि ड्ग रेसिसटेनट।

LIST OF ACRONYMS AND ABBREVIATIONS

AST	Antibiotic Susceptibility Testing
ATCC	American Type Culture Collection
BIS	Bureau of Indian Standards
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CLSI	Clinical Laboratory and Standard Institute
CoNS	Coagulase negative *Staphylococcus species*
DFTQC	Department of Food and Technology and Quality Control
ETEC	Enterotoxigenic *E. coli*
FAO	Food and Agricultural Organization
FDA	U.S. Food and Drug Administration
GMP	Good manufacturing practice
HACCP	Hazard analysis and critical control points
MDR	Multi-drug Resistant
MHA	Mueller Hinton Agar
MRSA	Methicillin Resistant *S. aureus*
MSA	Mannitol Salt Agar
NCHS	National Center for Health Statistics
PCA	Plate Count Agar
TBC	Total Bacterial Count
TSC	Total Staphylococcus Count
WHO	World Health Organization

LIST OF SYMBOLS

%	Percentage
<	Less than
≤	Less than or equal to
>	More than
℃	Degree Celsius

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CHAPTER 1

1. INTRODUCTION

1.1 General Introduction

Milk is a daily diet requirement of many people but it can become microbiologically hazardous to consumers when the principles of hygiene and sanitation are not met. It is an important source of carbohydrate, proteins with all ten amino acids, immunoglobulins, essential fatty acids, and other micronutrients (Hossain *et* al., 2011). Pasteurized milk is one of the better milk product which has nutrition composition and taste similar to fresh milk. But it need a strict quality control to guarantee the composition of nutrition and food safety (Varnam and Suthan, 1994). Similarly, raw milk is a nutrient rich beverage that may benefit your health in several ways. It is packed with important, vital nutrients like calcium, healthy fats, vitamins, protein, minerals and ions. Milk is a nutrient rich beverage that may benefit your health in several ways.

Contamination of milk may occur through various sources. Maybe through infected cow with tuberculosis, brucellosis, and mastitis and also from milk handlers infected with typhoid fever, diphtheria, dysentery, and scarlet fever (Jay *et* al., 2005). It is common that dairy cattle and their farm's surroundings may contain many pathogens such as *Listeria, Salmonella* and pathogenic *Escherichia coli*. Raw or inadequately pasteurized milk may contain toxin producing *E. coli, Campylobacter, Listeria, Monocytogenes* and others (Pal *et* al., 2016). Inappropriate handling may cause an outbreak to public health problems and economic losses, thus hygienic vigilance is essential throughout the entire milk chain starting from producer to consumer (Hayes and Boor, 2001). Most of the people in the world consume pasteurized milk and few people prefer raw milk as they believe that raw milk is more beneficial, tastier and convenient than pasteurized one (Altalhi and Hassan 2009).

More than 90 % of all reported cases of dairy related illness are of bacterial origin, with at least 21 milk-borne or potentially milk-borne diseases being recognized (Bean

NH *et* al., 1988-1992). *S. aureus* is a significant cause of food borne disease, causing an estimated 241,000 illnesses per year in the United States (Scallan *et* al 2011).The first epidemic report of foodborne outbreaks from Staphylococci was made in 1884 by Vaughan and Sternberg in Michigan (USA) (Spanu *et* al 2012, Nunes et al 2016, Fox *et* al 2017). According to the US FDA regulations as well as ICMSF-1996, the number of *S. aureus* in raw milk or other dairy products needs to be $\leq$ 104 CFU/gm (Yu *et* al 2016). In a survey of raw milk in Nepal, it was found that out of the 129 samples, 25%, 37.2%, 5.4%, 7.7%, 18.6%, 1.6%, were positive for *E. coli, Salmonella spp., Shigella spp., Klebsiella sp., Citrobacter spp., Pseudomonas spp.* respectively (Regmi *et* al., 2001).

Similarly, *Staphylococcus aureus* is another important human pathogen that causes food borne infections including milk and milk products (Bergdoll and Lee Wong 2006). In US antibiotic resistant outbreaks have been catalogued since the early 1970s dairy products accounting for the significant numbers of the outbreaks. And among different resistant bacteria causing outbreaks, enterotoxigenic *Escherichia coli* (ETEC) and *S. aureus* were also responsible for the number of outbreaks (Redfield 2019). In addition to that resistances towards the important human medicine are increasing (Van et al 2007). Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most successful modern pathogens (Turner *et* al 2019). Methicillin-resistance *Staphylococcus aureus* (MRSA) is a major pathogen worldwide; MRSA infections are associated with increase morbidity and mortality, in comparison with other *S. aureus*. In the 1940s, penicillin was introduced for the treatment of infection; as early 1942, strain of *S. aureus* resistant to penicillin had been detected in hospitals. The introduction of methicillin in 1961 was rapidly followed by reports of methicillin resistance in *S. aureus*. Today, MRSA strains are found worldwide, and most are multidrug resistance (Appelbaum PC.MRSA 2006).

Presence of *S. aureus* and an intestinal commensal *E. coli* indicates the alarming public health concern. To minimize the risk of milk-borne diseases, an intense study should be done to determine the microbiological quality of milk and other chemical adulterants and their public health impact (Arjyal *et* al., 2004). Thus, objectives of this study were to determine the bacterial contaminants as well as an admixture of adulterants in milk marketed in Kathmandu. However, Kilango *et* al.(2012) observed that while boiling generally makes milk safer by eliminating most microorganisms, it still carries the risk of consumer exposure to pathogenic bacteria.

1.2 Rationale

The prevalence of *S. aureus* in milk was detected and the antimicrobial susceptibility testing was done to determine MRSA and MDR isolated from raw and pasteurized milk samples. Milk borne disease are one of the recurrent foodborne illness between 1993-2012 over 12 outbreaks related to raw milk were recorded in U.S. with approximately 1,900 illness and 140 hospitalization. *S.aureus* is one of the leading causes of food borne illness. Therefore, routine monitoring of milk at the farm level and in dairy industry, monitoring at pre-production and post production level is important. So, with an aim to assess the bacterial analysis of milk available in Kathmandu , this project was conducted. This study gives the information about the current microbial status of the milk sold in Kathmandu and prevalence of Anti-microbial resistant.

This study helps to aware people about the bacterial quality of milk. It gives the information for the policy makers to prepare policy. Researcher can get the information about the current status and further research can be done in molecular level.

1.3 Objective

1.3.1 General objective

To assess the bacterial analysis of milk samples from different localities of Kathmandu, Nepal, and antibiotic susceptibility testing of bacteria isolated from milk samples.

1.3.2 Specific objectives

- To determine the total bacterial load (TPC) and total Staphylococcus count (TSC) in milk (raw & pasteurized) samples.
- To isolate and identify *S. aureus* from milk samples.
- To determine antibiotic susceptibility pattern of *Staphylococcus species* isolated from milk samples.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Definition and composition of milk

Milk is a nutrient rich liquid food produced by the mammary glands of the mammals. It is an important source of carbohydrate, proteins with all ten amino acids, immunoglobulins, essential fatty acids, and other micronutrients (Hossain *et al*., 2011). Milk is synthesized in specialized cells of the mammary gland and is virtually sterile when secreted into the alveoli of the udder (Tolle *et al.,* 1980). Milk is a compulsory part of daily diet for the expectant mothers as well as growing children (Javaid, S.B. *et* al 2009).

Milk is a nutrient-rich beverage that may benefit your health in several ways. It's packed with important nutrients like calcium, vitamins D, protein, healthy fats, minerals, antioxidants etc. These reduce the risk of osteoporosis and bone-factures later in life. Milk and dairy products can be important in diversifying the diet. They are nutrient dense and provide high quality of protein and micronutrient in an easily absorbed form that can benefit both nutritionally vulnerable people and healthy people when consumed in appropriate amount (FAO). The average composition of milk are: Water (87.20%), Protein (3.50%), Fat (3.70%), Milk sugar or Lactose (4.90%), Ash (0.70) and Dry matter (12.80%).

2.2 Types of milk and their importance

The primary types of milk available are whole milk, reduced-fat milk, low-fat milk and fat-free milk also known as skim milk. Types of milk vary by percentage of milk fat or the amount of fat that is in the milk by weight. Milk is available with different fat content including whole (3.25%), reduced fat (2%), low-fat or light (0.5, 1%), non-fat/fat-free/skim (<0.5%) (Vaclavik and Christian 2007).

2.2.1 Raw milk

Raw milk contain alkaline phosphatase enzyme, which is associated with decreased inflammation and lower rates of cardiovascular disease and Type-2 diabetes. Raw

milk is an excellent source of vitamin A, thiamine (B1), magnesium, zinc etc. Raw milk advocates argue that it is a complete natural food containing more amino acids, vitamins, minerals, fatty acid, antimicrobials than pasteurized milk. For the reason it may cut your risk of osteoporosis and reduce blood pressure.

2.2.2 Pasteurized milk

FAO/WHO defined pasteurization as "A microbicidal heat treatment aimed at reducing the number of any pathogenic microorganisms in milk and liquid milk products, if present, to a level at which they do not constitute a significant health hazard. Pasteurized milk is raw milk that has been heated to a specified temperature and time to kill pathogens that may be found in the raw milk. The most commonly used pasteurization methods are low-temperature/long-time (LTLT) pasteurization, in which the milk is heated at 62.5°C in a water bath for 30 minute (Updegrove 2010), and high-temperature/short-time (HTST) heating at 71.7°C for 15 second (Ball 2006). Pasteurization is the widely adopted milk process to ensure completely destruction of all pathogenic and spoilage microorganisms, commonly found in milk and inactivation or reduction of other nonpathogenic spoilage bacteria and certain undesirable enzymes to safeguard the food value of milk (Teka, 1997).

2.3 Microbial contamination and source of milk

Milk taken from the udder's of healthy animals is free of pathogenic bacteria, however certain animals in the field may be suffering from sub-clinical mastitis and excreting the causative agent in milk, contaminating the bulk milk. Air, feed, grass, soil, milking equipment and faeces may be the primary sources, through whose contact other sources may lead to contaminate raw milk with different microorganisms (Swai and Schoonman, 2011). Poor pre-milking udder hygiene that fails adequately to clean dirty udders may also result in the introduction of vegetation, soil, and bedding material and their associated microorganisms into the milk (Hayes and Boor, 2001). Such foreign matters and contaminations in the milk may lead to concerns regarding consumer health (Lemma *et al*., 2018). Bacterial contamination of raw milk can originate from sources such as air, milking equipment, feed, soil, faces and grass. Differences in feeding housing strategies of cows may also influence the microbial quality of milk (Torkar, K.G *et* al., 2008). Indigenous sweet milk products

are highly susceptible to variety of microorganism because of high nutritive value and complex chemical composition (Kumar *et* al.,2011).

According to different literature, the main cause of microbial contamination of milk is due to milking from infected udder of the cow, improper storage and packaging of milk, unclean equipment's, transportation, poor milk handling practices and the surrounding environment. If good hygiene is not maintained, contamination of milk may come from vegetation, soil, utensils, packaging materials and beddings (Lemma et al., 2018). It is emphasized that good manufacturing practice (GMP), critical control point, good hygienic practice, hazard analysis should be implemented in dairy industry to prevent the contamination of dairy products (Shrestha *et al* 2012; Pal *et al* 2016).

2.4 milk borne diseases and Pathogenic microorganism

Since milk is such a excellent nutrient source and because milk-producing animals may harbor organisms that cause human disease, it is not surprising that raw milk can be a source of diseases. Some of the most obvious are the animals diseases below to which human susceptible and which may occur in milk of cows (Jay *et*., al). milk borne disease are any diseases caused by the consumption of milk or dairy product contaminated by pathogens. Milk borne pathogenic bacteria pose a serious threat to human health. Milk borne pathogens cause 90% of the dairy related diseases. It is caused by the contamination occur from poor hygiene, contaminated utensils, milk-handlers and diseases like typhoid fever, diphtheria, dysentery, and scarlet fever (Jay et al., 2005). Milk borne diseases are given in Table-1.

Table 1: Diseases caused by milk-borne pathogens

Milk-borne diseases	
Brucellosis	Anthrax
Tuberculosis	Listeriosis
Salmonellosis	Q fever

Foodborne illness is a common and pervasive problem around the world. Foodborne illnesses account for 48 million infection per year in the United States America, with *Norovirus*, *Salmonella* spp. (nontyphoidal), *Clostridium* perfringens, *Campylobacter* spp and *Staphylococcus aureus* ranking as the top five pathogens contributing to

domestically-acquired foodborne illnesses (CDC 2011). The tests of coliform and *S. aureus* counts as an indicator of post-process contamination revealed dairy industry and its sanitary control (Flowers et al 1992). Some outbreaks, death rate by foodborne diseases are given below.

Table 2: Death rate by foodborne diseases (WHO)

Foodborne diseases in the WHO region	People falling ill	People dying South East
Asia region	>150 million	>175000
American region	77 million	9000
Western pacific region	125 million	>50000

Source: WHO estimation of the global burden of foodborne diseases 2015

Various sources are responsible for contamination of the milk that may be through cattle suffered with tuberculosis, brucellosis, and mastitis (Jay et al., 2005). It is common that dairy cattle and their farm's surroundings may consist of several pathogens such as *Listeria* spp., *Salmonella* and pathogenic *E. coli.* Raw or inadequately pasteurized milk may contain toxin-producing *E. coli, Salmonella, Listeria monocytogenes* and others (Pal et al., 2016).

2.5 *Staphylococcus species*

Staphylococcus aureus are Gram-positive cocci ranging from 0.5µm to 1.5µm in diameter, which may or may not contain a polysaccharide capsule. They are non-motile, non-spore forming facultative anaerobes that produce catalase and coagulase enzymes (Ho et al 2014). It is often positive for catalase and nitrate reduction and is facultative anaerobic that can grow with the need of oxygen (Masalha *et* al 2001). *Staphylococcus aureus* is a normal flora of the skin. It causes infection most commonly at sites of lowered host resistance, such as damaged skin or mucous membrane. *S. aureus* produces many virulence factors such as capsular polysaccharide, cell wall associated polymers, super antigen exotoxins, leucocidin, hemolysin, extracellular enzyme and protein receptors (Oogai et al 2011, Chakraborty 2016).

Staphylococcus aureus is a contagious pathogens that cause mastitis in dairy cattle and is an opportunistic pathogens in human and many other animal species. Dairy animals with mastitis frequently shed *S. aureus* into the milk supply which can lead to food poisoning in humans. *S. aureus* is a pathogenic bacterium contaminating milk and milk products causing food poisoning primarily due to its enterotoxins. *Staphylococcus aureus* is the most pathogenic. It typically cause pneumonia, endocarditis and osteomyelitis. Some strain elaborate toxins that cause gastroenteritis and toxic shock syndrome.

Epidemiology: *Staphylococcus aureus* is a significant cause of foodborne disease, causing an estimated 241,000 illnesses per year in United States (Scallan et al 2011).The CDC estimates that S. aureus causes about 185,000 cases of foodborne intoxications in the US (Mead et al 1999).

In the United States (US) the notification rate for vancomycin-intermediate *S. aureus* was 0.04 cases per 100,000 population in 2010, which was increase from the 2009 rate of 0.03 NCHS (2013). It is estimated that in the US, *S. aureus* accounts for 2.6% of foodborne illness caused by 31 major pathogens (Scallan et al 2011).

Arjyal. C, et al (2004) reported different milk brands were collected from different outlets in valley and analyzed for microbial quality. The samples were cultured using the standard microbiological techniques. Almost all sample showed the presence of bacterial growth including coliform bacteria. *Escherichia coli* was the most frequently isolated organism(92%) followed by coagulase negative Staphylococci (CONS) (24%).Thus, it was concluded that the market milk available in Kathmandu is contaminated with various type of microorganisms and does not meet the required standard.

Out of total 150 milk samples collected; *S. aureus* was prevalent in 56.67% of the samples (Mausam et al 2016).

Limbu, et *al* (2020) studied the microbiological quality and adulteration of pasteurized and raw milk marketed in Dharan, Nepal. Chemical analysis of the samples reveled that, most of the milk were adulterated with table sugar. Among the pasteurized milk and raw milk (total 40 sample) *E.coli*, Total coliforms, Thermoduric bacteria, *S. aureus* were detected in 30%, 80%, 75% , 20% and 55%, 95%, 45%, 95% respectively.

2.5.1 Source to milk

Staphylococcus aureus is one of the most important contagious pathogen in milk. The main reservoir of *S. aureus* are infected udders, teat canals, teat lesions, but these bacteria have been found on teat skin, muzzles and nostrils. Poor pre-milking udder hygiene that fails adequately to clean dirty udders may also result in the introduction of vegetation, soil, and bedding material and their associated microorganisms into the milk (Hayes and Boor, 2001). Such foreign matters and contaminations in the milk may lead to concerns regarding consumer health (Lemma et al., 2018).

2.6 Prevention and control of microbial contamination in milk

Food producers are responsible for the safety of their products, and to guarantee food safety of dairy products, the dairy industry has implemented hazard analysis of critical points (HACCP) system. Prevention and control of microbial quality of milk is through elimination of organisms from human carriers by general improvement in public health education, personal and environmental hygiene, water supplies.

The quality and safety of milk is related to the contamination of milk with microorganisms, chemical residues and other contamination. To ensure a good microbial quality of bulk tank milk, quality assurance systems for dairy farms are being developed and bacteriological schemes are being implemented in payment system of farm raw bulk milk (IDF, 2006). The primary controls of the microbes in raw and pasteurized milk are limiting the time and temperature of storage, ensuring any processing is performed effectively and paying close attention to equipment cleaning and sanitation. The strategy on the farm to reduce contamination by foodborne pathogens is to establish hygienic practices on the farm in various component of milk production chain. In the dairy industry, the ultimate control is by heat treatment. The presence of bacteria in pasteurized milk invites several speculations, ranging from faulty processing, to post-pasteurization contamination. Pasteurization is an effective technique to reduce and eliminate food-borne pathogens and other bacteria from milk. To upgrade the quality of raw and pasteurized milk, legal enforcement on the microbial guideline of marketed milk, routine monitoring of dairy industries and raw milk vendors, awareness campaign and good hygienic practice should be promoted.

2.7 Antibiotic and their resistance

Antibiotics are medicines used to prevent and treat bacterial infections. Antibiotic resistance occurs when bacteria change in response to the use of these medicines. The main aim of antibiotic susceptibility testing is to detect possible drug resistant in common pathogens and to assure susceptibility to drugs of choice for the particular disease or problem. Most widely used antibiotic susceptibility testing methods include broth dilution test, antimicrobial gradient method, disc diffusion method (Barth et al 2009).

The Kirby–Bauer disc diffusion method is most common antibiotic susceptibility testing method done for determination of susceptibility or resistance of microorganisms. A wide range of the biochemical and physiological mechanisms may be responsible for resistance (Davies and Devies 2010). The culture of standard bacteria, American Type Culture Collection (ATCC), has been used for decades as a control reference, some of which are mandated or recommended in both FDA (2012) regulations and in the standard put out in Clinical and Laboratory Standards Institute (CLSI) (Simione 2011).

2.7.1 Drug resistance in *Staphylococcus species*

S. aureus were isolated from 119 (29.7%) milk samples. MRSA were found in 45 (11.25%) milk samples. *S. aureus* isolates were found sensitive to ciprofloxacin (97.47%), gentamycin (94.95%), ceftriaxone (91.59%) and tetracycline (89.91%) in descending order while they were found least sensitive to cefoxitin (62.18%) (Joshi et al 2014).According to Karki *et al* (2014) among the 1173 clinical specimen, 100 *S. aureus* were isolated of which 19% cases were MRSA. *S. aureus* showed resistance towards Vancomycin 100%, Amikacin (90%), Gentamycin (83%), and Tetracycline (81%). Out of 70 total milk samples, 30 *S. aureus* isolates were subjected to antibiotic susceptibility test using Cefoxitin, Ciprofloxacin, Ceftriaxone, Chloramphenicol and Nalidixic acid antibiotic disc. All the isolates showed 100% susceptibility towards Chloramphenicol, while the susceptibility for Tetracycline, Ciprofloxacin, Cefoxitin and Ceftriaxone were 93.33%, 70%, 60% and 30% respectively and 12 MDR *S. aureus* (40%) were identified and they were confirmed as MRSA due to their resistance towards Cefoxitin (Karki.B, *et al* 2019).

2.7.2 Multi-drug Resistant (MDR)

Multi-drug resistant or multiresistance is antimicrobial resistance shown by the species of microorganism to at least two or more than two drug. It is insensitivity of a microorganism to the administered antimicrobial medicine despite earlier sensitivity to it. 100% pathogens isolated from raw and pasteurized milk exhibit multi-drug resistant (Marjan et al 2014).

2.7.3 Methicillin-resistant *S. aureus* (MRSA)

Methicillin-resistant *S .aureus* is a group of gram positive bacteria that are genetically distinct from other strains of *Staphylococcus aureus.* MRSA is responsible for the several difficult-to –treat infection in humans. It caused more than 100,000 deaths attributable to antimicrobial resistant.33.33% of the milk samples were confirmed as MRSA sue to their resistant towards Cefoxitin (Parajuli et al 2018).

CHAPTER 3

3. METHODOLOGY

3.1 Materials

The materials and equipment's required for this study are listed in Appendix-I .

3.2 Methods

3.2.1 Study site / Sampling site

This study was conducted in Kathmandu district which is in Province 3 of Nepal. Sampling and analysis of the milk samples were performed during April to June 2022. Kathmandu district covers an area of 395 kilometers square. It is densely populated district of Nepal with 30,152,366 people in 2022. There is 1 Metropolitan and 10-Municipalities.

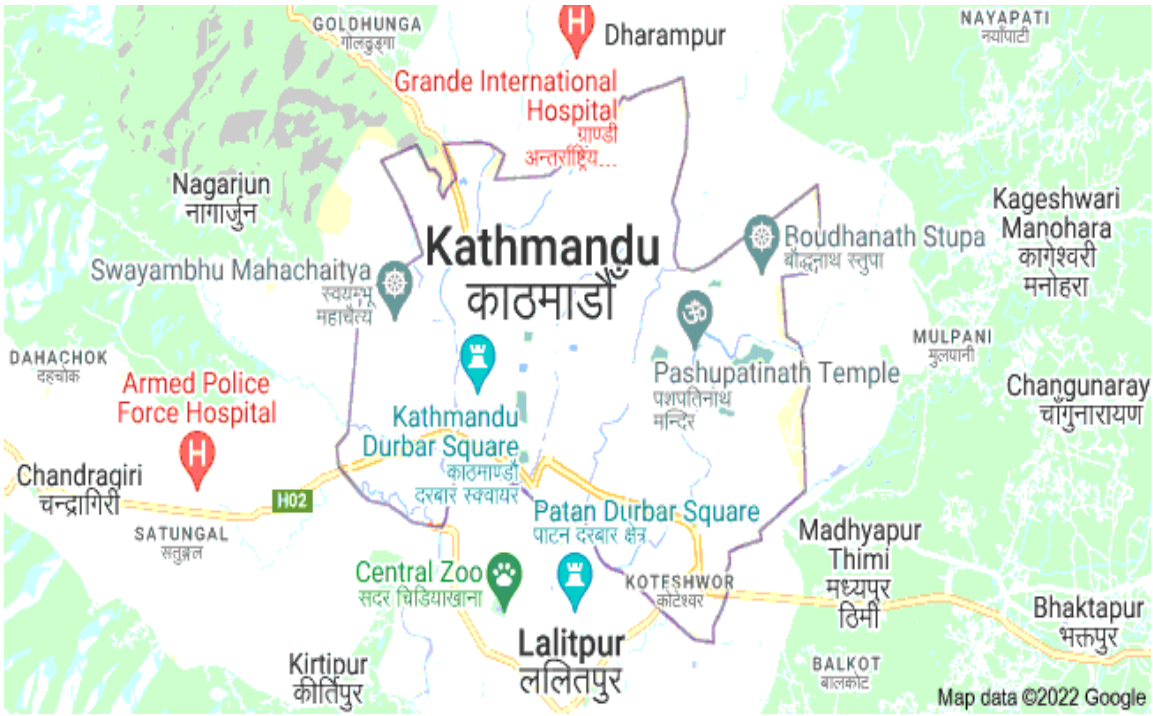


Figure1: Map of study area

Pasteurized milk was collected from local shops of Kathmandu district while raw milk was collected from Budhanilkantha Municipality, which were selected according to

population of characteristics and availability of commercial farms and small stock holders, characteristics and availability of commercial farms and small stock holders.

3.2.2 Study design

The present study employed a cross sectional study design to establish the magnitude of the microbial contaminants in raw and pasteurized milk from selected dairy farm's and local shop in Kathmandu district.

3.2.3 Sample source, Size and Sampling method

Two types of milk sample viz. Pasteurized milk and raw milk samples were included in the study. Raw milk samples from the selected study site were collected from cow's farm and small livestock keeper while pasteurized milk samples were collected from the shop of Kathmandu district. Convenient sampling was followed for sample collection. A total of 30 milk samples; 15 raw and 15 pasteurized milk samples were selected for the study.

3.2.4 Sample collection and transportation

30 milk samples were collected among them 15 were raw samples and 15 were pasteurized from different localities of Kathmandu district. For raw milk sampling, 30 ml samples was taken from the site milked from container containing milk that were milk on a particular day aseptically and kept into a sterile screw cap tubes which were directly transported into laboratory in ice-box. Similarly for pasteurized milk, 500ml packaged available in market were purchased and transported in plastic bag to the laboratory. Pasteurized milk were brought to room temperature and processed with in 2 hour of collection.

3.3 Sample evaluation and inclusion criteria

Raw milk was evaluated for different kind of debris like insects dust flooding on milk and evaluated for single type (milk from single cow) or mixed type (mixed milk of more than two cow) of milk. Buffalo's milk were excluded in this study. Pasteurized milk packets were evaluated well before taking it as a sample. It was evaluated for leaking manufacture and expiry date. Those packaged which is only processed and homogenized milk, leaking and expired was excluded during sampling.

3.4 Sample processing

Laboratory analysis was carried out in the Microbiology Laboratory of Amrit campus, Thamel, Kathmandu. Bacterial quality of milk samples was determined by standard guideline of BIS (1992). The Total plate count (TPC), Total Staphylococcus count (TSC) and the isolation of *Staphylococcus species*. Determination of antimicrobial susceptibility of the isolated *Staphylococcus spp* were done.

3.4.1 Sample preparation and dilution

For sample preparation, 1ml of sample was poured in sterile test tube containing 9ml of sterile normal saline after which, dilution up to 10^{-8} were done for raw milk and 10^{-6} were done for pasteurized milk.

3.4.2 Total Plate Count

The total bacterial count was carried out by the pour plate technique. The sample (1ml) of each dilution was taken onto each sterile Petriplate and evenly poured PCA and incubated at 37°C for 24 hours. The plate were screened for the presence of discrete colonies after incubation period and the actual number of bacteria were estimated as colony forming unit per ml (CFU/ml). Then the result per dilution were recorded.

3.4.3 Total Staphylococcus Count

The total staphylococcus Count was carried out by the pour plate technique. The sample (1ml) of each dilution was taken onto each sterile petridish and evenly poured MSA and incubated at 37°C for 24 hours. The plated were screened for the presence of discrete colonies and golden yellow colonies after incubation period and the actual number of *Staphylococcus spp* were estimated as colony forming unit per ml (CFU/ml). Then the result per dilution were recorded.

3.4.4 Isolation and identification of the *Staphylococcus spp*.

Isolation and identification of *Staphylococcus spp.* was done. From each sample single golden colony on Mannitol salt agar (MSA) were subjected to identification of *Staphylococcus spp*. A loopful of isolated golden colony was inoculated into nutrient broth from the MSA plate. NB were incubated for 4 hours at 37°C. A loopful of inoculum from NB was streak on nutrient agar plate as well as inoculate in

biochemical set. Identification was based on gram staining, biochemical characteristics and growth pattern on selective media according to the procedures recommended in Chakraborty (2011). Biochemical tests included in this study to identify and confirmed *Staphylococcus spp* were Catalase test, Oxidase test, Oxidation-Fermentation (OF)test and Coagulase test, as described by Cheesbrough (2006). *Staphylococcus aureus* were differentiated from *Staphylococcus species* and confirmed by coagulase or DNase test.

3.4.5 Antibiotic susceptibility pattern of the isolates

The susceptibility of the isolates to different antibiotics was done by kirby Bauer disk diffusion method as described by Clinical and Laboratory Standard institute using commercial discs of Hi Media Pvt. Ltd. The antimicrobial agents tested against *Staphylococcus spp* were Ciprofloxacin (5mcg), Ceftriaxone (30mcg), Tetracycline (30mcg), Cotrimoxazole (25mcg), Cefoxitin (30mcg), Ampicillin (10mcg), Amikacin (30mcg), Levofloxacin (5mcg), Penicillin G (10mcg), Chloramphenicol (30mcg). The zone of inhibition diameter was measured in millimeter scale. The zone of diameter for individual antimicrobial agents was then translated into sensitive, intermediate and resistant categories according to recommended interpretation guideline for common pathogen (CLSI 2018). Intermediate were placed in resistant group in this study.

3.5 Data collection and analysis

Data will be collected in laboratory by observational method. These collected data were tabulated in observational table and entered in MS excel data sheet and analyzed by interpreting the outcome or result. Analysis was done by SPSS software. Primary data were collected from experiment and secondary data were collected from published articles and books.

3.6 Quality control

ATCC culture were used for the Antibiotic susceptibility test. We can determine the quality of the lawn culture, preparation of MHA plate, quality of antibiotic disc by comparing the results with ATCC culture. To maintain the quality of experiment, instrument should be optimized and maintain the sterile condition while doing lab work.

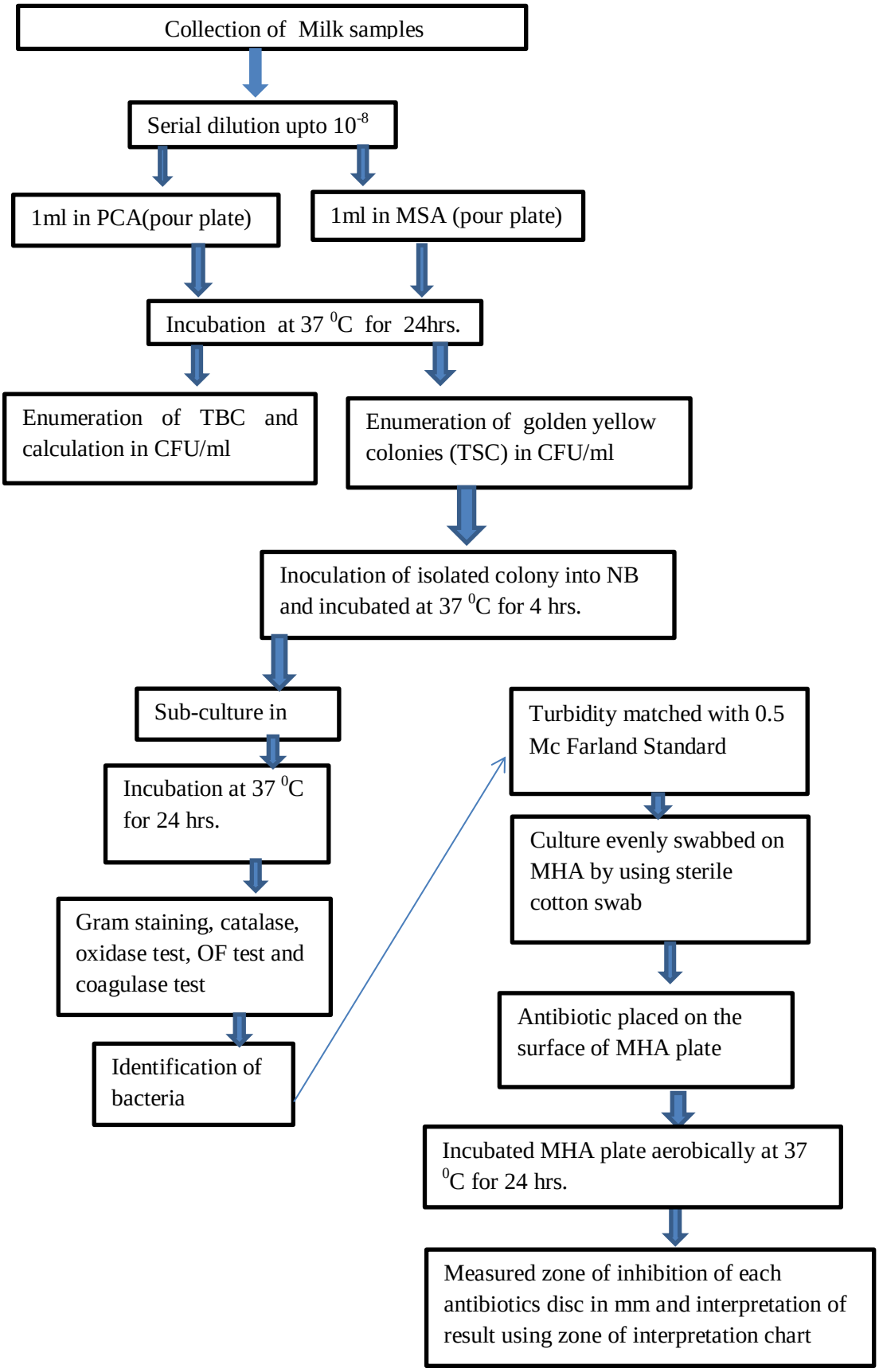


Figure2: Flow chart for isolation and identification & AST of bacteria (Manandhar *et al.*, 2013, Phattepuri *et al.*,2020 and CLSI 2018

CHAPTER 4

4. RESULTS AND DISCUSSION

In this study, the bacterial quality of the total 30 milk sample (raw and pasteurized) were evaluated by determining the bacterial load of the milk consumed in Kathmandu district. The samples were then subjected for the isolation and identification followed by the antibiotic susceptibility testing.

4.1 Microbial quality of the raw milk and pasteurized milk

4.1.1 Total Bacterial count

Total Bacterial Count (TBC) ranged from 0.0173×10^5 to 1.798×10^5 CFU/ml and the TBC was found to be 0.277×10^5 CFU/ml.

In 15 raw milk samples, the Total Bacterial Count (TBC) ranged from 0.0325×10^5 to 1.798×10^5 CFU/ml and the mean TBC was 0.443×10^5 CFU/ml which is lower than the recommended level of 5×10^6 CFU/ml. Out of 15 raw samples in average, all of them were graded as good quality of milk according to BIS 1992(Appendix XI).

In 15 pasteurized milk samples, the Total Bacterial Count (TBC) ranged from 0.0173×10^5 to 0.4×10^5 CFU/ml and the mean TBC was 0.1089×10^5 CFU/ml which is lower than the maximum recommended level of 3×10^4 CFU/ml.

The distribution of Total bacterial count is not same across raw and pasteurized milk sample type ($p<0.05$) (Appendix XII). The descriptive statistics is given below.

Table 3: Descriptive Statistics of TBC

	N	Minimum	Maximum	Mean	Std. Deviation
Total bacterial count	30	1730	179800	27656.00	33174.796
Valid N (list wise)					

Among 15 pasteurized milk samples in average all were graded as good according to Quality standard given by (BIS 1992) but in this case, few sample showed the microbial load above range which is shown in Figure-3.

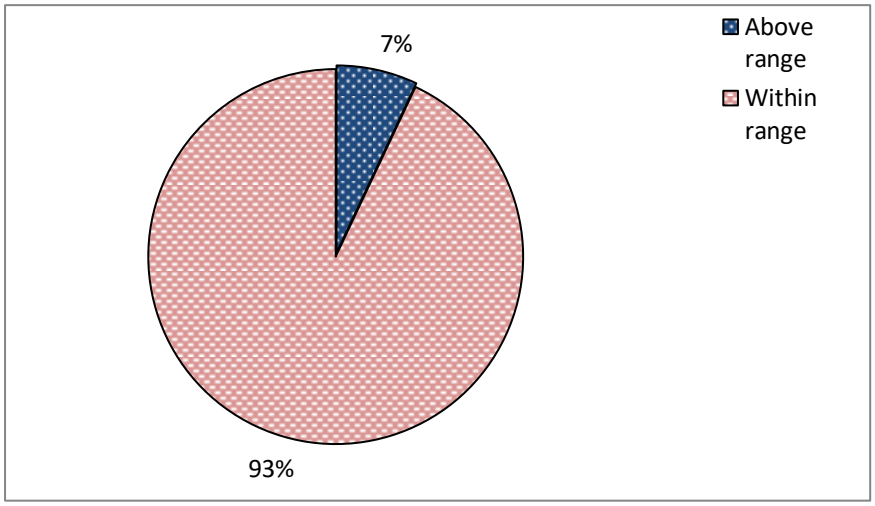


Figure 3: Microbial quality of pasteurized samples based on BIS guideline (1992)

4.1.2 Total *Staphylococcus* count

Out of 30 milk samples, the Total *Staphylococcus* count (TSC) ranged from 2.07×10^3 to 45×10^3 CFU/ml and the mean TSC was 1.064824×10^4 CFU/ml.

Out of 15 raw milk samples, the Total *Staphylococcus* count (TSC) ranged from 3.0×10^3 CFU/ml to 45×10^3 CFU/ml and the mean TSC was 1.310769×10^4 CFU/ml. It was found that 86.67% of raw milk samples were positive for *Staphylococcus spp*.

Out of 15 pasteurized milk samples, the Total Staphylococcus count (TSC) ranged from 2.07×10^3 CFU/ml to 3.0×10^3 CFU/ml and the mean TSC was 2.655×10^3 CFU/ml. It was found that 26.67% of pasteurized milk samples were positive for *Staphylococcus spp*.

The distribution of Total *Staphylococcus* count is not same across raw and pasteurized milk ($p<0.05$) (Appendix XII). The descriptive statistics of TSC is given below in Table-4

Table 4: Descriptive Statistics of TSC

	N	Minimum	Maximum	Mean	Std. Deviation
Only TSC Valid (listwise)	17	2070	45000	10648.24	11585.535

4.2 Isolation and identification of *Staphylococcus species*

Out of 30 milk samples, 17 (56.66%) samples were found to be *Staphylococcus species*. Among them 88.24% isolate were identified as *Staphylococcus aureus* while 11.76% were identified as other *Staphylococcus spp.*

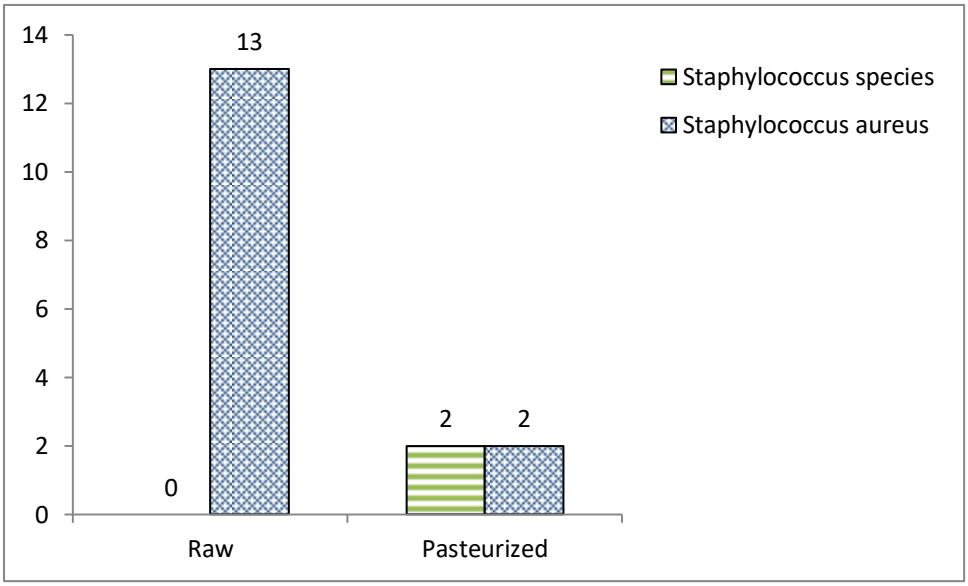


Figure 4: Staphylococcus aureus were confirmed from Staphylococcus species (n=17)

4.3 Antibiotic susceptibility pattern of *Staphylococcus species*

Out of 17 isolates of *Staphylococcus spp*, all showed 100% sensitivity towards Cotrimoxazole, Amikacin, and Levofloxacin. Whereas, resistance was observed in

Penicillin G (100%), Ampicillin (76.82%), Tetracycline (17.68%), Ceftriaxone (52.95%), Cefoxitin (23.58%), Ciprofloxacin (17.68%), Chloramphenicol (11.79%).

The Antibiotic susceptibility pattern of *Staphylococcus species* of raw milk and pasteurized milk are given in Figure-5 and 6 respectively.

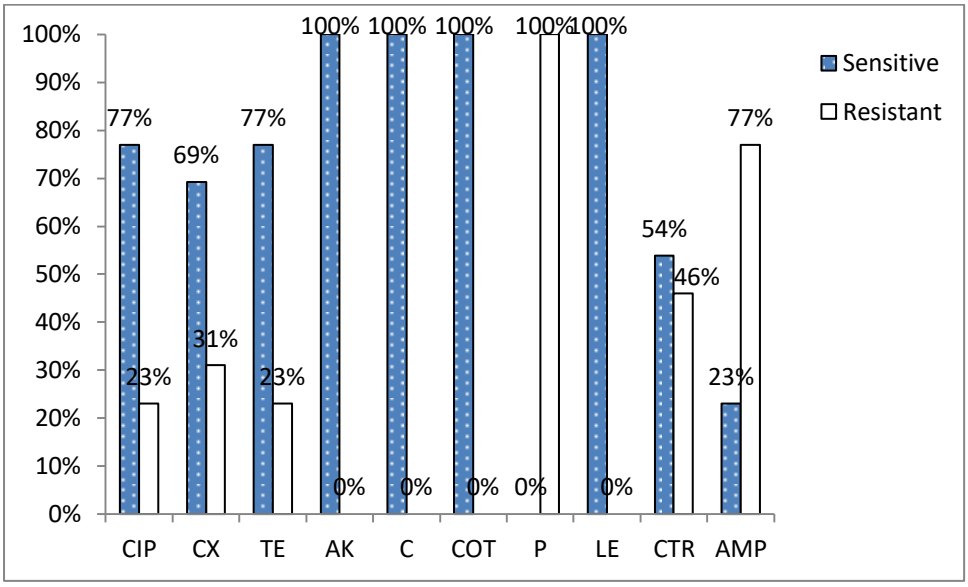


Figure 5: Antibiotic susceptibility Pattern of *Staphylococcus species* of raw milk (n=13)

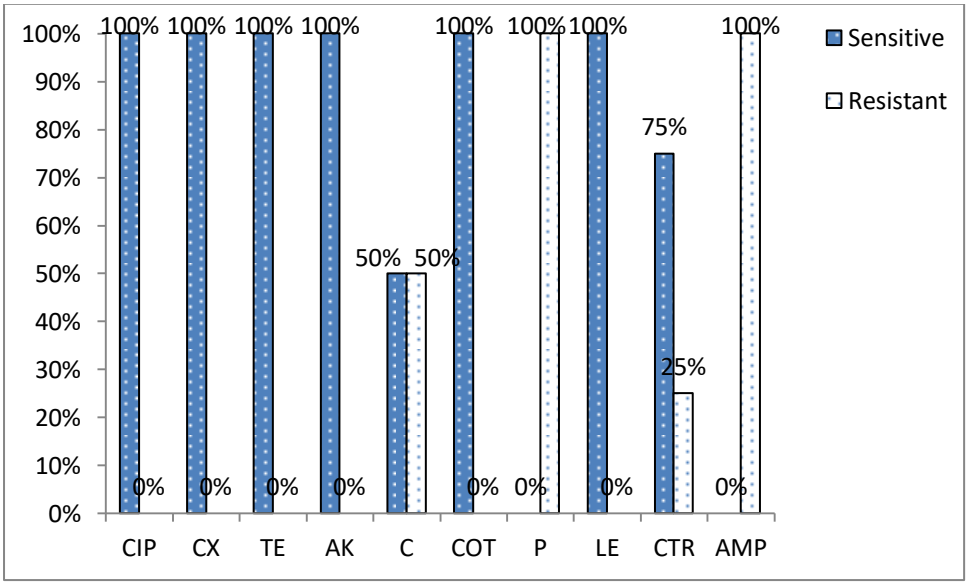


Figure 6: Antibiotic susceptibility Pattern of *Staphylococcus species* of pasteurized milk(n=4)

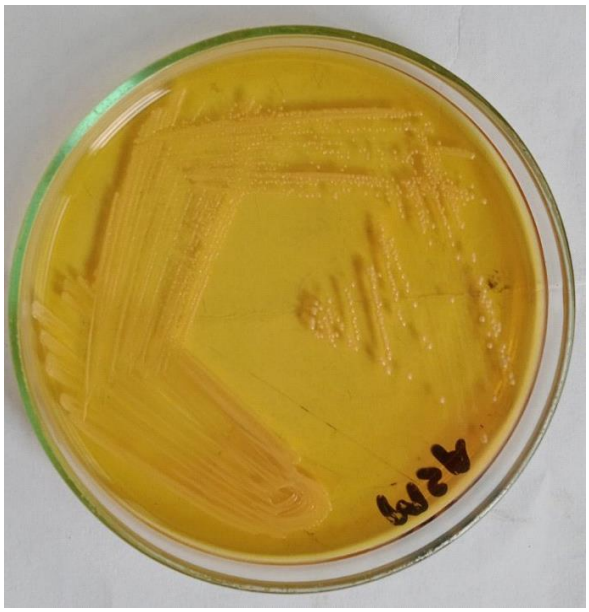
Out of 17 *Staphylococcus species* isolates, 5(29.47%) of *Staphylococcus species* from milk samples showed multi-drug resistance (MDR). 3(17.68%) isolates of *Staphylococcus aureus* from raw milk showed MDR and 2(11.79%) isolates of *Staphylococcus species* from pasteurized milk showed MDR. *Staphylococcus aureus* that showed MDR of Raw milk sample is given in Table-.5

Table 5 : *Staphylococcus aureus* that showed Multi-drug resistant (MDR) of Raw milk samples.

Sample	Resistant	Number of isolates	Organism
Raw	AMP. P, CIP, TE,CTR	1	*Staphylococcus aureus*
	AMP. P, CIP, CX, TE, CTR	2	*Staphylococcus aureus*

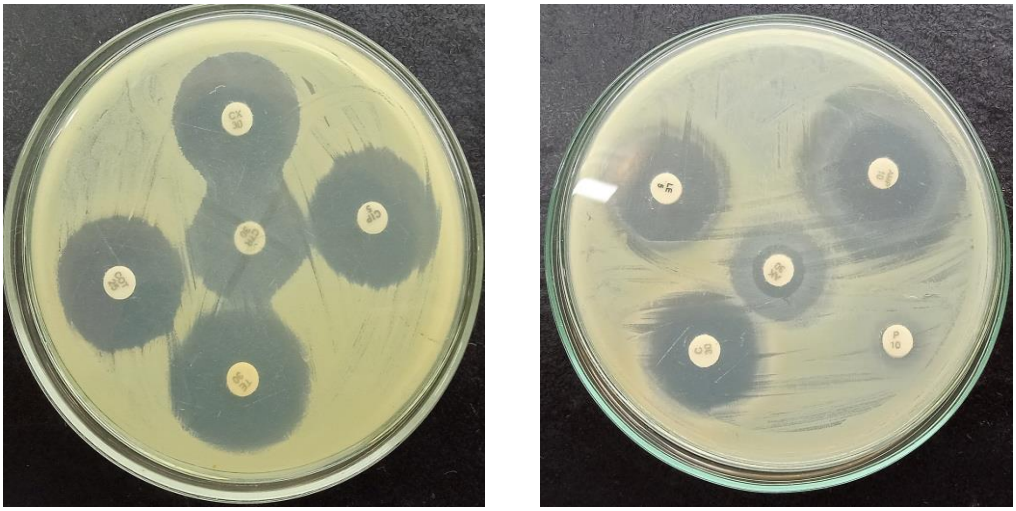
Identification of MRSA according to resistant towards Cefoxitin showed 4(23.58%) of *S. aureus* were MRSA. Only the isolates of raw milk samples has shown resistance toward Cefoxitin. Therefore, the pasteurized milk doesn't contain Methicillin-resistant *Staphylococcus aureus.*

PHOTOGRAPHS



Photograph-1: Streaking of *Staphylococcus species* on MSA (Golden yellow colonies)

Photograph-2: Biochemical test result of *Staphylococcus species* (OF-Fermentative)



(a) **(b)**

Photograph-3: Antibiotic susceptibility test of *Staphylococcus species* (a) : (Top to right CX, CIP, TE, COT and Middle: CTR) and (b) :(Top to right: LE, AMP, P, C and Middle: AK)

Photograph-5: Sample processing in the Microbiological laboratory of Amrit Campus

4.4 Discussion

Pathogenic bacteria in milk have been a major factor for public health concern since early days of dairy industry. This study was conducted with the aim to assess the bacterial quality of milk consumed by people of Kathmandu.

The Total Bacterial Count and Total Staphylococcus Count was used as an important indicator of the bacterial analysis of the raw and pasteurized milk. The TBC of most of the milk samples were within the range (Figure-3). The reason behind all samples being in the range could be related to good sanitary condition, proper pasteurization, hygienic practice, proper storage. In contrast to this result, Acharya et al (2018) reported the mean value of TBC of milk samples exceed the standard guideline in his study. The distribution of Total bacterial count is not same across raw and pasteurized milk sample. The significance level is ($p<0.05$).

The TBC in raw milk samples which was found to be lower than the maximum recommended level given by the Indian Standard (BIS 1992). This result shows that the average quality of raw milk were good and suitable for the consumption. The lower number of bacterial load in this study could be attributed to hygienic practice, lactating cows or good quality of water used for cleanliness, clean equipments are used as well as washed and sanitized their hands before lactating cows. The TBC of all of the raw milk samples were within range which is lower than the finding of some former studies in Nepal by Dahal et al (2010) and Phattepuri et al (2020). the result was similar to the study done by Acharya et al (2017) in Kathmandu in which the bacterial count of raw milk was found to be 1.2×10^5 CFU/ml which doesn't exceed the value recommend by Indian standard.

But in case of pasteurized milk samples, TBC was found to be higher than the recommended microbial load (Figure-3) in some of the samples (7%). The result of bacterial count doesn't agree with Acharya et al (2017) and Rai et al (2020), which reported that most of their samples have exceed all the standard values given by the guideline. In total 93% of pasteurized samples were within the range so the presence of lower number of organism in milk indicates safe for consumption. The reason behind the less occurrence rate could be related to the difference in time place and season of research. The annual report published by the DFTQC (2011/2012) reported that out of 65 milk and milk product analyzed ,31(47%) milk samples were found to be microbiological unsafe (DFTQC, 2011). Hence, the result of this study compile with the study done by DFTQC showing that more than 50% of the milk being sold in

Nepal were microbiologically safe for the consumption. But this assumption is based on results from total bacterial count only.

Regarding Staphylococcal Count, the TSC ranged from 2.07×10^3 to 45×10^3 CFU/ml which is lesser than the Phattepuri *et al* (2020) reported TSC ranged from 14×10^5 to 8×10^6 CFU/ml. Present study showed that, 86.67% of raw milk samples were confirmed to be *Staphylococcus aureus*. Our findings were in contrast with study of Limbu, *et al* (2022), in which 45% of raw milk samples were confirmed to be *S. aureus*. However, our findings were quite in agreement with Rai, *et al* (2020), in which 67.50% of raw milk samples were positive for *S. aureus*. The distribution of Total Staphylococcus count is not same across raw and pasteurized milk ($p<0.05$).

Although *Staphylococcus aureus* can be found in milk by the udder of cows, hands of the milkers. Presence of *S. aureus* in raw milk may be due to poor hygiene of milkers, utensils and milk handlers, Udder of cow. Expressed breast milk contains commensal bacteria, which inhibit *Staphylococcus aureus.*

While in pasteurized milk samples 13.33% were confirmed to be *S. aureus*. Our result were similar to Achary *et al* (2018) and Arjyal et al (2004), in which 15%, 12.5% of pasteurized milk were positive for *S. aureus*. *Staphylococcus aureus* contamination was detected in 20% pasteurized milk (Limbu et al 2020) and this result is a bit higher than current study (Figure-3). The higher prevalence of *S. aureus* in pasteurized milk might be due to unhygienic processing, improper cleaning, deficient handling, and post-processing contamination of packaging material from the polluted environment (Sankhar,2015). *Staphylococcus aureus* can be effectively killed by pasteurization, but the enterotoxins produce by *S. aureus* retain their biological activity even after pasteurization, which is becoming a hazard for consumers (Asao et al 2003). Mastitis is a major problem affecting dairy herds in the tropical countries like Nepal, in particular, small scale dairy farms. A major causative pathogen is *S. aureus*, which can contaminate milk from sick cows or from handlers. Human and sick dairy cows are the main carriers of *S. aureus*, presenting as a mucosal or cutaneous lesions. Detection of high *S. aureus* count in milk indicates the danger of food intoxication, as strain of S. *aureus* could produce enterotoxins A, B, C, D and E under favorable conditions.

Antimicrobial susceptibility testing was done to detect possible drug resistance in the isolated bacteria and to assure susceptibility to drug of choice for particular infections caused by them. Antimicrobial resistance may arise either spontaneously or by

selective pressure or due to antimicrobial misuse in humans or animals (Schroder et al 2016). AST of *S. aureus* from milk sample has shown 100% sensitive towards Cotrimoxazole, Amikacin, Levofloxacin whereas, resistance toward Penicillin G(100%), Ampicillin (76.82%), Tetracycline(17.68%), Ceftriaxone(52.95%), Cefoxitin (23.58%), Ciprofloxacin(17.68%) and Chloramphenicol (11.79%).

According to Sudhanthirakodi S.*et al* (2015), milk samples showed resistance toward Penicillin G (86.04%), Ampicilin (74.42%), Cefoxitin (4.65%), Cotrimoxazole (4.65%), and Ciprofloxacin (9.30%) which is quite agreement with our result. Likewise, Joshi *et al* (2014) found that S. aureus isolates were sensitive to Ciprofloxacin (97.5%), Ceftriaxone (91.6%) and Tetracycline (89.9%) while least sensitivity was seen in Cefoxitin (62.2%). Similarly, Matallah *et al* (2019) observed that S. aureus was 49.7% resistance to Penicillin G, 5.3% to Tetracycline, 2.1% to Cefoxitin, which showed less resistant than our result where *Staphylococcus aureus* showed 100% resistant to Penicillin G, 20% to Tetracycline and 26.67% to Cefoxitin. Matallah *et al* showed 100% sensitive to Chloramphenicol which contradict with our result. Similarly, the research carried out in Kathmandu valley by Parajuli et al (2018), showed 100% susceptibility to the antibiotics Amikacin, Levofloxacin which is similar to this study. However, Parajuli et al (2018) susceptibility results of Chloramphenicol (100%) and resistant in Penicillin (33.33%), Cefoxitin(67.7%) contradicts with our result.

Similarly, the result of Rai et al (2020), where raw milk sample showed sensitive toward Chloramphenicol (100%), Tetracycline (96.30%), Cefoxitin(62.30%), Ceftrixone(74.10%) and Ciprofloxacin (74.10%) (Figure- 4) is similar to our finding. While it contradicts with sensitive showed by pasteurized milk Chloramphenicol (100%), Tetracycline (66.66%), Cefoxitin(33.33%), Ceftrixone (100%) and Ciprofloxacin (33.33%) (Figure-5).

This difference in the results of various study indicates antibiotics resistant pattern of *Staphylococcus species* are changing. By the means of different mechanism, *Staphylococcus species* are developing resistant to different antibiotics day by day. Isolation of antibiotics resistant *Staphylococcus species* from milk samples against these drugs poses a major challenge in human medicine because these drugs are commonly used in the treatment of human and animals. Numbers of factors contribute to increase in the rate of resistance against antibiotics and some of those are poor government regulations regarding antibiotics, unnecessary prescribe of antibiotic,

easy and over the counter availability without prescription, unqualified personnel, prescribing unnecessary antibiotics, sub lethal use taking incomplete course of antibiotic by patient. This all leads to the haphazard use of antibiotics leading to antibiotic resistance (Hafsa *et al* 2013).

In this study, multi-drug resistant *S. aureus* was also isolated from both raw and pasteurized milk. Altogether 17 (29.47%) of *Staphylococcus species* were identified as MDR. Among them 2(11.79%) from pasteurized milk were identified as *Staphylococcus species* and 3 (17.68%) from raw milk sample were identified as *Staphylococcus aureus.* However this finding contradicts with the finding of Marjan et al (2014) where almost all 100% pathogens isolates include *S. aureus* from both raw and pasteurized milk exhibited multi-drug resistance. This findings is nearly in agreement with the finding observed by Rai et al (2020) and Jahan et al (2014) where 29.63%, 20-25% of *S. aureus* was isolated from raw milk samples respectively. Antimicrobial agents used in livestock production are no longer effective due to the presence of MDR isolates. MDR pathogens and MRSA isolates from milk can be a major health problem for the consumers. As a result, such illness should not be taken lightly, and should be encouraged to inform others about the risks associated with MDR and encourage more responsible antibiotic use.

Furthermore, 23.58% isolated *S. aureus* were of methicillin-resistance *S. aureus* (MRSA) from raw milk sample. Isolates having zone of inhibition size less or equal to 21mm for cefoxitin disks were confirmed as MRSA as suggested by CLSI 2007. MRSA was reported 33.33% in milk samples by Parajuli et al (2018) which is just bit higher than this study. This results were also on the contrary by Acharya *et al* (2017) where, *S. aureus* isolated from both raw and pasteurized milk consumed in Kathmandu valley were resistant to cefoxitin with an average of 33.30%. MRSA poses a formidable clinical threat, with persistently high morbidity and mortality. The finding of Rai et al (2020) contradicts with our finding, in which 66.67% and 37.70% MRSA was detected in pasteurized and raw milk samples respectively during this study.

Therefore, Antibiotic susceptibility test helps us to find out the effective antibiotics for treating the diseases in human and animals.

CHAPTER 5

5. CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Most of the milks solds in Kathmandu were found to be good based on Total bacterial count. Though the TBC value were lower than the guideline suggesting the samples to be of good quality but the presence of *S. aureus* questions the bacterial quality. Further, antimicrobial susceptibility test showed a clear evidence of multi-drug resistance and MRSA. MRSA isolates limit the therapy options, which might be life threatening.

Considering the result of this study, it can be concluded that the average milk sold in different places of Kathmandu district cannot be considered as good quality based on TBC because resistant *S. aureus* was detected which can cause milk-borne illness and food poisoning, thus all milk reaching to the consumers' needs a strict monitoring of bacterial contamination..

5.2 Novelty and National Prosperity aspect of Project work

Total staphylococcal count was not performed as routine analysis in dairy industry. From TSC, we can determine the bacteriological quality of milk. So, TSC should be included in routine analysis.

Milk consumptions estimated at billions of liters worldwide, therefore the consumption of contaminated milk can cause health hazard and cause food-borne illness. The data of AST of this project can be used in suggesting the effectiveness of the respective drugs which help in providing the proper treatment to the patients. This will help in improving the public health aspect at the national level.

5.3 Limitation of the work

The time duration of the project work was insufficient so, only a limited number of samples were studied. This project is also limited by finance, so molecular level analysis of isolated pathogens could not be done

5.4 Recommendation for further work

From this study conducted the following recommendation are suggested

- Cleanliness should be maintained at all times in the farm area to reduce microbial contamination in collected milk. Milking equipment's, utensils, containers should be cleaned in order to avoid cross-contamination.

- Infected animals or animals under treatment should be kept in isolation in the farm to minimized the spread of diseases. Also, milk from infected animals should not be sold or consumed.

- More research work has to be conducted in different areas of Kathmandu with the aim of quantifying the magnitude of milk borne pathogens as it may be present in small-scale livestock keeper's and developing resistance to antimicrobial agents.

- Good manufacturing practice, good hygiene practice, hazard analysis and critical control point should be implemented in dairy industry to prevent the contamination of the dairy products.

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Appendix I : List of materials Equipment

1. Autoclave (Life, Indian)
2. Incubator (Leader, UK and Memmert, Germany)
3. Hot air oven (Ambassador, India)
4. Electronic balance (Phoenix instrument, Germany)
5. Refrigerators (LG and Whirlpool, India)
6. Microscope (COSLAB, India)

Glass-wares

1. Beakers
2. Conical flasks
3. Petri dish
4. Pipettes
5. Measuring cylinders

Miscellaneous

1. Aluminium foil
2. Cotton
3. Forceps
4. Pipette filler
5. Labeling tags

Chemical and reagent

1. Crystal Violet
2. Gram's Iodine
3. Acetone - Alcohol
4. Safranine
5. Paraffin oil
6. Normal saline
7. Rosalic acid
8. Methylene blue dye

Media (Hi-Media Laboratories Pvt. Ltd.)

1. Mannitol Salt Agar
2. Nutrient Agar
3. Nutrient Broth
4. Muller Hinton Agar
5. Hugh and Leifson's Media
6. Plate Count Agar

Appendix II: Composition and preparation of sample diluent

Normal Saline

Ingredients	Amount
Sodium Chloride	8.5 gm
Distilled Water (D/W)	1 litre

Preparation: 8.5 gm of sodium chloride was weighed and transferred to a beaker. Then, around 100 mL distilled water was added to a beaker and dissolved completely and transferred to 1 litre volumetric flask. Then, distilled water was added upto 1 litre mark, and mixed well. Then the solution was dispensed on a test-tube and conical flask and then sterilize by autoclaving at 15 psi pressure for (121o C) 15 minutes.

Appendix III: Composition and preparation of staining reagents

Gram staining reagents:

Crystal Violet Composition	Amount
Crystal Violet	20.00 gm
Ammonium Oxalate	9.00 gm
Ethanol (absolute)	95.00 mL
Distilled Water (D/W)	1000 mL

Preparation: In a piece of clean paper, 20 gm of crystal violet was weighed and transferred to a clean brown bottle. Then 95 mL of ethanol was added and mixed until the dye dissolved completely. Then 9 gm of ammonium oxalate was weighed and dissolved in about 200 mL distilled water. Then it was added to the stain. Finally, the volume was made 1 litre by adding distilled water.

Gram's Iodine Composition	Amount
Potassium Iodide	2 gm
Iodine	1 gm
Distilled Water (D/W)	100 mL

Preparation: 2 gm of Potassium Iodide was taken and transferred to a brown bottle premarked to hold 100 mL. Then about 30 mL distilled water was added and mixed well until the Potassium Iodine was dissolved completely. Then, 1 gm iodine was added to Potassium Iodide solution and mix well until the iodine was dissolved completely. Then the solution was make upto 100 mL distilled water and mixed well

Composition	Amount
Acetone	500 mL
Ethanol (absolute)	475 mL
Distilled Water (D/W)	25 mL

Preparation: To 25 mL of distilled water, 475 mL of absolute alcohol was mixed and transferred in to a leaked-proof screw-cap clean bottle. Then immediately, 500 mL of acetone was added to the bottle and mixed well.

Safranine Composition	Amount
Safranine (2.5% solution in 95% ethanol)	10.0 mL
Distilled Water (D/W)	100 mL

Preparation: To 10 mL of safranine solution, 100 mL of distilled water was added and mixed well until safranine dissolved completely

Appendix IV: Composition and preparation of microbiological media

Nutrient Agar (NA)

Ingredients	Amount (gm/L)
Peptic digest of animal tissue	5.00
Sodium Chloride	5.00
Beef Extract	1.50
Yeast Extract	1.50
Agar	15.00

Final pH (at 25^{o} C) 7.4 ± 0.2

Preparation: 28 gm of the media was suspended in 1000 mL distilled water and boiled to dissolve completely then sterilized by autoclaving at 15 psi pressure (121^{o} C) for 15 minutes and then poured 18-20 mL into sterile petriplates at around 45°C temperature. Then the media was cooled to around 45°C and poured to sterilized 90 mm petriplate around 18-20 mL.

Nutrient Broth (NB)

Ingredients	Amount (gm/L)
Peptic digest of animal tissue	5.00
Yeast Extract	1.50
Beef Extract	1.50
NaCl	5.00

Final pH (at 25°C) 7.4 ± 0.2

Preparation: 25 gm of NB broth media was suspended in 1000 mL distilled water and boiled to dissolve completely. Then, the media was sterilized by autoclaving at 15 psi pressure (121°C) for 15 minutes.

Muller Hinton Agar (MHA)

Ingredients	Amount (gm/L)
Beef infusion	300.00
Acid hydrolysate of casein	17.50
Starch	1.50

Agar	17.00

Final pH (at 250 C) 7.3 ± 0.1 IX

Preparation: 38 gm of the media was suspended in 1000 mL distilled water and boiled to dissolve completely. It was sterilized by autoclaving at 15 psi pressure (121o C) for 15 minutes. Then the media was cooled to around 45°C and poured to sterilized 90 mm petriplate around 18-20 mL.

(Hugh Leifson's) Basal Media

Ingredients	Amount (gm/L)
Casein enzymic hydrolysate	2.00
NaCl	5.00
Dipotassium Phosphate	0.30
Bromothymol blue	0.08
Agar	2.00

Final pH (at 25°C) 6.8 ± 0.2

Preparation (100 mL): 0.938 gm of Hugh Leifson's media and 2 gm 1% dextrose was suspended in 100 mL distilled water and boiled to dissolve completely. The media was dispensed in test tubes about 4-5 mL and sterilized by autoclaving at 10 psi pressure (110°C) for 15 minutes.

Mannitol Salt Agar (MSA)

Ingredients	Amount (gm/L)
Protease peptone	10.00
Meat extracts	1.00
NaCl	75.00
D-Mannitol	10.00
Phenol red	0.025
Agar	15.00

Final pH (at 25 C) 7.4 ± 0.2

Preparation: 111 gm of the media was suspended in 1000 mL distilled water and boiled to dissolve completely, it was sterilized by autoclaving at 15 psi pressure (121o C) for 15 minutes

Appendix V: Composition and preparation of biochemical reagents

Catalase Reagent (3% H_2O_2)

Composition	Amount
Hydrogen Peroxide solution (30% H_2O_2)	10 mL
Distilled water (D/W)	90 mL

Preparation: To 90 mL distilled water, 10 mL of hydrogen peroxide was added and mixed well.

Oxidase Reagent

Ingredients	Amount
Tetra methyl-p- phenyl diamine dihydrochloride (TPD)	1.00 gm
Distilled water (D/W)	100 mL

Preparation: This reagent solution was made by dissolving 1 gm TPD in 100mL distilled water. The strips of Whatman's No. 1 filter paper were soaked and drained for about 30 seconds. Then strips were freeze dried and stored in a dark bottle tightly sealed with a screw cap

Appendix VI: Principle and procedure for Gram staining and biochemical properties

Gram staining:

Principle of Gram staining:-

Gram staining is a very useful differential staining technique for identifying and classifying bacteria into two major groups: Gram positive and Gram negative. In this process, the bacterial smear is subject to four different reagents in the order: crystal violet (primary stain), Gram's iodine solution (mordant), acetone alcohol (decolorizing agent) and safranine (counter stain). The bacteria which retain the primary stain (appear dark blue or violet) are called Gram positive, whereas those that lose the crystal violet and counter satined by safranine (appear red/pink) are referred as Gram negative.

Procedure for Gram staining: -

1. A clean grease free slide was taken and a thin smear sample was made on the slide. Then the slide was air dried and heat fixed.
2. The smear was covered with crystal violet for 1 minute and then washed with water.
3. Then the smear was covered with Gram's iodine for 1 minute and washed.
4. Then decolorized with acetone alcohol (10-15 seconds) and then washed with water.
5. Finally, the slide was covered with safranin for 1 minute and washed with water.
6. Then the slide was air dried and observed under microscope at 10x, 40x and 100X (oil immersion).

Catalase test Principle: -

This test is used to differentiate those bacteria that produce the enzyme catalase, such as Staphylococci, from non-catalase producing bacteria such as Streptococci. XVI Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen water. An organism is tested for catalase production by bringing it into contact with hydrogen peroxide. Bubbles of oxygen are released if the organism is a catalase producer. The culture should not be more than 24 hours old.

Procedure:-

1. Using wooden stick, a portion of colony was transferred to the surface of clean, dry glass slide.
2. A drop of 3% H_2O_2 was added over the transferred colony.
3. Then effervescence of the gas was marked by bubbles in case of positive result within 10 seconds.

Oxidase test Principle:-

The oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. When present, the cytochrome c oxidase oxidizes the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple colour end product. The oxidase test is used to assist in the identification of *Pseudomonas, Neisseria, Vibrio, Brucella,* and *Pasteureella species*, all of which produce the enzyme cytochrome oxidase.

Procedure:-

1. A piece of oxidase paper was taken on clean glass slide (filter paper soaked with the oxidase reagent 1% tetra methyl- p- pheny lenediamine dihydrochloride).
2. A small portion of bacterial culture was taken with the help of wooden applicator and rubbed on the reagent paper.
3. Then oxidase paper was observed for the development of purple color within 15 seconds.

Oxidative-fermentative test Principle:-

The oxidative-fermentative test determines metabolism of glucose by fermentation or aerobic respiration (oxidatively). During the anaerobic process of fermentation, pyruvate is converted to a variety of mixed acids depending on the type of fermentation. The high concentration of acid produced during fermentation will turn the bromothymol blue indicator in Hugh-leifson media from green to yellow in the presence or absence of oxygen. During aerobic repiration, organisms metabolize glucose producing weak acid during glycolysis and Krebs cycle which in turn change the bromothymol blue indicator in Hugh-leifson media from green to yellow in the presence of oxygen.

Procedure:

1. Two tubes containing Hugh-leifson media were taken and the organism was stabbed into both media using sterile inoculating wire.
2. One of the tubes was sealed with paraffin oil to create anaerobic condition.
3. Both the tubes were incubated at 37°C for 24-48 hrs and observed for color change in both the tubes.

Coagulase test Principle:

The coagulase test identifies whether an organism produces the exoenzyme coagulase, which causes the fibrin of blood plasma to clot.

Procedure:- Tube test method

1. Three small test tubes were taken and labelled as 'T' (Test organism, 18-24 hours' broth culture), 'Pos' (Positive control, 18-24 hours S. aureus broth culture) and 'Neg' (Negative control, sterile broth).
2. Then, 0.2 mL of plasma was pipetted into each tube.
3. After that, 0.8 mL of the test broth culture was added to tube 'T'.
4. 0.8 mL of the *S. aureus* culture was added to the tube 'Pos' and 0.8 mL of the sterile broth to the tube labelled 'Neg'.
5. After mixing gently, all three tubes were incubated at 35-37°C for 1 hrs.
6. Clotting was then examined in each tube after 1 hour of incubation.
7. If no clotting occurred, tubes were examined after 3 hours. If the test showed negative, then the tubes were left at room temperature overnight and examined again.

Appendix VII: Procedure for antibiotics susceptibility testing (CLSI 2013, CLSI 2018)

1. Using a sterile inoculating loop, single isolated colony of the test organism was inoculated into 2-3 mL of nutrient broth and was incubated at 37°C for 4 hours.
2. Then, the turbidity of the prepared inoculum was compared and adjusted with the MacFarland turbidity standard no. 0.5.
3. Then, sterile cotton swab was dipped into the nutrient broth containing inoculum. Excess inoculum was removed by pressing and rotating the swab against the side of the tube wall above the level of suspension.
4. Then, the inoculum was swabbed evenly over the surface of the MHA media in three directions, rotating the plate approximately 60° to ensure even distribution to obtain lawn culture.
5. With the petri-dish lid close, the surface of the agar was allowed to dry for 3-5 minutes.
6. Then, the antibiotics were placed on the surface of agar with the help of sterile forceps and pressed gently.
7. Within 30 minutes of applying the discs, the plate was inverted and then it was incubated at 35 ± 0.2o C for 16-18 hours. 8. The susceptibility pattern was noted following overnight incubation by measuring zone of inhibition as sensitive, intermediate and resistant as according to zone size interpretative chart (Appendix I)

Appendix VIII: Preparation of McFarland turbidity standards

McFarland standard No.	1.0% Barium chloride(mL)	1.0% Sulfuric acid (mL)	Approx. Cell density (1×108 CFU/mL)
0.5	0.05	9.95	1.5
1	0.1	9.9	3.0
2	0.2	9.8	6.0

Preparation of McFarland standard no. 0.5

1. 1% w/v solution of barium chloride was prepared by dissolving 0.5gm of dehydrate barium chloride ($BaCl_2$. $2H_2O$) in 50 mL of distilled water.
2. . 1% w/v solution of barium chloride was prepared by dissolving 0.5gm of dehydrate barium chloride ($BaCl_2$. $2H_2O$) in 50 mL of distilled water.
3. 0.6 mL of the barium chloride solution was added to 99.4 mL of the sulphuric acid solution and mixed.
4. A small volume of the turbid solution was then transferred to a screw capped bottle.
5. To standardize the inoculum density for a susceptibility test, a $BaCl_2$ turbidity standard, equivalent to a 0.5 McFarland standard was used.

Zone of interpretation chart of Antibiotics

For *Staphylococcus aureus*

Antibiotics Used	Concentration (mcg)	Diameter of zone size (mm)			ATCC culture S. aureus 25923 target zone
		Resistant Mm or less	Intermediate	Sensitive mm or more	
Ciprofloxacin (CIP)	5	15	16-20	21	22-30
Ceftriaxone (CTR)	30	13	14-20	21	22-28
Tetracycline(TE)	30	14	15-18	19	24-30
Cotrimoxazole (COT)	25	14	14-16	17	24-32
Cefoxitin (CX)	30	21	-	22	23-29
Ampicillin (AMP)	10	28	-	29	27-35
Amikacin (AK)	30	14	15-16	17	20-26
Levofloxacin(LE)	5	15	16-18	19	25-30
Penicillin-G (P)	10	28	-	29	26-37
Chloramphenicol(C)	30	18	-	18	19-26

Appendix IX: Morphological and cultural characteristics of bacteria

BACTERIA	MORPHOLOGICAL CHARACTERISTICS	CULTURAL CHARACTERSTICS
Staphylococcus aureus	Gram positive, non-sporing, non-motile, usually non capsulated, facultatively anaerobic cocci , characteristically arranged in cluster.	Mannitol fermenter, round, smooth, raised and glistening yellow colonies on MSA

Source: Chakraborty (2019), Cheesebrough (2006) and Baily and Scott's (2014)

Appendix X: Biochemical tests for identification of bacteria

Test organism	Catalase test	Oxidase test	Oxidation-fermentative test	Coagulase test
Staphylococcus aureus	+	-	F	

+ positive

- Negative

F Fermentative

Source: Chakraborty (2019), Cheesebrough (2006) and Baily and Scott's (2014)

Appendix XI: Standards of Milk

Nepal Standard of DFTOC

०१:०१ दुध दुध भन्नाले स्वस्य गाई वा भैसीको थुनबाट दुहेको रर स्वभाविक अवस्थाको स्वच्छ ताजा श्रावलाई सम्झनुपर्छ ।

०१.०२ गाईको दुध (Cow milk): गाईको दुध भन्नाले ३.५ प्रतिशत दुधको चिल्लो (Milk fat) र सो चिल्लो बाहेक दुधको ठोस पदार्थ ९:षपि कयष्मि लयत ावत० ७.५ प्रतिशत भन्दा घटि नएको दुध सम्झनु पर्छ ।

०१.०५ प्रशोधित दुध (Processed Milk): प्रशोधित दुध भन्नाले आंशिक रुपमा दूधको चिल्लो झिकी वा नझिकी वा दूधको चिल्लो रहित दुध ठोस पर्दाथ घोली वा नघोली दूधको चिललो ३.० प्रतिश दूधको चिल्लो वाहेक दुध ठोस पदार्थ ८.० प्रतिशतमा घटी नभएको तथा निरोगन प्रक्रिया (Pasteurization) वा जिवाणु हनन प्रक्रिया (Sterilization) सम्पन्न गरी तयार गरिएको तरल दूधलाई सम्झनुपर्छ । यसमा निरोगन प्रक्रिया गरिएको प्रशोधित दूधलाई प्रशोधित तथा पास्चुराइज्ड दुध र जिवाणु हनन प्रक्रिया सम्पन्न प्रशोधित दुधलाई प्रशोधित तथा स्टरिलाइज्ड दुध भन्न वा लेख्न सकिनेछ ।

द्रष्टव्य: उपर्युक्त सव्केत नम्बर ०१.०५ मा उल्लिखित दूध वा क्रिमको निरोगन प्रक्रिया (Pasteurization) गर्दा दूध वा क्रिमलाई कम्तीमा ६३ सेण्टिग्रेडमा ३० मिनेटको समयभन्दा कम नहुने गरी सम्पूर्ण दूध वा क्रिमलाई तताई वा अन्य कुनै बढी तापक्रममा निरोगन क्रिया पुग्ने समयसम्म राखी त्यसपछि तुरुन्त सम्पूर्ण दूध वा क्रिमलाई १० सेन्टिग्रेड वा त्यो भन्दा कम तापक्रम सम्म चिस्याईएको हुनुपर्नेछ र देहाय मापदण्ड बमोजिम निरोगन प्रक्रिया पूरा गेको हुनुपर्नेछ ।

१. फोस्फाटेज टेस्ट नेगेटिभ हुनुपर्नेछ ।

२. निरोगन गरिएको दूध तथा अन्य दुग्ध तरल पर्दाथ विक्रिमा राखिएको भए वा तयार गरिएको भएमा प्रति मितितिटरमा कोलीफर्म (Coliform) गणना शुन्य हुनुपर्नेछ ।

Quality Standard of milk on the basis of microbial load (DDC Nepal)

Standard plate count per ml

< 2,00,000	very good
2,00,000	Good
10,00,000	Fair
>50,000,000	Poor

Indian Standards

1. Pasteurized milk

The bacterial criteria prescribed by the Bureau of Indian Standards (BIS 1992) stipulated that the plate count of pasteurized milk, at the plant in the final container, should not exceed 30,000 per ml and the coliform should be absent in 1:10 dilution of pasteurized milk.

2. Raw milk

The Bureau of Indian Standards (BIS 1992) prescribed the following criteria as a guideline for grading of the milk based on the Total viable count.

Grade	Total viable count (Lakh/ml)
Very good	less than 2 lakh
Good	2-10 Lakh
Fair	10-50 Lakh
Poor	More than 50 Lakh

Appendix XII: Total Plate Count and Total Staphylococcus Count of Raw (R) and Pasteurized (P) milk

Sample code	TPC (CFU/ml)	BIS grading	TSC (CFU/ml)
R_1	3.25×10^3	Very good	-
R_2	22×10^3	Very good	-
R_3	40×10^3	Very good	1.96×10^4
R_4	36.75×10^3	Very good	2.16×10^4
R_5	30.25×10^3	Very good	2.18×10^4
R_6	37.8×10^3	Very good	1.94×10^4
R_7	56.5×10^3	Very good	4.5×10^4
R_8	32.05×10^3	Very good	0.45×10^4
R_9	28×10^3	Very good	0.31×10^4
R_{10}	50×10^3	Very good	0.32×10^4
R_{11}	25×10^3	Very good	0.92×10
R_{12}	38.95×10^3	Very good	0.71×10^4
R_{13}	30.7×10^3	Very good	-
R_{14}	179.8×10^3	Very good	-
R_{15}	54×10^3	Very good	-
P_1	2.1×10^3	within range	2.07×10^4
P_2	7×10^3	within range	3.0×10^4
P_3	1.73×10^3	within range	-
P_4	12.9×10^3	within range	-
P_5	7.9×10^3	within range	-
P_6	3×10^3	within range	-
P_7	19×10^3	within range	-
P_8	4.1×10^3	within range	-
P_9	8.2×10^3	within range	-
P_{10}	10×10^3	within range	2.85×10^4

P_{11}	5.5×10^{3}	within range	2.70×10^{4}
P_{12}	5.0×10^{3}	within range	-
P_{13}	22×10^{3}	within range	-
P_{14}	40×10^{3}	above range	-
P_{15}	16.2×10^{3}	within range	-

Appendix XIII: Hypothesis of test summary of TBC and TSC

S.N	Null Hypothesis	Test	Significane level P- value	Decision
1.	The distribution of Total bacterial count is not same across raw and pasteurized samples.	Independent samples Mann-Whitney U Test	P<0.05	Reject the null hypothesis
2.	The distribution of Total Staphylococcus count is not same across raw and pasteurized samples.	Independent samples Mann-Whitney U Test	P<0.05	Reject the null hypothesis