ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

BACTERIOLOGICAL ANALYSIS OF COW MILK IN HAWASSA TOWN, SNNPRS ETHIOPIA

BY:

Deresse Daka (BSc)

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A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR MASTERS OF SCIENCES DEGREE IN MEDICAL MICROBIOLOGY

Principal Investigator: Deresse Daka (Bsc)

School: Medicine

Department: Microbiology, Immunology and Parasitology

ADVISORS:

Dr. Solomon Gebre-Selassie (MD, MSc): Department of Microbiology, Immunology and Parasitology School of Medicine, Addis Ababa University

Dr. Kassaye Aragawu (DVM, MSc): Faculty of animal sciences, Hawassa University

Mr. Dawit Yehedego (BSc, MSc): Faculty of Medicine, Department of Medical Laboratory, Hawassa University

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List of acronym

AAU: Addis Ababa University

CCP: critical control points

EPA: Environmental Protection Agency

FDA: Food and Drug Administration

HACCP: Hazard Analysis and Critical Control Point

ILDP: integrated livestock development project

SNNPRS: Southern Nations and Nationality Peoples Regional States

Abstract

Modern dairy processing is a recent phenomenon in Ethiopia for the very reason that the country has not been producing enough milk at commercial scale. The establishment of state owned and private dairy farms in big cities resulted in the realization of the few dairy processing plants. In addition to poor transport system and infrastructure of the country, the dairy processing plants are located far away from farms. This is further aggravated by other facts like poor dairy management, poor milking hygiene and lack of good milk cooling and transport facilities and therefore the quality of milk is greatly depreciated by the time it arrives to processing plants.

Thus, the specific objectives of the study are to assess the bacteriological quality of raw milk from the farm to the processing plant, to determine the bacteriological quality of pasteurized milk, to identify public health important bacteria from the raw and pasteurized milk and to identify the critical control points through the milk collection and processing practices.

The study was conducted mainly bacteriological quality tests and bacteriological isolation and identification of sampled milk at different points before pasteurization and at different time points of pasteurized milk and subordinately used questionnaire. Detailed microbiological analysis was carried out on selected pathogens of public health importance. For selection of the samples, simple random sampling technique was used. The data to be generated from the questionnaire and the laboratory tests were summarized and analyzed using appropriate descriptive and analytical statistics.

As the result of the study showing that 326 bacterial strains were isolated from CCP1 up to CCP6. There was different species of bacteria in different HACCP levels of milk. In CCP1, there were 51 bacteria of different species and in CCP2, CCP3, CCP4, CCP5 and CCP6, was 64, 73, 80, 20, and 38 respectively. The predominant bacteria species in all (CCP1-CCP5) levels of the milk was S. aureus which accounts 14(27.5%), 20(31.3%), 21(28.8%), 17(21.3%), and 6(30.0%) respectively.

In line with this the remedial solution for aforementioned identified findings the effective drugs was Ciprofloxacin, Gentamycin, TMP-SMZ, and Ceftriaxone, whereas, Ampicillin and Penicillin were resistant for many of bacteria.

Key words: *milk, bacterial plate count, coliforms, milk hygiene, raw milk, pasteurized milk, antibiotic sensitivity.*

Chapter I

Introduction

Background of the study

Milk is used throughout the world as a human food at least one form or more. The demand of consumers for safe and high quality milk has placed a significant responsibility on dairy producers, retailers and manufacturers to produce and market safe milk and milk products (Adesiyun *et al.,* 1995; Hahn, 1996; Mennane *et al.*, 2007).

Milk is a good growth medium for many microorganisms because of its high water content, nearly neutral pH, and variety of available essential nutrients that renders it as one of the good media for microbial growth and multiplication (Gudeta, 1987; O'Mohany, 1988; Ashenafi and Beyene, 1994; Soomro *et al* 1996; Teka, 1997; DeGraaf *et al* 1997). Mainly because of this reason, milk and milk products are more prone to the harboring and proliferation of microorganisms. Microorganisms may contaminate milk at various stages of procurement, processing and distribution. The ill health of the cow and its environment, improperly cleaned and sanitized milk handling equipment, and unhygienic workers who milk the cow, come in contact with milk due to a number of reasons could serve as sources of contamination for the milk. These have inevitably increased the risk of infection of many people from common source. Lack of refrigeration facilities at farm and household level in developing countries of tropical regions, with high ambient temperature implies that raw milk will easily be spoiled during storage and transportation (Kurwijilla *et al* 1992; Ombui *et al* 1995; Gilmour, 1999; Godefay and Molla, 2000).

Although milk is known to possess several anti-microbial agents, bacterial number will be doubled in less than three hours in unchilled milk. The rate of microbial growth will depend on initial numbers and the temperature at which milk is held immediately after milking and thereafter (Kurwijilla *et al* 1992; Wolfson *et al.*, 1993). There is often sufficient time between milk collection and consumption for psychrophilic bacteria to grow. Many of the flavor defects detected by milk consumers' results from this growth. Milking equipment, utensils, and storage tanks are the major source for psychrotropic contamination of raw milk. Milk residues on unclean equipment provide a growth niche for these bacteria, which enter milking machines, pipelines, and holding tanks with water rinses or milk. Generation times in most milk of the most rapidly growing psychrotrophic bacteria are 5.5 to 10.5 hours at $3^{o}C$ to $5^{o}C$. These growth rates are sufficient to cause spoilage within 5 days if the milk initially contains only one cell/ml (Suhren 1989). Preventing products defect that result from the growth of psychrotrophic bacteria in raw milk involves limiting contamination levels, rapid cooling immediately after milking and maintenance of cold storage temperatures. Limitation of populations of bacteria primarily involves cleaning, sanitizing, and drying cow's teats and udder before milking and using cleaned and sanitized equipment. Removal of residual milk solids from milk contact surfaces is critical to psychrotroph control (Frank and Koffi. 1990). These bacteria commonly found in raw milk are inactivated by pasteurization (Doyle *et al.*, 2001).

Milk and dairy products are an excellent source of calcium, phosphorus and magnesium. These minerals in optimum ratio are present in milk and are required for optimum growth and maintenance of bones (Aneja *et al.*, 2002). However, in spite of this, milk can also serve not only as a potential vehicle for transmission of some pathogens but also allows these organisms to grow, multiply and produce toxins. A variety of pathogenic organisms may gain access in to milk and milk products from different sources and cause different types of food borne illnesses. Milk and milk products may carry toxic metabolites of different organisms growing in it. Ingestion of such products, contaminated with these metabolites, cause food poisoning. On the other hand the ingestion of viable pathogenic bacteria along with the food product leads to food borne infection. Sometime these organisms undergo lysis in the gastrointestinal tract and liberate toxic substances from inside the cells which are detrimental to the health of the consumers (Aneja *et al.*, 2002). Recent development regarding Quality and safety management systems such as ISO and Hazard Analysis Critical Control Point (HACCP) has reduced such incidences.

Spoilage of milk and dairy products resulting from growth of acid-producing fermentative bacteria occurs when storage temperatures are sufficiently high for these microorganisms to outgrow psychrotrophic bacteria, or when product composition is inhibitory to gram-negative aerobic organisms. Non-spore-forming bacteria responsible for fermentative spoilage of dairy products are mostly in either the lactic acid producing or coliform groups. Defects in fluid milk caused by coliforms and lactic acid bacteria are controlled by good sanitation practices below

7^{o}c, pasteurization and refrigeration of pasteurized products (Slaughuis, 1996; Doyle. *et al.*, 2001).

Pasteurized milk is expected to have shelf life of 14 to 20 days. So, consumption of the contents with even one rapidly growing microorganism can lead to spoilage. Pasteurized milk products become contaminated with psychrotrophic bacteria by exposure of contaminated equipment or air. Proper cleaning and sanitizing procedures can effectively reduce contamination from these sources (Schroder 1984).

In general in order to make milk free from bacterial pathogenic agents, Hazard Analysis and Critical Control Point (HACCP) system should be done starting from milk collection through milk processing to milk storage. Once the significant hazards and control measures are identified, critical control points (CCP) within the process scheme are identified. Risk (hazard) analysis is the overall process of defining a problem, collecting information, calculating results, risk and uncertainties, evaluating potential mitigations, and deciding on the course of action. Microbial exposure assessments are critical component of risk analysis (McClue *et al.*, 1994; McMeekin and Ross, 1996; Elliot 1996; FAO/WHO 1997; NACMF 1998).

As modern dairy processing is not widely practiced in Ethiopia, milk is mostly produced for own consumption. However, small-scale dairy farms have been established in urban and peri urban areas to supply milk to the consumers in these areas. In central part of the country, producers have organized themselves in to cooperatives and established milk collecting centers. The actual methods of storage at the farm and mode of transportation to the processing plant do not prevent microbial activity in the raw milk in the country. Pasteurization and post pasteurization handling and marketing of milk has its own problems. Hence the keeping quality is also low. Due to either surviving thermoduric bacteria and/or post pasteurization contamination, the shelf life of pasteurized milk at ambient temperature or refrigeration temperature decreases through time (Mehari, 1988). Hygienic control of milk and milk products in Ethiopia 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 (Godefay and Molla, 2000).

Moreover, most of the studies conducted yet concerning the bacteriological quality of either raw or pasteurized milk are on milk collecting centers and processing plants in Addis Ababa and its vicinity towns (Alehegn, 2004). But there is no study yet conducted on quality of raw milk collected from dairy cooperatives, which were established by integrated livestock development project (ILDP) in and around Hawassa town and processed milk at different Milk Processing Plant. So far there is no document how far the raw milk is bacteriologically safe for the public and the average shelf life of the pasteurized milk of different milk processing plant.

Statement of the problem

As some studies undertaken on the topics illustrated that basically it focused on the kinds of milk processing ways and its impacts on human health were studied so far. Studies by (Ashenafi and Beyene, 1994) account mainly focused on microbial load, micro flora, and keeping quality of raw and pasteurized milk from a dairy Farm; (Godefay and Molla 2000) again showed the Bacteriological quality of raw milk from four dairy farms and milk collection center in and around Addis Ababa; (Mehari, 1988) also saw the Thermoduric and Psychrophilic Bacteria from Raw Milk; (Teka, 1997) on Food Hygiene Principles and Food Borne Disease Control with Special Reference to Ethiopia. And also (Gudeta, 1987) Isolation and Identification of Enteric Bacteria in Raw Milk Produced by Three Dairy Farms at Bahir Dar. All these reports were not worth fully emphasized the issue of milk related impact on the livelihood of the society and couldn't see the bacteriological analysis of both pasteurized and unpasteurized milk in the country as well as in the study areas.

Therefore, the basic theme of this study is to fill this gap of the proceeding writers. In light of the above drawbacks this study will help to develop a the bacteriological quality of raw milk collected from cooperatives on the way from the farm to the site of pasteurization and the shelf life of the pasteurized milk and also will analyze any risk of contamination based on the concept of HACCP. Furthermore, the study will identify the critical control point (CCP) in the process of milk production from the farm to the consumer to take appropriate measures.

Chapter II

Literature Review

Theoretically, milk that is secreted in the udder of a healthy cow should be free of microorganisms. However, freshly drawn milk is generally not free of microorganisms. Numbers of several hundred to several thousand cfu/ml are often found in freshly drawn milk, and they represent the movement up the teat canal of some and the presence of others at the lower ends of teats. Although the APC of milk from healthy cows is generally $<10^3$ cfu/ml, numbers of 10^4/ml are not uncommon (James, 2005).

Fresh milk is neutral or slightly alkaline but on souring becomes acid because of the lactic acid forming by bacterial action on lactose. It has a water content of 88% and 12% of solids which constitute of 4.8% sugars, 3.5% fats, 3.1% protein and 0.6% salts (Stewart, 1978). It has a wide range of positive nutritional benefits and supplies a variety of nutrients including protein for bodybuilding, vitamins, minerals (especially calcium), fat and carbohydrate for energy (Harding, 1995).

There are numerous proteins found in milk. The major groups of milk proteins are caseins and whey proteins. Milk provides easily digested protein of a high nutritional value and is a rich source of essential amino acids which also supports the growth of bacteria (Harding, 1995). In addition casein is the principal protein of cow's milk. Also whey proteins are now well known for high nutritional value for bacteria and versatile functional properties in food products (Wit, 1998).

Many trace elements essential for health and growth of microorganisms present in milk. Na, Ca, K, P and some of the trace minerals are, Zn, Co, I, Fe, etc (Stewart, 1978; Harding, 1995). Also vitamins are important both for bacterial growth and humans (Harding, 1995) such as vitamin A, vitamin D, vitamin E, vitamin K (Bendicho *et al.*, 2002), vitamins B complex, vitamin C (Bendicho *et al.*, 2002).

Also milk is a good growth medium for many microorganisms because of its high water content, nearly neutral pH and variety of available essential nutrients that renders it as one of the good media for microbial growth and multiplication (Gudeta, 1987; O'Mohany, 1988; Ashenafi and Beyene, 1994; Soomro *et al* 1996; Teka, 1997; DeGraaf *et al* 1997).

The quality of the starting raw milk has a very definite effect on the yield and quality of products made from it. The compositional quality, the hygienic quality, the health of the cow and the level of contaminants present can all have an impact on the yield and quality, and hence financial return from products made from milk (Harding, 1995). Milk drawn aseptically from the udder of a healthy cow contains only a small number of microorganisms, these being of little importance commercially and presenting no danger to the consumer. While some contamination with bacteria from the milking environment and equipment is inevitable, the total bacterial count of cooled milk, produced under good hygienic conditions, should be lower than 10^3cfu/ml. If the bacterial count of milk was allowed to increase significantly, e.g. to over 10^3 cfu/ml this could lead to significant degradation of the fat, protein and lactose causing off-flavours and would significantly reduce the flexibility the processor has with respect to storage and use of milk. In order to achieve a high bacteriological quality at farm level, it is important for farmers to be aware of the sources of contamination and to understand how they can be controlled (Harding, 1995).

Milk is synthesized in specialized cells of the mammary gland and is virtually sterile when secreted into the alveoli of the udder. Beyond this stage of milk production, microbial contamination can generally occur from three main sources (Bramley and McKinnon, 1990); from within the udder, from the exterior of the udder and from the surface of milk handling and storage equipment (IDF, 1996). The health and hygiene of the cow, the environment in which the cow is housed and milked, and the procedures used in cleaning and sanitizing the milking and storage equipment are all important in influencing the level of microbial contamination of raw milk. Equally important are the temperature and length of time of storage, which allow microbial contaminants to multiply and increase in numbers. All these factors will influence the total bacteria count or Standard Plate Count (SPC) and the types of bacteria present in bulk raw milk.

Pasteurization is defined by the International Dairy Federation (IDF) as “a process applied to a milk product with the object of minimizing possible health hazards arising from pathogenic microorganisms associated with milk, by heat treatment which is consistent with minimal chemical, physical and organoleptic change of the product”.

The object of pasteurization primarily is to render milk safe by inactivating microorganisms and enzymes, followed by cooling and holding at low temperature. There are numerous combinations of time and temperature having the desired effect of destroying bacteria, but in practice there are limits for both parameters, which, if exceeded produce undesirable effects such as the destruction of the cream line or caramelization of the lactose in milk (Hayes, 1981). Milk is often pasteurized using two methods, the high temperature short time (HTST) and the holder method. The holder method involves holding fixed batches of milk for at least 30 minutes at not less than 62.8°C and not more than 65.6°C. The HTST method is a 72°C, 15 seconds process. The whole of the heating, holding and cooling of the milk is done as the milk flows in a continuous stream through a single unit composed of a series of plates arranged in parallel (Chalmers, 1955). This is designed to kill all pathogenic bacteria such as Salmonella and Brucella species at levels expected in fresh milk. However, there are microorganisms that survive pasteurization such as *Streptococcus thermophilus* and *Micrococcus luteus* to name a few, and they are called thermoduric organisms (Forsythe, 2000).

Many factors are influencing the growth bacteria on milk such as temperature, pH of milk, food supply, moisture, air, light preservatives, and concentration of sugars. The growth of bacteria on milk produce enzymes, decomposition products (fats, proteins, sugars) pigments, toxins, miscellaneous changes.

Souring is a most common, result of bacterial growth due to transformation of lactose into lactic acid and other volatile acids and compounds, principally by lactic acid bacteria. This is mostly occurred by *Streptococus lactis*, *E. coli* and other bacteria’s. Moreover the contamination of milk results is souring and gassiness, aroma production, proteoloysis, ropiness, sweet curdling (Shojaei**,** 2008**).**

Raw milk can be a large source of diseases. Some of the most obvious are the animal diseases below to which humans are susceptible and which may occur in milk of cows are Brucellosis, Anthrax, Tuberculosis, Listeriosis Salmonellosis, Q fever, Campylobacteriosis, Enterohemorrhagic colitis, *Staphylococcus* and *Streptococcus mastitis* (James, 2005). Also common bacterial contaminants of milk (milk borne pathogens) are *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* spp. (Isabel 2009) *Klebsiella pneumoniae, Proteus spp, Campylobacter jejuni,* and *Bacillus cereus* (Shekhar, 2010). Milk was also the source of both human respiratory diseases (e.g., diphtheria) as well as enteric infections (e.g., typhoid fever). It was a relation between raw milk and cases of human scarlet fever, diphtheria, and typhoid fever in the early 1900s that led the New York to require milk pasteurization in 1910 (James, 2005).

Also new bacteria which is Gram-negative, rod-shaped, oxidase-positive, aerobic, non-motile and orange-pigmented bacterial strain, containing flexirubin-type pigments, designated H8T, was isolated from raw cow's milk in Israel. 16S rRNA gene sequence analysis indicated that the isolate should be placed in the genus Chryseobacterium (family Flavobacteriaceae, phylum Bacteroidetes) (Elionora et al.2008).

Chapter III

Significance of the study

Milk is secreted by cells within the mammary glands and is virtually sterile when secreted into the alveoli of the udder. Beyond this stage of milk production, 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 and storage. Cow health, environment, milking procedures and equipment sanitation can influence the level of microbial contamination of raw milk. Equally important is the milk holding temperature and length of time milk is stored before testing and processing that allow bacterial contaminants to multiply. All these factors will influence the total bacteria count (SPC) and the types of bacteria present in raw milk. There are plenty of pathogenic and non-pathogenic bacteria causing infection due to the contamination of milk pre-pasteurization and post pasteurization. This is mostly critical issue in developing countries as well as developed countries especially where HIV/AIDS infection is highly dominated area.

Therefore, this study helped to develop base line information on the bacteriological quality of raw milk collected from cooperatives on the way from the farm to the site of pasteurization and the shelf life of the pasteurized milk and also was analyzed any risk of contamination based on the concept of HACCP. Furthermore, the study was identified the critical control point (CCP) in the process of milk production from the farm to the consumer to take appropriate measures.

Chapter IV

Objective of the study

General objective

To identify common pathogenic bacteria from the raw and pasteurized milk consumed in and around Hawassa; assess their antimicrobial susceptibility patterns and identify the critical control points through the milk collection, processing and storage practices

Specific objective

- To isolate public health important aerobic bacteria from the raw and pasteurized milk
- To assess the antimicrobial susceptibility patterns of bacterial isolates
- To evaluate the hygienic condition of milking, milk collection, transportation, pasteurization and post pasteurization storage conditions
- To identify the critical control points through the raw milk collection, processing and storage practices

Chapter V

Methodology

Study area

The study was conducted in Hawassa town and its surroundings. Milk samples were collected from individual farmer's cows and dairy cooperatives established under the Integrated Livestock Development Project (ILDP).

Study design

A cross-sectional study design was used to determine the bacteriological analysis of cow milk in Hawassa town. Questionnaire and various laboratory tests were used for bacterial isolation and identification.

Study population

The study population was cow's milk of small holder dairy producers from cooperatives coordinated or established under Integrated Livestock Development Project (ILDP) in and around Hawassa town. Each member of the cooperatives does have one or more lactating local and/or crossbred cows which are a source of milk. The study was also conducted on pasteurized and packed milk from Milk Processing Plant at different time points of its shelf life.

Sample size determination

The questionnaire was administered to randomly selected members of the cooperatives in the *sub city* and milk sample were taken for bacteriological investigation at intervals from selected collection centers, and 32 samples of different ages of pasteurized milk. Random sampling was used for collecting the milk samples from individual farmers herd (which mean the family might be contained more than one cow so that randomly included in study participant) at milk collecting centers. The herd samples (10ml) were taken from individual cow during milking directly from the teat and farmers' utensil. The 5ml of milk was pasteurized as standard method to check the effect of pasteurization and 5ml was used as raw milk to culture. Side by side commercially pasteurized milks were compared to evaluate the bacteriological quality. Sample size was determined by considering 50% bacterial contamination prevalence since there is no prevalence study before. 95% CI, precision of 5% power of 80%, etc

This is calculated by using the following formula:

$$n = \frac{1.96^2 \times P_{exp}(1-P_{exp})}{d^2}$$

$$= \frac{1.96^2 \times 0.5(1-0.5)}{0.05^2}$$

$$= 384$$

Where; n= required sample size

d= desired absolute precision (usually 0.05) (Thrusfied, 1995)

P_{exp}= expected prevalence

There is a considerable time and financial constraints during these studies, so that since the population in the sub city was less than 10,000 i.e., as maximum as 250, the above values for n was corrected as follows:

$$n_f = \frac{n}{1+\frac{n}{N}}$$

Where; n_f = *final sample*

$$= \frac{384}{1+\frac{384}{250}} = 152$$

N = is maximum population

For non respondents and unwillingness of providing the sample at different level 20% were added to be 192 samples from different CCP. Since each source of milk has six collection points, 192 divided by 6 and become 32 families or sectors was selected to collect milk sample. From this 32 immediate pasteurized and 32 from different shelf life in different shops and storages were collected. In addition all collection, transportation and temporary storage containers (tanks) were sampled. Frequency of sampling was three working days within a week. Totally 192 samples of milk were analyzed for bacteriological and some risk factors. In this sample 64 pasteurized and 128 raw milk were collected. If in a single family more than 1 cow's were available the systematic random selection method was carried. The sampling tubes were formerly sterilized to minimize the contamination level. Also quality controls were performed in each level of the sample collection.

Inclusion and exclusion criteria

The organization and individuals who working and providing milk and milk products to the public services like Hospitals at most five times per week were all included. Those who do not provide milk at fresh status was also excluded like Yogurt milk.

Definition of the terms

Hazard analysis critical control point (HACCP) means a systematic approach to the identification, evaluation, and control of significant milk or milk product safety hazards.

Lot: means a quantity of food, which is prepared or packed under essentially the same conditions, usually, from a particular preparation or packing unit; and • during a particular time ordinarily not exceeding 24 hours.

Milk: is a major component in human diet all over the world, but it also serves as a good medium of the growth of many microorganisms, especially pathogenic bacteria. Thus, the quality control of milk is considered essential to the health and welfare of a community. Also, all cases of dairy illness continued to be of bacterial origin, pathogens that have involved in communicable diseases associated with the consumption of milk include *Salmonella*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Campylobacter*, *Yersinia*, pathogenic *Escherichia coli* and *Clostridium botulinum* (Adesiyun *et al.*, 1995; Hahn, 1996; Mennane *et al.*, 2007).

Abnormal Milk: Milk that is visibly changed in color, odor and/or texture.

Undesirable Milk: Milk that, prior to the milking of the animal, is known to be unsuitable for sale, such as colostrum.

Contaminated Milk: Milk that is un-saleable or unfit for human consumption following treatment of the animal with veterinary products, i.e. antibiotics, which have withhold requirements, or treatment with medicines or insecticides not approved for use on dairy animals by the U.S. Food and Drug Administration (FDA) or the U.S. Environmental Protection Agency (EPA).

Pasteurization: means the process of heating every particle of milk or milk product in properly designed and operated equipment, to one of the temperatures given in the following table and held continuously at or above that temperature for at least the corresponding specified time:

Temperature	Time
63ºC (145ºF)	30 minutes
72ºC (161ºF)	15 seconds

Sanitization: means the application of any effective method or substance to a clean surface for the destruction of pathogens, and of other organisms as far as is practicable.

Variables

Dependent variables

- Pasteurized milk
- Raw milk (unpasteurized milk)
- Bacteria

Independent variables

- Sized of herd
- Sex
- Age

Data collection

A questionnaire was prepared and administered to identify potential risk factors and evaluate their effect on the quality of the milk. Data on each farmer's herd were collected in a properly designed data collection sheet (Annex 1). Risk factors to be considered was hygienic quality of the udder and the milker during milking, means of cleaning of the container, hygienic condition of transporting container, duration of transportation to the collection center and to the processing plant, and storage facilities and shelf life of the pasteurized milk.

Milk sample collection and transport

After collection, the milk samples were placed in icebox and immediately transported to the laboratory and processed. The sample was either cultured soon or stored at 4^{o}C until cultured within few hours. Icebox or dry ice pack was used for the samples shipment to the diagnostic laboratory. The samples were taken from points considered to be associated with contamination or critical control points (CCP).

- At the point of milking directly from the teat (CCP1).
- From the bucket at farm level (CCP2).
- From storage containers at milk collection centers (CCP3).
- From transportation containers up on arrival at the processing plant (CCP4).
- After cooling at the pasteurization plant (CCP5)
- At different shelf life/time points of the pasteurized milk from distributors (CCP6).

Procedure

Quality control methods

While dealing with micro-organisms, every possible safety measures was taken using precautions and safety measure devices. Waste disposal were also taken according to the standard procedures.

- Using standardized sterile tube of sample collection and transport system
- Use of quality culture Media
- Collection of specimens, processing and analysis of the data (sample and questionnaire) was monitored by the investigator to minimize bias.

Bacterial count

Decimal dilutions of milk samples was platted on nutrient agar and violent red bile agar for standard plate count and coliform count respectively following the standard procedures recommended by American Public Health Association (1992).

Bacterial isolation

The milk sample was centrifuged using sterile test tubes for 20 minutes at 1500 rpm to concentrate the bacteria. By discarding the supernatant, a loop full of the sediment milk was inoculated into a sterile screw capped test tube containing Peptone water at 37^{o}C and incubated for 24hours aerobically. Then a loop full of peptone water culture after homogenization was inoculated into 5ml brain heart infusion (BHI) broth and it was incubated loose capped aerobically at 37^{o}C for 24hours.

After 24 hours of incubation, a loop full broth culture was plated onto the sheep blood agar by quadrant streaking method and incubated aerobically at 37^oC for 24 hours (Quinn *et al.,* 2002). After 24 hours incubation, the plates were observed for presence of bacterial colony. Morphological characteristics and size of the colony, presence or absence of the haemolysis and pigment production was observed and noted. The type of haemolysis were also examined and recorded. Then the isolated colonies was sub-cultured by half plating on blood agar and MacConkey agar and incubated at 37^oC for 24hours aerobically. After 24hours, growth or absence of growth and lactose fermentation on MacConkey agar was evaluated. Then the single isolated colony was sub-cultured on BHI agar slant and aerobically incubated for 24 hours at 37^oC. Finally, these slants were preserved at refrigerator temperature for further biochemical analysis.

Bacterial identification was done according to the methods of Barrow and Feltham (1995). Colony Characteristics on blood agar, Manitol salt agar, Gram's Staining reaction and morphological characteristics, Growth on MacConkey agar, Catalase and Oxidase tests, Hydrogen Sulfide (H_2S) production, Indole, Methyl Red (MR), Voges-Proskauer (VP), Citrate Utilization, Urease, Oxidation-Fermentation, Motility and different Carbohydrate Fermentation tests (TSI), depending on the primary culture indication, were used for characterization and identification of bacteria to species level (Annexes 2, 3 and 4).

Data Analysis

The questionnaire and bacteriological data was entered to Microsoft Excel Spreadsheet and was analyzed using suitable software like SPSS and descriptive statistics was used.

Ethical considerations

The proposal was submitted to DMIP, Addis Ababa University School of Medicine, and the Department Research and Ethics Committee approved it.

Benefits and Beneficiaries of the proposed study

The bacteriological quality of raw and pasteurized milk was established, critical control points was identified and possible continuous monitoring procedures was forwarded after the completion of the study. In addition the bacteria involved in contaminating the milk and their public health impact was known. Knowing this all will enable farmers, processing plants, researchers, and institutions to suggest appropriate measures in the effort of insuring the quality of raw and pasteurized milk. Milk consumers were the line of first beneficiary of this project; determination of the shelf life of pasteurized milk has economic significance to utilize the milk prior to its deterioration. The farmers, processing plants, researchers, academic institutions, health and agricultural policy makers, and individuals working on milk hygiene was also benefit a lot from this study.

Chapter VI
Results

1. Risk factors

This study included 32 families with maximum and minimum age of 12 and 2 years with SD of 3.6, respectively. From which 28(87.5%) of them use barn cleaning one times per day. Half of these families bedding condition were good in quality according to the animal health sciences specialist description. In this community the teat cleaning materials were mostly cold tap water 23(71.9%), warm well water 4(12.5%), cold well water 4(12.5%) and cold river water.

More than 56% of the participants used disinfectant and 37.5% of them used detergents. None of the respondents used the combined and almost 2% of these families used neither disinfectant nor detergent but other mechanisms.

Not less likely they used bactericidal treatment for milk utensils cleaning. However, more than 90% of the respondents used cold water to clean utensils after each usage. The largest size 28(87.5%) did not apply traditional favorants to clean the transport equipment. On the other hand, few of them used local grass called “Nech Sar” which is a grass used to clean and give good smells of utensils.

Due to the herd size and some economic factors machine milking technique was less likely carried. Even though, there were no machines, 90.6% of the participants were able to milking twice per day manually. More than 93.0% of the milkers used tap water predominantly (78.1%) to wash their hands. Some of them used well water (21.9%). None of the milkers clean their hands between cows in order.

More than 81% of the respondents used a cooling system for milk at room temperature and mainly they provided to the processing plant organization every day.

Few of the milkers (6.2%) respond to illness or symptoms for diarrhea, skin disease and other cases. However, there was no plan to test the quality of milk before adding to the pool at study area. Moreover, the public health aspects also were not considered completely to test, Tb and brucellosis. Not more than 25% of the families used mastitis test. The raw milk consumption habit was common in this study area.

2. Bacterial analysis

Pasteurized and unpasteurized milk was assessed for bacteriological quality from different sources around Hawassa town. Thirty two farm level families were randomly selected and provided questionnaire to obtain milk sample from teat (CCP1), from bucket at farm level (CCP2), from storage containers at milk collection center (CCP3), from transportation container (CCP4), after cooling at the pasteurization plant (CCP5), and different shelf life/time from market level (CCP6).

One hundred ninety two (192) samples were collected from different sites. Table 1 indicates bacterial species isolated from cow milk at different hazard analysis and critical control point (HACCP) levels. According to this result the bacterial species that found in the different level were 14, 16, 22, 22, 9, and 11 different species were obtained from CCP1, CCP2, CCP3, CCP4, CCP5 and CCP6 respectively.

Gram positive bacilli were also isolated from CCP2, CCP3, and CCP4. Except CCP1 level there was undefined bacterial species in different level of milk source due to a shortage of some laboratory reagent and biochemical tests. In addition to this, *Klebsella rhinosclermatis*$^{-}$*/S. Sonnei*$^{+}$ and *E. aerognes*$^{+}$*/Hafnia*$^{-}$ were obtained without differentiation due to absence of ornithine and inositol tests respectively to differentiate each other. Moreover CONS also were not specifically differentiated due the same reason.

Table 1: Commonly human pathogenic bacteria species isolated from milk at different HACCP levels

CCP1	CCP2	CCP3	CCP4	CCP5	CCP6
S. aureus *E. coli* *Shigella dysentery* CONS *K. pneumoniae* *P. vulgaris*	*S. aureus* *E. coli* *S. dysentery* CONS *K. pneumoniae*	*S. aureus* *E. coli* *S. dysentery* CONS *K. pneumoniae* *P. aerogenosa* *P. mirabilis*	*S. aureus* *E. coli* CONS *S. dysentery* *K. pneumoniae* *P. aerogenosa* *P. mirabilis* Shigella species	*S. aureus* CONS *S. dysentery*	*P. areogunesa* *E. coli* *P. vulgaris* *S. aureus* CONS

Table 2: Other bacteria species isolated from raw milk at different HACCP levels

CCP1	CCP2	CCP3	CCP4
C. diversus *K. rhinoscleromatis/S. Sonnei* *E. aerogenes/Hafnia* *Morganella morganii* *C. freundii* *E. cloacae* Serratia species Arizona species	*C. diversus* *K. rhinoscleromatis/S. Sonnei* *E. aerogenes/Hafnia* *Morganella morganii* *Serratia species* *E. cloacae* *C. freundii* *K. oxytoca* *Enterobacter agglomerans* (Erwinia) Gram positive bacillus Undefined bacteria	*C. diversus* *K. rhinoscleromatis/S. Sonnei* *E. aerogenes/Hafnia* *Morganella morganii* *Serratia species* *E. cloacae* *C. freundii* *K. ozenae* Arizona Edwardsiella Shigella species *Klebsella oxytoca* *P. rettigeri* Gram positive bacillus Undefined bacteria	*C. diversus* *K. rhinoscleromatis/S. Sonnei* *E. aerogenes/Hafnia* *Morganella morganii* Serratia species *E. cloacae* *K. ozenae* Arizona Edwardsiella *K. oxytoca* *P. rettigeri* *Providinics stuartii* Gram positive bacillus Undefined bacteria

Table 3: A bacteria species isolated from pasteurized milk at different HACCP levels

CCP5	**CCP6**
K. rhinoscleromatis/S. Sonnei *K. ozenae* *K. oxytoca* *C. freundii* Serratia spp Undefined bacteria	*C. freundii* *E. cloacae* *C. diversus* *K. oxytoca* *Providinics alkalifaciens* Undefined bacteria

A total of 326 bacterial species were isolated from CCP1 up to CCP6. There was different species of bacteria in different HACCP levels of milk. In CCP1, there were 51 bacteria of different species and in CCP2, CCP3, CCP4, CCP5, and CCP6, was 64, 73, 80, 20, and 38, respectively. The predominant bacteria species in all (CCP1-CCP5) levels of the milk was *S. aureus* which accounts 14(27.5%), 20(31.3%), 21(28.8%), 17(21.3%), and 6(30.0%), respectively.

Out of 326 bacteria 58(17.8%) were from pasteurized milk and 268(82.2%) were obtained from raw milks.

Within 32 teat milk samples the top five bacteria were *Shigella dysentery* 7(13.7%), E. coli 5(9.8), CONS 4(7.8), GPB (gram positive bacilli), *Ent. aerognes/Hafnia*, *C. diversus* 3(5.9%) each. The remaining accounts less than 4.0% (*Arizona* spp, *K. pneumonia*, *K. rihinoscleromais/S. Sonnei*, *Morganella morganii, P. mirabilis, Providencia rettgeri*, *P. vulgaris, and Serratia* species).

Sixty four bacterial species were obtained form 32 samples of bucket storage at farm level (CCP2). In this sample next to *S. aureus*, the five leading bacteria were *Shigella dysentery* and *E. coli* 6(9.4%) each, *K. pneumoniae* and *C. diversus* 5(7.8%) each and *C. freundi* 4(6.3%). The rest species that found in this sample were accounts less than 5% (CONS, *Ent. aerognes/Hafnia, K. rihinoscler/S. Sonnei, Morganella morganii, P. mirabilis, Proteus rettigeri, P. vulgaris, Serratia*, *K. oxytoca, Enterobacter agglomerans* (*Erwinia*). Moreover gram positive bacillus and undetermined gram negative bacteria also were seen in this sample.

From storage containers at milk collection center (CCP3) the predominant bacteria's were *S. auerus, Shigella dysentery, P. aerogenes, K. pneumonia, E. coli accounts, C. freundii* 21(28.8%), 8(11%), 5(6.8%), 5(6.8%), 5(6.8) and 4(5.5%) respectively. About 5.5% of the organism was not determined to their species categories under this setup (Table 4)

More than 80 bacteria were determined from transportation container (CCP4). From which the most frequently obtained bacteria was *S. aureus* 17(21.3%), *E. coli* 10 (12.5%), *Shigella dysentery* 7(8.8%), *K. pneumoniae* 5(6.3%), *C. freundii* 5(6.3%), *C. diversus* 4(5.0%). The remaining organisms such as *P. aeruginosa, K. oxytoca, Serratia*, CONS, *Arizona* spp, *Ediwardsela*, *E. cloacae, P. vulgaris, Providencia stuartii, Morganella morganii, K. rhinoscleromatis/S. Sonnei* and Gram positive bacillus were less than 5%.

From immediate pasteurized milk *S. aureus* 6(30.0%), CONS 4(20.0%), *Shigella dysentery* 3(15.0%) and *Serratia* 2(10.0%) were isolated. The others like *C. freundii, K. rhinoscleromatis/S. Sonnei, K. oxytoca, K. ozane*, and Gram positive bacillus were determined.

Thirty two pasteurized milk samples were collected from different shop at different shelf life. The commonly found organisms were *P. vulgaris* 9(23.7%), *P. aeruginosa* 6(15.8%), *K. pneumoniae* 6(15.8%), *K. oxytoca* 6(15.8%), *E. coli* 5(13.2%), *C. diversus* 2(5.3%), *C. freundii* 1(2.6%) and *Providencia alkalifaciens* 1(2.6%). Some of the unspecified bacteria were also found in this pasteurized shelf life bacteria (Table 4).

Table 4. Prevalence of bacterial species in different level of milk

Organisms	HACCP Level					
	CCP1	**CCP2**	**CCP3**	**CCP4**	**CCP5**	**CCP6**
	Nº (%)	Nº (%)	Nº (%)	Nº (%)	Nº (%)	Nº (%)
Arizona spp	2(3.9)	0(0.0)	3(4.11)	3(3.8)	0(0.0)	0(0.0)
C. freundi	1(2.0)	4(6.3)	4(5.48)	5(6.3)	1(5.0)	1(2.6)
C. diversus	3(5.9)	5(7.8)	3(4.11)	4(5.0)	0(0.0)	2(5.3)
CONS	4(7.8)	3(4.7)	1(1.37)	3(3.8)	4(20.0)	0(0.0)
E. coli	5(9.8)	6(9.4)	5(6.85)	10(12.5)	0(0.0)	5(13.2)
Ent. aeruginosa/Hafnia	3(5.9)	1(1.6)	2(2.74)	0(0.0)	0(0.0)	0(0.0)
K. pneumoniae	1(2.0)	5(8.2)	5(6.85)	5(6.3)	0(0.0)	6(15.8)
K. rhinoscleromatis/S. Sonnei	2(3.9)	1(1.6)	2(2.74)	1(1.3)	1(5.0)	0(0.0)
Morganella morganii	1(2.0)	1(1.6)	2(2.74)	1(1.3)	0(0.0)	0(0.0)
P. mirabilis	2(3.9)	0(0.0)	1(1.37)	0(0.0)	0(0.0)	0(0.0)
Providencia rettigeri	1(2.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
P. vulgaris	1(2.0)	0(0.0)	0(0.0)	2(2.5)	0(0.0)	9(23.7)
S. aureus	14(27.5)	20(31.3)	21(28.8)	17(21.3)	6(30.0)	0(0.0)
Serratia spp	1(2.0)	2(3.1)	1(1.37)	3(3.8)	2(10.0)	0(0.0)
Shigella dysentery	7(13.7)	6(9.4)	8(11.0)	7(8.8)	3(15.0)	0(0.0)
E. cloacae	0(0.0)	3(4.7)	2(2.74)	2(2.5)	0(0.0)	0(0.0)
Enterobacter agglomerans (Erwinia)	0(0.0)	1(1.6)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
K. oxytoca	0(0.0)	1(1.6)	1(1.37)	3(3.8)	1(5.0)	6(15.8)
Ediwardsela spp	0(0.0)	0(0.0)	1(1.37)	2(2.5)	0(0.0)	0(0.0)
K. ozane	0(0.0)	0(0.0)	1(1.37)	0(0.0)	1(5.0)	0(0.0)
P. aeruginosa	0(0.0)	0(0.0)	5(6.85)	3(3.8)	0(0.0)	6(15.8)
Providencia stuartii	0(0.0)	0(0.0)	0(0.0)	1(1.3)	0(0.0)	0(0.0)
Providencia alkalifaciens	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(2.6)
Gram positive bacillus	3(5.9)	3(4.7)	1(1.37)	2(2.5)	0(0.0)	0(0.0)
Undetermined bacteria	0(0.0)	2(3.1)	4(5.48)	6(7.5)	1(5.0)	2(5.3)
Total	**51(100)**	**64(100)**	**73(100)**	**80(100)**	**20(100)**	**38(100)**

CONS = Coagulase negative staphylococcus

3. Colony count

A total of 32 pasteurized milk samples upon storage were examined for total viable count and total coliform count. TVC in this sample was found to be in the range of 0.63-9.24x10^8CFU/ml. The TCC were detected in this entire sample to be in the range of 1.01x10^8 - 1.51x10^8 CFU/ml. The mean TVC and TCC were 3.65x10^8 and 2.39x 10^8. The same procedure was applied for 32 raw milks to examine for TVC and TCC. The TVC and TCC in raw milk sample were found to be in the range of 1.78x10^6-21.77x10^8 CFU/ml and 2.15x10^7-15.25x10^7 respectively. The mean TVC and TCC were 6.57x10^8 and 4.64x10^7CFU/ml respectively. Under this condition, 32 immediately pasteurized milk samples were examined for TVC and TCC. The minimum and maximum cell count in total viable and total coliform count was 1x10^4- 8x10^5 and 8x10^2-10x10^2 CFU/ml. The mean TVC and TCC were 7x10^5 and 10x10^2 CFU/ml respectively (Table 5).

Table 5. Total viable count and total coliform count in raw and pasteurized milk

Sample	**Sample size**	**Total viable count**			**Total coliform count**		
		Minimum	**Maximum**	**Mean**	**Minimum**	**Maximum**	**Mean**
Raw milk	32	1.78 x10^8	21.77 x10^8	6.57x 10^8	2.15x 10^7	15.25x10^7	4.64 x 10^7
Pasteurized milk	32	0.63 x10^8	9.24 x10^8	3.65x 10^8	1.01x 10^8	1.51x10^8	2.39 x 10^8
Immediate pasteurized milk	32	1 x10^4	8 x10^5	7x 10^5	10x10^2	8x10^2	10x10^2

4. Antimicrobial profile

In this study Penicillin (Pen) 10µg, Ampicillin (Amp) 10µg, Amoxicillin-Clavulanic acid (Amox) 30µg, Ceftriaxone (CRO) 30µg, ciprofloxacin (CIP) 5µg, Chloramphenicol (CAF) 30µg, Gentamicin (Gen) 10µg, Oxacillin (Oxa) 1µg, Vancomicin (Van) 30µg, TMP-SMZ 25µg and Erytheromycin (Ery) 15µg was included to test the susceptibility pattern.

Based on culture 78 *S. aureus* strains were identified from total milk sample. These organisms were examined for Ampcillin, Penicillin, Gentamicin, Amoxicillin-Clavulanic acid and Vancomycin. Out of 78 *S. aureus* strain 67.9% were resistance to Ampiciline, 67.9% were resistance to Penicillin, 38.5% were resistance to Vancomycin, 30.8% were resistance to Amoxicillin-Clavulanic acid and 3.8% were resistance to Gentamycin, According to 24th edition of Medical microbiology (Brooks *et al*, 2007) the 1st choices of the drug for the treatment of *S. aureus* were Vancomycin and/or Gentamycin, Penicillin and other drugs.

Moreover these organisms also develop resistance to TMP-SMZ (1.64%), Ceftriaxone (23.2%), CAF (28.2%), Erythromycin (32.1%) and Oxacillin (60.3%). Contrarily there was no resistance strain to Ciprofloxacin antibiotics. Intermediate resistance development of this organism was seen for Ciprofloxacin, CAF, Erytheromycin, Ceftriaxone and TMP-SMZ about 16.7%, 20.5%, 3.8%, 42.3% and 19.2% respectively (fig 2).

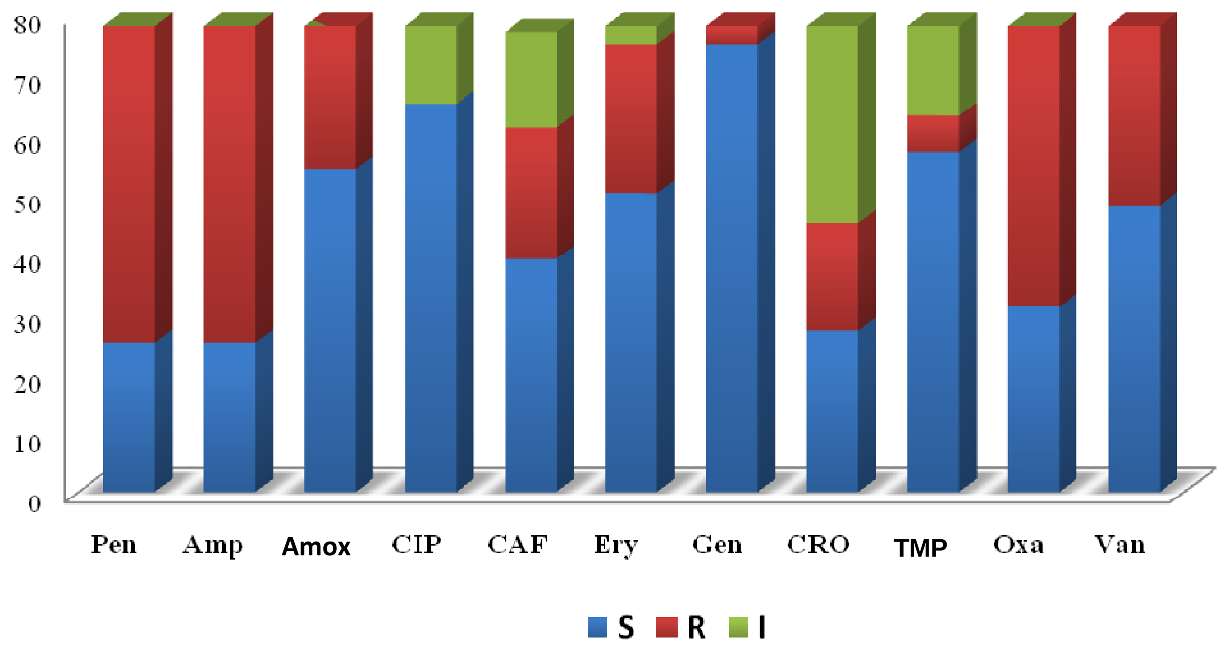

Fig 7: Antimicrobial resistance level of *S. aureus* in milk sample

Pen*=penicillin,* ***Amp****= Ampcilin,* ***Amox****= Amoxicilline-Clavulanic acid,* ***CIP****= ciprofloxacilin,* ***CAF****= chloramphenicol,* ***Ery****= Erythromycin,* ***Gen****= Gentamicin,* ***CRO****= Ceftriaxone,* ***TMP****= Trimethoprime-Sulphamethoxazole,* ***Oxa****= oxacilin,* ***Van****= vancomycin,* ***S****=Susceptible,* ***R****=resistant,* ***I****=intermediate*

Thirty one isolates of *E. coli* were examined for antibiotic susceptibility against Penicillin, Ampicillin, Amoxicillin-Clavulanic acid, Erythromycin, Ciprofloxacin, Gentamycin, Ceftriaxone and TMP-SMZ. *E. coli* isolated from milk sample was resistance to Penicillin 31(100%), Ampicillin 29(93.5%), Erythromycin 21(67.7%), Amoxicillin-Clavulanic acid 6(19.4%), Ceftriaxone 6(19.5%), TMP-SMZ 5(16.10%) and Gentamycin 2(6.5%). There was no resistance strain of *E. coli* for Ciprofloxacin antibiotics which is second generation of synthetic analogs of Nalidixic acid called quinolones. However, 11(35.50%), 1(3.20%), 8(25.80%), 6(19.30%), 6(19.40%) of E. coli developed intermediate resistance to Amoxicillin-Clavulanic acid, Ciprofloxacin, Erythromycin, Ceftriaxone and TMP-SMZ respectively. E. coli is sensitive to 30(96.80%), 29(93.50%), 20(64.50%), 19(61.30%), 14(45.10%), 2(6.50%) for Ciprofloxacin, Gentamycin, TMP-SMZ, Ceftriaxone, Amoxicilline-Clavulanic acid, Ampicillin and Erythromycin, respectively.

Out of 17 isolates of *K. pneumoniae* from milk sample 17(100%) of them are resistant to Penicillin 16(94.1%) to Ampicillin, 4(82.4%) to CAF and 11(64.7%) for Erythromycin. For the remaining antibiotics like Amoxicilline-Clavulanic acid, Ciprofloxacin, and Ceftriaxone, *K. pneumonia*e develop resistance for less than 30% resistance. However, there was no K. pneumonia strain which developed resistance pattern to Gentamycin with in 17 isolates. Among this isolates there was no *K. pneumonia*e species which is sensitive to CAF and Erythromycin. This species is sensitive to Gentamycine, Ciprofloxacin and Amoxicillin-Clavulanic acid for 76-88%.

Commonly used antibiotics for the treatment of *P.aeruginosa* were Gentamicin, Penicillin, ceftriaxone, and Ciprofloxacin. Also TMP-SMZ and Ampicillin frequently prescribed elsewhere. However, the antibiotics resistance profiles of *P. aeruginosa* in this study were compiled as 14(100%) to Penicillin. In addition *P. aeruginosa* developed resistance to Ampicillin, ceftriaxone and TMP-SMZ by 78.6%, 64.3% and 71.4% respectively. Few species (7.1%) were developing resistance to ciprofloxacin. Meanwhile *P.aeruginosa* was susceptible for Qunolones such as ciprofloxacin and Amynoglycosides such as Gentamicin 92.9% and 100% respectively.

Ciprofloxacin, Ampicillin, trimethoprim-sulfamethoxazole, Ceftriaxone are most commonly inhibitory for shigella isolates and can suppress acute clinical attacks of dysentery and shorten the duration of symptoms. Therefore, these and another antibiotic like Penicillin, Amoxicillin-

Clavulanic acid, and Gentamicin were examined for its resistance level. Twenty seven isolate of S. dysentery 66.7%, 37%, and 92.6% were resistance to Ampicillin, TMP-SMZ and Penicillin respectively. Compared with other antibiotics Genatmicin, Ciprofloxacin, Amoxicillin-Clavulanic acid, and TMP-SMZ were sensitive for S. dysentery about 100%, 77.8%, 77.8% and 63.0% respectively. However, for TMP-SMZ, Gentamicin, and Ciprofloxacin other Shigella species which identified from milk samples were 100% susceptible and for Amoxicillin-Clavulanic acid and ceftriaxone 50% and 75% susceptible. But 100% of these species were resistance to Ampicillin.

Ceftriaxone, TMP-SMZ, Ciprofloxacin, Gentamicin, Ampicillin and Penicillin were examined for the resistance level of *Serratia* species. No *Serratia* species were developed resistance pattern for gentamicin however, 88.9% of these species were develop resistance to Ampicillin and penicillin. So that gentamicin were 100% effective for this species, in addition ciprofloxacin, ceftriaxone and TMP-SMZ were effective for 88.9% species.

Ampcillin, TMP-SMZ, Gentamicin, ceftriaxone, ciprofloxacin and Amoxicillin-Clavulanic acid were examined for resistant level of *P. vulgaris, P. mirablis, Providencia rettgeri, Providencia alcalifaciens, Morganella morganii and Edwardsiella* species. All of them are resistance to Ampicillin 100% except for *P. vulgaris* which is 50% resistance.

According to this work *P. rettgeri, M. morganii, Providencia alcalifaciens,* and *P. vulgaris* were resistance to Amoxicilline-Clavulanic acid 100%, 60%, 100%, and 25% respectively. But *P. vulgaris* and *Edwardsiella* species were susceptible for this antibiotic fully.

There was no resistance developed group for ciprofloxacin and gentamicin with in *P. mirablis, Providencia rettgeri,* and *Edwardsiella* species which mean 100% susceptible. But some strain of *M. morganii* and *Providencia alcalifaciens* resistance to ciprofloxacin 20 and 50% respectively. However, *M. morganii* is susceptible to gentamicin but *Providencia alcalifaciens* were resistance.

Except *P. vulgaris* and *Providencia alcalifaciens* all of these microorganisms were less susceptible to ceftriaxone. *P. mirablis* and *Providencia alcalifaciens,* and *Edwardsiella* species were 100% susceptible for TMP-SMZ but *P. vulgaris* and *M. morganii* were susceptible only 66.7% and 40%. There were no susceptible groups of *P. rettgari* for TMP-SMZ.

C. diversus, C. freundii, E. cloacae, E. aerognese/Hafnia and *Arizona* species were tested for antibiotic resistance level of penicillin, Ampicillin, Amoxicilline-Clavulanic acid, ciprofloxacin, CAF, gentamicin, ceftriaxone and TMP-SMZ. All of these microorganisms were resistance to Penicillin and Ampicillin 100%. But some strains of *C. freundii* and Arizona species were resistance about 60% and 87.5% for Ampicillin respectively. Moreover, more than 50% of these organisms were also resistance to Amoxicillin-Clavulanic acid. There were no resistance patterns of these organisms for ciprofloxacin except less than 20% of these strains. CAF was less effective against these organisms because it showed more than 75% resistance. According to this result gentamicin and ceftriaxone were good antibiotics to treat these organisms because they showed 100% susceptible. TMP-SMZ were 100% effective for *C. freundii,* E. cloacae, *E. aerognese/Hafnia* however, *C. diversus* but *Arizona* species were not.

K. oxytoca were 100% resistance to Ampicillin and Penicillin but more than 90% was susceptible to ciprofloxacin, gentamicin, ceftriaxon and TMP-SMZ. However, few species less than 10% develop intermediate resistance pattern.

More than 47% of microorganisms from milk were less sensitive to the common antibiotics. But *S. aureus, E.coli, Citrobacter diversus, Shigella dysentery, K. pneumoniae,* and *P. aeruginosa* were the most resistant groups of organism in a given antibiotics. Contrarily, *K. ozanae, Edwardeisela*, and *P. alkalifiens* were least resistance to their antibiograms. Because this is might be due to the community acquired drug resistance pattern gradually increasing.

Table 4: Prevalence of microorganisms and level of resistance

Organisms	**Antibiotics**														
	Penicillin			**Ampcillin**			**Amoxicillin-Clavulanic acid**			**Ciprofloxacin**			**Chloranphinecol**		
	R	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**
Arizona spp	100	0	0	87.5	0	12.5	37.5	37.5	25	12.5	37.5	50	25	12.5	62.5
C. freundi	100	0	0	60	6.7	33.3	46.7	13.3	40	20	0	80	46.7	20	33.3
C. diversus	100	0	0	100	0	0	70.6	11.8	17.6	17.6	0	82.4	70.6	23.5	5.9
E. coli	100	0	0	93.5	0	6.5	19.4	35.5	45.1	0	3.2	96.8	16.2	41.9	41.9
Ent. aeruginosa/Hafnia	100	0	0	100	0	0	75	0	25	0	0	100	62.5	12.5	25
K. pneumonia	100	0	0	94.1	0	5.9	23.5	0	76.5	23.5	0	76.5	82.4	17.6	0
K. rhinoscleromatis/S. Sonnei	83.3	0	16.7	50	50	0	50	0	50	0	0	100	50	16.7	33.3
Morganella morganii	100	0	0	100	0	0	60	0	40	20	0	80	60	0	40
P. mirabilis	100	0	0	100	0	0	0	0	100	0	0	100	100	0	0
Providencia rettgeri	100	0	0	100	0	0	100	0	0	0	0	100	100	0	0
P. vulgaris	100	0	0	50	0	50	25	8.3	66.7	0	16.7	83.3	50	25	25
S. aureus	67.9	0	32.1	67.9	0	32.1	30.8	0	69.2	0	16.7	83.3	28.2	21.8	50
Serratia spp	88.9	0	11.1	88.9	0	11.1	0	77.8	22.2	11.1	0	88.9	66.7	11.1	22.2
Shigella dysentery	92.6	3.7	3.7	66.7	22.1	11.1	14.8	7.4	77.8	7.4	14.8	77.8	18.5	25.9	51.9
E. cloacae	100	0	0	100	0	0	42.9	0	57.1	0	0	100	0	100	0
Enterobacter agglomerans (Erwinia)	100	0	0	100	0	0	100	0	0	0	0	100	0	0	100
K. oxytoca	100	0	0	100	0	0	16.7	16.7	66.6	8.3	25	66.7	16.7	25	58.3
Ediwardsela spp	100	0	0	100	0	0	0	33.3	66.7	0	0	100	33.3	0	66.7
K. ozane	100	0	0	50	50	0	0	0	100	0	0	100	0	0	100
P. aeruginosa	100	0	0	78.6	0	21.4	71.4	0	28.6	7.1	0	92.9	78.6	21.4	0
Providencia alkalifaciens	100	0	0	100	0	0	50	0	50	0	0	100	0	0	100

Table 4: (*cont.*)

Organisms	**Antibiotics**																	
	Erytromycin			**Gentamycin**			**Ceftriaxone**			**TMP-SMZ**			**Oxacilin**			**Vancomycin**		
	R	I	S	R	I	S	R	I	S	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**
Arizona spp	87.5	0	12.5	0	0	100	0	0	100	37.5	25	37.5	100	0	0	100	0	0
C. freundi	100	0	0	0	0	100	0	0	100	0	0	100	100	0	0	100	0	0
C. diversus	100	0	0	0	0	100	47.1	0	52.9	58.8	17.6	23.6	100	0	0	100	0	0
E. coli	67.7	25.8	6.5	6.5	0	93.5	19.4	19.3	61.3	16.1	19.4	64.5	100	0	0	100	0	0
Ent. aeruginosa/Hafnia	50	37.5	12.5	0	12.5	87.5	0	62.5	37.5	0	0	100	100	0	0	100	0	0
K. pneumonia	64.7	35.3	0	0	11.8	88.2	29.4	47.1	23.5	41.2	5.9	52.9	100	0	0	100	0	0
K. rhinoscleromatis/S. Sonnei	66.7	0	33.3	0	0	100	16.7	66.7	16.7	0	0	100	100	0	0	100	0	0
Morganella morganii	60	0	40	0	20	80	40	20	40	60	0	40	100	0	0	100	0	0
P. mirabilis	100	0	0	0	0	100	0	100	0	0	0	100	100	0	0	100	0	0
Providencia rettgeri	100	0	0	0	0	100	0	0	100	100	0	0	100	0	0	100	0	0
P. vulgaris	83.3	0	17.7	0	0	100	16.7	8.3	75	16.7	16.7	66.6	100	0	0	100	0	0
S. aureus	32.1	3.8	64.1	3.8	0	96.2	23.1	42.3	34.6	7.7	19.2	73.1	60.3	0	39.7	38.5	0	61.5
Serratia spp	0	66.7	33.3	0	0	100	0	11.1	89.9	11.1	0	88.9	100	0	0	100	0	0
Shigella dysentery	29.6	14.8	55.6	0	0	100	18.5	11.1	70.4	37	0	63	92.6	0	7.4	100	0	0
E. cloacae	42.9	0	57.1	0	0	100	0	28.6	71.4	0	0	100	100	0	0	100	0	0
Enterobacter agglomerans (Erwinia)	100	0	0	0	0	100	0	0	100	0	0	100	100	0	0	100	0	0
K. oxytoca	91.7	0	8.3	0	8.3	91.7	8.3	0	91.7	41.7	8.3	50	100	0	0	100	0	0
Ediwardsela spp	66.7	33.3	0	0	0	100	0	66.7	33.3	0	0	100	100	0	0	100	0	0
K. ozane	0	0	100	0	0	100	50	0	50	0	0	100	50	0	50	100	0	0
P. aeruginosa	78.6	0	21.4	0	0	100	64.3	7.1	28.6	71.4	0	28.6	100	0	0	100	0	0
Providencia alkalifaciens	100	0	0	0	50	50	0	0	100	0	0	100	100	0	0	100	0	0

S= sensitive, I= intermediate, R= Resistance

Chapter VI

Discussion

Milk contains relatively few bacteria when it is secreted from the udder of healthy animal. However, during milking operations it gets contaminated from the exterior of the upper and the adjacent areas, dairy utensils, milking machines, the hands of the milkers from the soil and dust (Harding 1995).

In this 32 incorporated families barn cleaning habit was one times per day. In contrast to this study others work done on Ethiopia, Oromiya zone, were used barn cleanig more than one times (Care-Ethiopia, 2009). This might be due to less practice around the study area or no documented rule for barn cleaning frequency. However, the recommended barn cleaning frequency per day is more than two times before milking and after milking. Only 50% of the study family seems good bedding condition. In contrast, other study confirmed that the bedding condition should be clean, concrete cemented and dray with effortlessness flow cows waste at the living area (Bramley, 1982; Bramley and McKinnon, 1990; Hogan *et al.*1989; Zehner *et al.*1986). Because, unclean bedding has been shown to harbor large numbers of microorganisms even more than10^8-10^{10} per gram.

According to this study almost all participant families have performed washing a teat before milking using cold tap water (71.9%), warm well water (12.5%), cold well water (12.5%) and cold river water. Therefore, one can easily conclude that the source of microorganism's in this study were might be due to the contaminated water by dust, animals, plants, people, and other agents http://www.microbiologyprocedure.com. Also Dahlberg *et al.* mentioned that the presence of microorganism in milk were influenced by unclean water, unhealthy cows, unclean utensils; inappropriate bactericidal treatment of utensils, insufficient cooling of milk to low temperature and minimum storage time (Dahlberg *et al.* 1953).

In this study more than 56 and 37.5% of participants were used disinfectant and detergents respectively. Few of them used neither detergents nor disinfectants but other mechanisms which was not mentioned. However, still the number of bacteria obtained from the sample indicates poor hygienic condition. This is may be due to misunderstanding how to apply the detergents and also insufficient disinfection. Moreover, the bacteria might be resisting to disinfectants and detergents like soaps (Walker, 1924; Eggerth, 1926; Avery, 1918). Also (Walker, 1924) confirmed that staphylococci were very resistant to soaps. Moreover, the increment of bacteria in this research might be due to unknown materials which they used (fig 2). The survival of microorganisms in application area might be also influenced the presence of bacteria in this study (Aquaox, 2010).

The milk utensil cleaning activity of this study was 90% by using cold water after each use. Some of them applied local agents like grass called "Nech Sar" to clean the utensils (Dahlberg *et al*. 1953). This might be also contributed the presence of microorganism in the milk. Moreover, there was no written document to justify how the mechanism of the "Nech Sar" influences bacterial contamination. But it has a valuable contribution of bacteria's from the environment where this material was collected.

The machine milking technique was less likely carried out. Even though, no machine, 90.6% of the participants were milked the cows twice per day by their hands. The same reports were generated from Ethiopia that indicated the milking condition was traditional at all (Care-Ethiopia, 2009). Therefore, hand milking might be contributed a great role in contamination of milk in this study area. Also unclean cloth wearing during milking obviously transmitting microorganism from milkers and environment to cows directly therefore, it is clear why the bacteria count was high in this study (Care-Ethiopia, 2009; Dahlberg *et al.* 1953; Bramley and McKinnon, 1990).

The pre-milking udder hygiene condition was poor in this study. Confirming this study several studies have investigated pre-milking udder hygiene techniques in relation to the bacteria count of milk (Bramley and McKinnon, 1990; Galton *et al.*1984; McKinnon *et al.*1990, Pankey, 1989). Therefore, poor pre-milking udder hygiene might be contributed the presence of the bacteria in this study.

This study confirmed 6.2% of the milkers and related persons showed a clinical symptom after a long contact with cows. Also FAO reported the infectious disease related with milk in Ethiopia more than this study (FAO, 1993) and (Sharp, 1989) reported the milk born infection in Scotland. In UK also there were a few outbreak reports due to milk related problem. However, in our study few of them realized that the symptoms originated after the contact of the cows for long time. But there were no clear reports over milk-born problems around the study area.

More than 81% of the respondents used room temperature cooling system. Only less than 19% of the subjects used refrigerator for cooling milk. About 78.1% of the farmers provide to the processing plant organization to pasteurization every day using aluminum container. Few of them (43.8%) used plastic bags. From this data one would expect the bacterial counts of the milk in this study area to be higher. Because of less effective cooling of milk on the farm level of this study area increased the bacterial count of the milk (Dahlberg *et al*. 1953). Moreover, the well water that they used to clean aluminum and plastic bag every day cause less effective handling of milk at farm level. Therefore, the milk supplied to the processing center might be increased with bacterial contamination of the milk.

The milk quality test before adding to the pool was not tested at this study area. Also the public health aspect and mastitis test was not considered. But as a work of (Ruegg, 2002 and Ott, 1999) a variety of diagnostic tests are routinely used to evaluate milk quality on dairy farms to check the quality of milk. Also in this study the raw milk consumption habit was very common. High raw milk consumption rate in USA and other developed countries were reported (Dahlberg *et al*. 1953, CSA 2005).

The predominant bacteria species in all (CCP1-CCP5) levels of the milk was *S. aureus* which accounts 14(29.2%), 20(31.3%), 21(28.8%), 17(21.3%), and 6(30.0%) respectively. There was no *S. aureus* species reported in CCP6 milk level. Similar idea was reported by (Bruces, 2010) that the prevalence of *S. aureus* in Senegal was 26% in raw milk which confirms poor milking hygiene. D'Amico and colleagues determined 67% of the *S. aureus* from farm levels raw milk. This is relatively worse than present study (D'Amico, Donnelly, 2010).

CCP1 (Teat milk)

From 32 teat milk samples the top five bacteria was *Shigella dysentery* 7(13.7%), *E. coli* 5(9.8%), CONS 4(7.8%), GPB (gram positive bacilli), *E. aerogenes/Hafnia*, *C. diversus* 3(5.9%) each. The remaining accounts less than 4.0% (*Arizona* spp, *K. pneumoniae, K. rihinoscleromatis/S. sonneii*, *Morganella morganii*, *P. mirabilis*, *Providencia rettgeri*, *P. vulgaris, S. aureus and Serratia spp*). The same research was conducted in Sudan by (Ibtisam and Owni, 2009) confirmed the presence of these organisms with higher prevalence. This is might be due to contamination of milk from milkers hand and external part of udder. Because they mostly used tap water and river water to clean the udder of the cows. Furthermore, during milking, tying of the cows tail usually advisable but were not applicable in this study area. Tying of the tail is important in the local setting because cows carry a lot of dust or mud from the stable on their body. During milking, a lot of this dust is dislodged by the constant waving of the tail to drive way flies. This might be constitutes one of the most direct methods of milk contamination. Also the milkers use a piece of cloth to dray the udders which seem clean. So that one can be easily conclude that the microbiological quality of the milk might be problematic. Quite the opposite of (Zemelman and Longeri, 1965) the prevalence of *S. aureus* was lower however; the overall percentage was equivalent with this study. Similar results were reported by (Loken and Hoyt, 1962).

The presence of *S. dysentery* may indicate a contamination of udder or teat from milkers hand or other objects. In view of the fact that, the milking time of the cows were 6:00 AM morning, so that the milkers habitually go to the toilet and might be contaminated with these organisms. Furthermore, *S. dysentery* might be carried to the teat or udder due to unhygienic area of the cow's, bedding, surrounded by houseflies. With the intention of that it is advisable to take great care of this organism while it has very low virulent dose.

Naturally *E. coli* found in the intestinal tract of human and animal, in soil and water. These organisms are present wherever there is faecal contamination, a phenomenon that is an indicator of faecal pollution of water sources, drinking water and food (Chart, 2007). Therefore, the incidence of *E. coli* in this study might be due to the contamination of water which they used to clean utensils and udder or teat as well as their hand. Furthermore, "fecal coliform" associated with manure/environmental contamination. So that it is possible to conclude the organisms that found in this sample might be due to fecal contamination, water source and other external materials. In general, these all organisms found in teat milk belong to enterobacteriaceae are clinically important (Chart, 2007) so that the milkers and other representative should design the rule and regulation to supply quality milk.

Klebsiella species, *Enterobacter* species, *Proteus* species, *Serratia* species and *Citrobacter* species were gram negative bacteria increasingly reported even in developed country like UK in different public samples such as in water, milk and other foods and clinical isolates (Health protection agency, 2002).

The dry season of the climate might be contributed a great roll in the presence of *Serratia* and other gram negative bacteria. Concomitant conclusions were reached by (Smith *et al*. 1985). Also (Oliver *et al.* 1983) and (Smith *et al.* 1985) confirmed the presence of *Serratia* species associated with the dry period. This is not unique for *Serratia* species but also there was report for *K. pneumoniae*. Moreover, contaminated teat dip, or unpracticed teat dipping, recycled manure and teat end exposure to recycled manure bedding contributed the presence of these organisms (Smith *et al.*, 1985).

CCP2 (bucket milk at farm level)

A milk sample from bucket at farm level showed more bacteria than a teat level. This is might be due to the presence of microorganisms in the bucket formerly that was slot in the crack of the pail or the air sole of the milking environment. Additionally, might be due to the cleaning materials like water application, bactericidal treatment insufficiency, and application of a piece of clothes to filter the milk. Also milkers at the site of study regularly used cold water after each usage of bucket and utensil (Ibtisam and Owni, 2009). Also contamination of the soil, water, manure, poor silage, dirty cows and unhygienic dairy environment is another agent of milk contamination (Murphy and Boor, 2000).

Due to the fact that milk is a good nutritive medium for the growth of microorganisms, especially with poor sanitary procedures (Adesiyun, *et al.*1997; Murphy and Boor, 2000) and lack of the cooling facilities (Murphy and Boor, 2000) microorgansims multiply and grow on it easily(IDF, 1994).

CCP3 (storage containers at farm level)

At the site of milk collection the individual farmer's collects milk together in one container until the recipient collates to their processing plant. In this position the milk stay maximum of four hours until the recipient deliver it. The number and species of the organism in this level more than any of before. This might be due to the contamination of the utensil that they used to collect milk and unhygienic condition of the utensil. The site of the study usually applied the river water, well water and tap water to clean the utensils. Moreover, the milk that collected from different cows in the same site may carry organism from contaminated udder and the milkers external hygiene.

The predominant bacteria's from storage containers at milk collection center (CCP3) was *S. auerus*, *Shigella dysentery, P. aeroginosa, K. pneumoniae, E. coli* and *C. freundii* accounts, 21(28.8%), 8(11%), 5(6.8%), 5(6.8%), 5(6.8) and 4(5.5%) respectively. Few organisms' found in this level accounts less than 5% like *Edwardsiella* species. Mostly this organism associated with fresh water environments and reptiles and gut of fish and fecal origin. In healthy individual faeces may not observe but high isolation rate has been found in patients with diarrhea (Greenwood *et al.* 2007). Therefore one can easily understand that the presence of *Edwardsiella* species in milk might be due to application of fresh water or river water to clean the milking utensils and fecal contamination. Since fish processing habit was common around study area in order that, the contamination of *Edwardsiella* spp. also might be facilitated due to this commotion.

Enterobacteriaceae family includes coliform group (*Escherichia*, *Enterobacter*, *Citrobacter* and *Klebsiella*) in addition to many other genera (*Salmonella*, *Shigella*, *Morganella*, *Providencia*, *Edwardsiella*, *Proteus* and *Serratia*) which isolated from this sample also are existing in animal intestine (Collins *et al.*1995; Hays *et al.* 2001). The existence of coliform bacteria may not necessary indicate a direct faecal contamination of milk, but precisely as an indicator for poor sanitary practices during milking and further handling processes.

Moreover, the higher incidence of isolated bacteria was found to be *S. aureus* followed by *S. dysentery*. As cited in (Ibtisam and Owni, 2009) this might be due to the improper hygiene and sanitation, poor cleaning and primitive system of transportation, handling system (Hutchison, 2004).

Also 6.85% of *P. aruginosa* was isolated form storage containers at milk collection center which was equivalent in percent like (Hutchison, 2004) report. This is might be because *P. aeruginosa* are ubiquitous in agricultural environments and can be isolated from feed, soils, water supplies, bedding and the surfaces of teats and udders.

CCP4 (milk sample for transportation container)

The most frequently obtained bacteria in this level was *S. aureus* 17(21.3%), *E. coli* 10 (12.5%), *Shigella dysentery* 7(8.8%), *K. pneumonia*e 5(6.3%), *C. freundii* 5(6.3%), *C. diversus* 4(5.0%). The remaining organisms such as *P. aeruginosa, K. oxytoca, Serratia,* CONS, *Arizona* spp, *Ediwardsela*, *E. cloacae, P. vulgaris, Providenicia stuartii*, *Morganella morganii, K. rhinoscleromatis/S. Sonnei* and Gram positive bacillus were less than 5%. This is might be due to a contaminated container to transport milk for processing plant and other place (Care-Ethiopia, 2009). Additionally, a piece of clothes used to filter milk before transporting to the processing plant, restaurants might be increased with these organisms.

The organisms that isolated in this level indicates that a contamination from environment, equipment, soil, water and external part of the cow's udder. This is might be due to insufficient application of cleaning agents and clothes that contaminated by soils, airs and other environmental agents. Furthermore, during transferring of milk from the local storage to processing plant contaminated from air and milkers hands. According to FAO report poor handling and transporting system affect the quality of milk in farm level (FAO, 1993). Hence, one can easily conclude that the transportation and storage container influences the microbiological quality of milk. It is advisable to give great care to milk equipment and utensils while using at the site of farm level or at processing plant.

CCP5 (after cooling at the pasteurization plant)

The leading bacteria in immediate pasteurized milk (CCP5) was *S. aureus* 6(30.0%), CONS 4(20.0%), *Shigella dysentery* 3(15.0%) and *Serratia* 2(10.0%). The others like *C. freundii*, *K. rhinoscleromatis/S. Sonnei*, *K. oxytoca*, *K. ozane*, and Gram positive bacillus. The presence of these organisms might be indication of poor post-pasteurization and/or recontamination of the utensils. On other hand the organisms might be survived the pasteurization heat (Robinson, 2002).

However, there was no pseudomonas because gram-negative psychrotrophs generally do not survive pasteurization, thus they occur in processed milk as post-pasteurization contaminants (Robinson, 2002). Furthermore, as (Macaulay *et al.* 1963) reported that the survival of psychrophilic bacteria in pasteurized milk appeared to depend on the initial number of organisms and partly on the length of time of storage at 3 to 5^{o}c. According to the report of the National study on the microbiological quality of and heat processing of cow's milk performed in UK before and after heat processing suggested that the bacteria being able to survive pasteurization (UK report, March 2003). For instance the presence of *S. aureus* indicates the survival of pasteurization temperature. Supplementary report was generated by (Walker and Harmon, 1966).

CCP6 (milk from different shelf life)

The commonly found organisms from different shop at different shelf life were *P. vulgaris* 9(23.7%), *P. aeruginosa* 6(15.8%), *K. pneumoniae* 6(15.8%), *K. oxytoca* 6(15.8%), *E. coli* 5(13.2%), *C. diversus* 2(5.3%), *C. freundii* 1(2.6%) and *Providencia alkalifaciens* 1(2.6%). Some of the undetermined bacteria were also found in this pasteurized shelf life bacteria (Table 2). In this level the organisms obtained was higher than that of fresh pasteurized milk. This is might be due to either post-pasteurization contamination or ineffective pasteurization. Moreover, the presence of Pseudomonas species is a main cause of reduced shelf-life due to post-pasteurization contamination (Robinson, 2002). Also (Esther *et al.* 2004) reported the same conclusion that the post-process contamination compromised the quality and shelf life of pasteurized milk.

Colony count from pasteurized milk

In this study 32 pasteurized milk samples was collected from different shelf life in commercial sites and examined for total viable count and total coliform count. Total viable count was found to be in the range of 0.63-9.24x10^8CFU/ml. The total coliforms were detected in this entire sample to be in the range of 1.01x10^8 - 1.51x10^8 CFU/ml. The mean total viable count and total coliform was 3.65x10^8 and 2.39x 10^8. This is also too high in number compared with USA standards which was 1.89x10^5 per ml and 14 per ml for total viable count and coliform bacteria respectively. Even though, in some area of the USA attain more than 10^6per ml that is very high for pasteurized milk as they concluded. As according to USA study the lowest value of the result was very far from our study result. No results of 32 samples were obtained lower than the lowest value of the result in USA.

Research conducted in Iran indicated that the pasteurized milk was 71x10^4 CFU/ml for total viable count and 800CFU/ml total coliform count. Compared to (Shojaei and Yadollahi, 2008) still present study was poorest of any others. This is might be due to high contamination of milk and poor hygienic condition in farm level and even the processing plant. Also the milk handling technique in this study area was poor so that the colony count was high. In this study there was no milk samples stayed more than five days in refrigerator. The maximum and minimum duration of milk was 5 and 2 days respectively were collected from different shops. But the number of bacteria count from this sample was very higher than even the sample examined from seven day refrigerated milk in USA. One can easily conclude that the refrigeration variation have their own effect in the bacterial count that is why this result were too high. This might be also due to post pasteurization contamination and the raw milk situation. Moreover, the pasteurization situation cleaning and handling process might not be standardized.

Colony count from raw milk

The total viable count and total coliform count of raw milk was found to be in the range of $1.78x10^6$-$21.77x10^8$ CFU/ml and $2.15x10^7$-$15.25x10^7$ respectively. The mean total viable count and total coliform count was $6.57x10^8$ and $4.64x10^7$CFU/ml respectively. The research conducted in Prince Edward Island dairy herd at 2005-2007, the average coliform count of the raw milk was 21CFU/ml, which is extremely higher than the report of (Elmoslemany *et al*. 2009). This is might be the milkers used unclean water for rinsing of the utensils and other milk equipment. Furthermore the bedding condition of the cow's and the teat cleaning condition was also poor. The application of river water and well water also contributed this result.

The overall mean total bacterial count of cows' milk produced in the study area was $6.57x10^8$cfu/ml. The total bacterial count obtained in this study is generally the same as the result of (Tassew and Seifu 2010) and higher than the acceptable level of 1×10^5 bacteria per ml of raw milk (O'Connor 1994). Beyene (1994) reported that the minimum and maximum total bacterial count of raw cows' milk produced in southern region to be 10^6 to 10^8cfu/ml. Similarly, (Tola, 2002) reported total bacterial count of cows' milk produced in Bila Sayo and Guto Wayu districts of eastern Wollega to be $7.4x\ 10^7$and 2.0×10^7 cfu/ml, respectively. In general, lack of knowledge about clean milk production and use of unclean milking equipment would be some of the factors contributed to the poor hygienic quality of milk produced in the study area.

The overall mean coliform count of milk produced in the area was $4.64x10^7$cfu/ml. The coliform count obtained in the current study is greater than that reported by (Tassew and Seifu 2010) who found $3.0x10^4$ cfu/ml. (Beyen, 1994) also reported a coliform counts of $6.3x10^3$, $1x10^4$ and $6.3x10^3$cfu/ml for cows' milk produced in Aneno, Gulgula and Dongora districts of southern region, respectively. On the other hand, (Yilma and Faye 2006) found higher coliform count of $3.7x10^6$cfu/ml for cows' milk collected from different producers in the central highland of Ethiopia. Study conducted in Iran indicated that the raw milk total coliform count was

1300cfu/ml and total viable count was $13x10^6$cfu/ml. Still present study outcome was poorest of any others (Shojaei and Yadollahi, 2008). The higher coliform count observed in this study may be due to the initial contamination of the milk samples either from the cows, the milkers, milk containers and the milking environment. Also (Isabel *et al*, 2009) suggested that this may be due to incorrect handling of milk and dairy products and inadequate cleaning and sensitization of milk container. Thus, extension services and training of farmers in improved milk handling practices are required to improve the raw milk quality in the study area.

Colony count from pasteurized milk after cooling at the pasteurization plant

In this study 32 immediate (fresh) pasteurized milk sample was examined for total viable count and total coliform count. The minimum and maximum cell count in total viable and total coliform count was $1x10^4$- $8x10^5$ and $6x10^2$-$10x10^2$ CFU/ml. The mean total viable count and total coliform count as $7x10^5$ and $10x10^2$ CFU/ml, respectively (Table 3). In USA the total viable count from immediate pasteurized milk was $2x10^4$CFU/ml for 100% sample with a maximum coliform count of zero CFU/ml. Furthermore, all total viable count was less than 10^4CFU/ml and there were no coliform bacteria detected on milk (Dahlberg *et al.* 1953). Comparing of USAs study the result of current study were most horrible than any of the result reported in USA cities. This is might be due to the milk supplies of pasteurized were re-contaminated with coliform to a very slight extent. Furthermore, one would easily understand that the utensils and other equipment might be not handled carefully. Also the working situation might not assurance not to be contaminated. Additionally, the effectiveness of the pasteurization technique, temperature applying and time holding also might be doubtful.

In this study Penicillin 10µg, Ampicillin 10µg, Amoxicillin-Clavulanic acid 30µg, Ceftriaxone 30µg, Ciprofloxacin 5µg, Chloramphenicol 30µg, Gentamicin 10µg, Oxacillin 1µg, Vancomicin 30µg, TMP-SMZ 25µg and Erytheromycin 15µg were used.

The susceptibility of 78 *S. aureus* organisms was examined for Ampcillin, Penicillin, Gentamicin, Amoxicillin-Clavulanic acid and Vancomycin and oxacillin. Out of which 38.5% were resistance to Vancomycin , 3.8% were resistance to Gentamycin, 30.8% were resistance to Amoxicillin-Clavulanic acid and 67.9% were resistance to Penicillin and 67.9% were resistance to Ampiciline. In others works 30% of *S. aureus* isolates were resistant to Penicillin (DANMAP, 2002). In others report *S. aureus* isolates were 94.4% resistance to Penicillin, 58.3% resistance to

TMP-SMZ. Also (Mekonnen *et al.*, 2005) and (Corrales *et al,* 1995) reported the resistance pattern of *S. aureus* was 75 and 83% to Ampicillin respectively. In this result Penicillin resistance *S. aureus* were high in the community that is why Denmark report on the trends of *S. aureus* resistance to Penicillin was increasing. This is might be due to the consumption of the resistance strain pattern of the *S. aureus* increased from time to time, however, it is not the solely explanation that why the resistance pattern were high. Furthermore, the resistance of *S. aureus* to Penicillin and Ampicillin may be attributed to the production of beta-lactamase, an enzyme that inactivates Penicillin and closely related antibiotics. It is believed that around 50% of mastitis causing *S. aureus* strains produce beta-lactamase (Green and Bradely, 2004).

For the past 20 years, methicillin-resistant *S. aureus* (MRSA) have represented less than 1% of *S. aureus* blood isolates and more than half of these MRSA strains have been acquired outside Denmark which was conducted Denmark. In contrast to this study during this era no report were generated to the Vancomycin resistance strain of *S. aureus* (DANMAP, 2002). However, worseness of Vancomycin resistance *S. aureus* was reported by (Sheen, 2010) was 5%. As of April 2006, 2007, 2008 and 2009, report CDC has confirmed six, seven, ten and nine VRSAs respectively in the U.S. Also 16 and 17 VISA was reported in 2006 and 2007 in USA. The reason that the VRSAs increment suggested as (Willems *et al*, 2007) was the vancomycin-resistance gene (vanA) has crossed genus boundaries to methicillin-resistant *S. aureus*.

The present study has demonstrated the existence of alarming levels of resistance of *S. aureus* to commonly used antibiotics in the study farms and the results are in accordance with reports from earlier studies in other countries (Edward *et al.*, 2002; Gentilini, 2000) suggesting a possible development of resistance from prolonged and indiscriminate usage of some antimicrobials. It is therefore, very important to implement a systemic application of an *in-vitro* antibiotic susceptibility test prior to the use of antibiotics in both treatment and prevention of infections.

According to 24th edition of Medical microbiology (Brooks *et al.*2007) the 1st choices of the drug for the treatment of *S. aureus* were Vancomycin and/or Gentamycin, Penicillin and other drugs. Moreover, *S. aureus* also develop resistance to Ceftriaxone 23.1%, CAF 28.2%, Erythromycin 32.1%, Oxacillin 60.3% and TMP-SMZ 7.7%. However there was no resistance strain to Ciprofloxacin antibiotics. Intermediate resistance development of this organism was seen for

Ciprofloxacin, CAF, Erytheromycin, Ceftriaxone and TMP-SMZ about 16.7%, 20.5%, 3.8%, 42.3% and 19.2% respectively.

From 31 isolates of *E. coli* high resistance were observed for Penicillin 100%, Ampicillin 93.5%, Erythromycin 67.7% and CAF 42%. The same study conducted in Peru and Bolivia (CDC, 2006) confirmed this result. Lower resistance was seen for Amoxicillin-Clavulanic acid 19.4%, Ceftriaxone 19.5%, TMP-SMZ 16.10% and Gentamycin 6.5%. In contrast to CDC report TMP-SMZ showed lower resistance for *E. coli*. This may be due to the sample variation in which our sample was not clinical sample. The same conclusion were done by (Scott, 1981) saying *E. coli* shown alarming resistance to many commonly used antibiotics, so infections caused by these bacteria are very difficult to treat.

Disparity of the result was seen in CDC report, that there were no resistance strains of *E. coli* for Ciprofloxacin antibiotics. However, 35.50%, 3.20%, 25.80%, 19.30%, 19.40% of *E. coli* developed intermediate resistance to Amoxicillin-Clavulanic acid, Ciprofloxacin, Erythromycin, Ceftriaxone and TMP-SMZ respectively. *E. coli* is sensitive to 96.80%, 93.50%, 64.50%, 61.30%, 45.10%, and 6.50% for Ciprofloxacin, Gentamycin, TMP-SMZ, Ceftriaxone, Amoxicillin-Clavulanic acid, Ampicillin and Erythromycin respectively. This is because *E. coli* that isolated from milk may acquire resistance gene from the environment. In this study *E. coli* is more resistance organism compared with other organisms to given antibiotics next to *S. aureus.*

Out of 17 isolate of *K. pneumoniae* from milk sample 17(100%) of them are resistance to Penicillin 16(94.1%) to Ampicillin, 4(82.4%) to CAF, 11(64.7%) for Erythromycin. For the remaining antibiotics like Amoxicillin-Clavulanic acid, Ciprofloxacin, and Ceftriaxone *K. pneumonia*e develop resistance for less than 30% of the isolate. However, there was no *K. pneumonia*e strain developed resistance pattern to Gentamycin with in 17 isolates. Among this isolates there was no *K. pneumonia*e species sensitive to CAF and Erythromycin. It is sensitive to Gentamycine, Ciprofloxacin and Amoxicillin-Clavulanic acid for 76-88%. Similarly as reported by (Kumar and Talwar, 2010) the resistant group of *K. pneumonia*e for Ciprofloxacin was only 2.2% and TMP-SMZ was 33.3%. More than 41.2% of the *K. pneumonia*e was resistant to TMP-SMZ higher than the report of Kumar and Talwar. Again the results were produced by this researcher for Ampicllin and penicillin. This is might be due to the resistant pattern consumption of the organism was increasing periodically. Moreover, application of some drugs to animals for meat and milk production might be influenced the microbial physiology. Also this might be indication of a tendency of organisms to develop resistance pattern gradually from time to time depending on geographical location. In this study Ampicillin, Penicillin and CAF were less effective for treatment. Especially *Klebsiella pneumoniae* is naturally resistant to Ampicillin and Amoxicillin, usually by the production of SHV-1 beta-lactamase encoded on the chromosome or a transferable plasmid (Nugent and Hedges; 1979).

The resistance profiles of *P. aeruginosa* in this study were compiled as 14(100%) to Penicillin. In addition *P. aeruginosa* developed resistance to Ampicillin, ceftriaxone and trimethoprim-sulfamethoxazole by 78.6%, 64.3% and 71.4% respectively. Few species (7.1%) were developing resistance to ciprofloxacin. Meanwhile *P. aeruginosa* was susceptible for Quinolones such as ciprofloxacin and Amynoglycosides such as Gentamycin 92.9% and 100%, respectively. This is might be due to the organisms developed the resistant gen for common antibiotics or the consumption of the resistant organism was acquired from the environment.

Ciprofloxacin, Ampicillin, trimethoprim-sulfamethoxazole, Ceftriaxone are most commonly inhibitory for *Shigella* isolates and can suppress acute clinical attacks of dysentery and shorten the duration of symptoms. Therefore, these and another antibiotic like Penicillin, Amoxicillin-Clavulanic acid, and Gentamicin were examined for its resistance level. Twenty seven isolate of *S. dysentery* 66.7%, 37%, and 92.6% were resistance to Ampicillin, trimethoprim-sulfamethoxazole and Penicillin respectively. Compared with other antibiotics Genatmicin, Ciprofloxacin, Amoxicillin-Clavulanic acid, and trimethoprim-sulfamethoxazole were sensitive for *S. dysentery* by 100%, 77.8%, 77.8% and 63.0% respectively. However, for trimethoprim-sulfamethoxazole, Gentamicin, and Ciprofloxacin other *Shigella* species identified from milk samples were 100% susceptible and for Amoxicillin-Clavulanic acid and ceftriaxone 50% and 75% susceptible. But 100% of these species were resistance to Ampicillin.

Ceftriaxone, trimethoprim-sulfamethoxazole, Ciprofloxacin, Gentamicin, Ampicillin and Penicillin were examined for the resistance level of *Serratia* species. Opposing Bowman *et al.* and Damme, there were no *Serratia* species developed resistance patterns for Gentamicin. According to (Harrison's, 2005) report a high proportion of *Serratia* strains greater than 80% are resistant to Ampicillin and the first-generation Cephalosporins as confirmed in laboratory result. This is might be due to resistance gene or enzyme called lactamases may be preexistent or may develop during therapy. More than 88% of these species were developed resistance to Ampicillin and penicillin. Gentamicin was 100% effective for this species; in addition ciprofloxacin, ceftriaxone and trimethoprim-sulfamethoxazole were effective for 88.9% species.

Ampcillin, trimethoprim-sulfamethoxazole, Gentamicin, ceftriaxone, ciprofloxacin and Amoxicillin-Clavulanic acid were examined for resistant level of *P. vulgaris, P. mirablis, Providencia rettgeri, Providencia alcalifaciens, Morganella morganii* and *Edwardsiella* species. All of them are resistance to Ampicillin 100% except for *P. vulgaris* which was 50% resistance.

C. diversus, C. freundii, E. cloacae, E. aerognese/Hafnia and *Arizona* species were tested for antibiotic resistance level of penicillin, Ampicillin, Amoxicillin-Clavulanic acid, ciprofloxacin, CAF, Gentamicin, ceftriaxone and trimethoprim-sulfamethoxazole. All of these microorganisms were resistance to Penicillin and Ampicillin 100%. But some strains of *C. freundii* and *Arizona* species were resistance about 60% and 87.5% for Ampicillin respectively. Moreover, more than 50% of these organisms were also resistance to Amoxicillin-Clavulanic acid. There were no resistance patterns of these organisms for ciprofloxacin except less than 20% of these strains. CAF was less effective against these organisms because it showed more than 75% resistance. According to this result Gentamicin and Ceftriaxone were good antibiotics to treat these organisms because they showed 100% susceptible. Trimethoprim-sulfamethoxazole were 100% effective for *C. freundii, E. cloacae, E. aerognese/Hafnia* however, *C. diversus* and *Arizona* species were not.

K. oxytoca were 100% resistance to Ampicillin and Penicillin more than 90% susceptible to ciprofloxacin, Gentamicin ceftriaxon and trimethoprim-sulfamethoxazole. However, few species less than 10% develop intermediate resistance pattern.

Chapter VII

Conclusion and Recommendation

The milk obtained in this town had no healthful milk supply of good sanitary quality. The health condition of the cows, mastitis control programs, Brucellosis and other public health aspect were not established at the milk production areas of the town. As a further means of preventing the contamination of milk with pathogenic bacteria such items as a safe water supply, sanitary disposal, of human excreta, health of personnel and fly control are of paramount significance from the public health viewpoint.

In this study area the cleanness of and effective bactericidal treatment given, utensils, and milking condition on farms in their producing areas had high bacteria counts in both raw and pasteurized milk. Furthermore, the clean barns, cows and milk houses were found generally on the farms unclean dairy utensils; hence it is concluded that these items should be required by town ordinance and state laws.

Milk processing plants should be inspected and samples of pasteurized milk should be tested for total bacterial count, coliform count. The immediate or fresh pasteurized milk of all samples had low average bacterial count per ml. The coliform count was 100 to 800 per ml. But the total viable bacterial count was 10,000-100,000 per ml. This study shows that milk produced on farm under most extensive was very poor. High bacteria counts were associated with bad sanitary practices and un-proper cooling of milk on the farm. This was true for the total numbers of bacteria and also for the numbers of coliform bacteria. The quality of the study area of this research was not good generally. Therefore, the raw milk never be given to babies, pregnant women on the farm, any of who suffering from chronic disease, immune suppressed patient because of large number of bacteria count was observed in this study.

Many organisms were developed resistant to some antibiotics because addition of some antibiotics into feed additive as growth promoters may enhance a large pool of resistant organisms and resistant genes, which may contribute to the development and transmission of antibiotic resistant bacteria. Chemiccal preservative which added to preserve milk might be induced the resistance of microorganisms.

The most contamination of the milk in this study was due to environmental hygiene. Their nutritional versatility means they are well-equipped to survive in a diverse range of niches such as contaminated water supplies, deteriorating milk hoses. The presence of *E. coli* (and other coliforms) in milk is usually associated with faecal contamination. However, the use of these organisms as faecal indicators is confounded because in milk and milk residues at ambient temperatures these bacterial can multiply quickly. Therefore, the levels of these organisms are likely to be related to farm hygiene conditions, the condition and effectiveness of cleaning of milking equipment, surfaces and lines and also the temperature that the milk has been held at.

Also the present study showed that a high values of total bacterial count (TBC) and coliform count (CC) percentage in the milk. Hence, adequate sanitary measures should be taken at stage of production to consumption such as proper handling of cows, personnel hygiene, use of hygienic milking and processing equipment, improving milk and milk handling environment among others. The poor bacteriological quality observed in the present study requires further investigation of the status of the animals' health, especially mastitis and the significance of the effect of containers to ascertain their contribution on microbial quality. Moreover, the public health aspect of the milk should be tested at the environment. This activity therefore needs the collaboration of health workers, agricultural officers, veterinary medicine workers, water and energy sources, electric service providers and research institutes to investigate the further problems. Moreover, the rule and regulation setup is very crucial to minimize the risk range due to milk and milk related problem. It is better to use antibiotics which are effective for treatment of the specific organisms. According to this study the effective drugs for organism found in this study were Vancomycin, Ciprofloxacillin, Gentamycin, Ceftriaxone and Amoxicillin-Clavulanic acid.

Recommendation

The government, health bureau, agricultural office and other direct and indirect related individuals should give emphasis to the quality of milk.

- Data clearly indicate that milk hygienic quality, under current is affected by milking and handling practices. Therefore, the milk quality handling system training should develop around the study area for individual farm level. Also the knowledge, attitude and practice activity is very important so that the concerned body needed to give emphasis on it.
- The processing plant should inspected frequently by relevant individuals to maintain the quality of milk. Moreover, the rule and regulation for milk quality based on the situation should establish.
- All together, these results show that urgent measures are needed to ensure safe milk at farm level, by the promotion of good hygiene practices. These would preferably focus on efficient cleaning of vessels, hands, udder and the whole building facilities with appropriate detergents and water. These measures should be applied massively because this criterion remains very poor in the majority of dairy systems.
- Bacteria found in manure, soil, and water may enter from this source. Such contamination can be reduced by clipping the cow, and washing the udder with water or a germicidal solution before milking, paving and draining barnyards, keeping cows from stagnant pools, and cleaning manure from the barns or milking parlors.
- Pasteurization kills pathogens that may enter the milk and improve the keeping quality of milk so that effective pasteurization mechanism under regulatory supervision is important.
- Bacteriological quality analysis is very important at the site of farm level and processing plant.
- Also in this study some organisms were not addressed so that standardized set up and those organisms like M. bovis and brucellosis should perform to minimize the risk factors related with milk contamination.

Limitation

- During this study there was a shortage of reagents and biochemical's and differential media to determine specific microorganism.
- Sero typing was not possible during this study
- Cooperation of the farm level and processing plant organization was also difficult issue

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Annex I

Questionnaire

The purpose of this questioner is to check the quality of cow milk

Dear;

This questioner is for the purpose of milk quality assessments, therefore I would like to acknowledge you that your good cooperation by providing true information.

1. **Description of the farm**

   Name ______________________________

   Kebele _____________________________

2. **Herd size and composition**

   Species __________________ Age _____________________Herd size _____________

3. **Barn hygiene**

   Barn cleaning frequency per day _________ bedding condition ______________

4. **Pre- milking udder preparation and hygiene**

   A. Are flanks, udder or teats washed before milking? Yes ___________ No _________

   If yes, udder or teat cleaning material

   - Cold tap water ☐
   - Warm tap water ☐
   - Lake Water ☐
   - Warm well water ☐
   - Warm River ☐
   - Cold river water ☐
   - Cold well water ☐
   - if any specify_________

   B. According to question #4 if any of your choice of water type which agent do you use?

   - Detergent
   - Disinfectant
   - Combination of the listed options
   - Other (specify) ____

C. How are teats or udder washed and dried? ______________________________

D. Do you use teat dips? Yes__________ No _________

If yes, when?

- Pre-milking
- Post-milking
- Both before and after milking ______________with what? _______________

5. Milk utensils

A. Do you use bactericidal treatment? Yes _____________ No __________

B. How often is it cleaned? ____

- After each usage using cold water ☐
- After each usage using warm water ☐
- Others (specify) ____

C.Do you use traditional favorants to clean the transport equipment?

If yes, what? ___________________

6. Milking technique, milk cooling and transport

A. Milking procedure used:

- Hand ☐
- Machine ☐
- Both ☐

B. Milking frequency per day:

- Once ☐
- Twice ☐
- three or more times ☐

C.Do milker's wash hands before milking? Yes __________No ____________

- If yes, hand cleaning mechanisms:
  - Tap water ☐
  - River water ☐
  - Well water ☐
  - Detergent ☐
  - A combination one of more of the options ☐
  - Other (specify) _____________________________________

D. Have you washed hands between cows? Yes ___ No ______

E. Have you drying your hands: Yes ___ No ______

F. Average number of cows milked by an individual milker_________________________

G. Milker's clothing

- o Clean outer garment ☐
- o Own cloth ☐
- o others (specify) ☐

Interviewer observation ______________________

H. Do you use cooling system for milk? Yes____ No____

If yes how?

a. refrigerator
b. At room temperature
c. Traditional cooling system
d. If others ____________________________________

I. For what purpose do you use milk?

- o To household consumption ☐
- o To processing plants ☐
- o To local consumers ☐
- o To restaurant ☐
- o Others (specify) ___________________________

J. If your answer on question alternative L is "to processing plant" how often?

- o Every day ☐
- o Every other day ☐
- o Other (specify) __________________________

K. Type of storage container used to transport milk to the processing plant

- o Aluminum cans ☐
- o Plastic bags ☐
- o Tanks ☐
- o Pot/jar ☐
- o Others (specify) ________________________

L. Do the workers at collecting centers test the quality of milk before adding to the pool?

Yes ____ No_______

7. Public health aspects

a. Are there tests for tuberculosis? If yes, frequency of the tests ______________________

b. Are there tests for brucellosis? If yes, frequency of the tests _______________________

c. Are there screening tests for mastitis? If yes, frequency of the tests __________________

d. Habit of raw milk consumption: Yes _______ No_______

e. Have you seen any illness/symptoms observed in milkers, family members etc?

yes____ No____

If yes, mention? ____

____

8. Mention the proposed solution for aforementioned problems?

____

____

____

9. Laboratory results

Bacterial count **Sample #----------------------**

Sample #	Dilution method	# of colony	Remark
	1: 10^1		
	1:10^2		
	1:10^3		
	1:10^4		
	1:10^5		
	1: 10^6		
	1:10^7		
	1:10^8		

Sample Number-------------------------

Sample Address--

Sample level -------------------

CCP1	CCP2	CCP3	CCP4	CCP5	CCP6	Remark

<u>Identified Organism</u>

1. --------------------------------------- 2. -------------------------------------3. ---------------------

Drug sensitivity test for---

Sample Number---------

Drug		**Zone of Inhibition (mm)**	**Result**			**Remark**
S. No	**Name**		**S**	**R**	**I**	
1.	Pen					
2.	Amp					
3.	Amox					
4.	CIP					
5	CAF					
6.	Ery					
7.	Gen					
8.	CRO					
9.	TMP-SMZ					
10.	Oxa					
11.	Van					

Annex II

Consent

Informed consent statement (English version)

My name is DERESSE DAKA. I am a Medical Microbiology Masters student. I am taking my study in Addis Ababa University. I am going to study ‘’bacteriological analysis of cow milk in Hawassa town, SNNPRS Ethiopia’’. I am inviting you to participate in this study. The findings of the study will help health professionals to make aware of the risks while stopping bacterial contamination. Also the finding of this study is expected to have important role in reducing the morbidity rate and other factors due to milk contamination. You are selected to participate in this study just by chance. Similarly, other study participants are also selected by chance. It is your full right not to be providing from the outset or to withdraw in the middle in the taking sample. This study will avoid providing information such as your and other study participants name, house number, Telephone number and Kebele for others. It is only I who what I take the sample only for this moment.

After that, the sample collecting will be mixed previously collected and impossible even myself to identify one from the other interims of specific source. More important one, confidentiality and anonymity of the sample collected from different study participants is going to be assured by analyzing and disseminating the research findings in aggregate. If you are not happy to be participating, you are welcome; and you will not loss any of social and medical services you deserve. If you agree, the sample will be collected as my procedure without affecting your privacy and marketing. You will be asked questions focusing on your opinion regarding *milk and milk processing in your home*. You are free to ask questions any time and your questions are respected. The findings of this research was disseminated in aggregate during medical conferences, published in international medical journals and may be publicized using mass media. The purpose of disseminating of the study findings is primarily to get the attention of policy makers to design evidence based strategy how to curtail bacteriological quality of the milk and milk products. You will not be provided any incentive for participating in this study.

Risks and discomforts are considered; that participants may feel uncomfortable or experience some emotional stress from being taken a samples, some of the questions; and that someone may accidentally learn of their responses to the questions or discussion, primarily as a result of a person from a focus group discussion telling others what was said. In these cases, participants will be free to refuse from answering any questions or stop the sample collection at any time.

Participants are expected to benefit from the interview by learning about bacteriological quality of milk, its handling methods, and the use. Results of this study will also be used to better explain the positive and negative aspects of milk quality, testing of bacteriological effects. What is learned from the study will be used to help others in the community and as a base for a large-scale investigation.

Fully informed consent will be taken from all participants with each method of data collection. The consent form will be written in Amharic, and read to those persons who are illiterate or passed for them to read for those who can read. Participants will be informed on how to contact the study staff, should any questions or concerns come up at a later time. Informed consents will be obtained privately and individually for each participant prior to the interview or sample collection.

Certificate of consent (English version)

I understand that the purpose of this study is to know ''bacteriological analysis of and cow milk'' in Hawassa town, SNNPRS Ethiopia. I understand that I will be given a sample for this study and as much as possible I consent voluntarily to participate in this study and to be interviewed. I have read the preceding information, or it has been read to me. I have been given a chance to ask questions and all my questions are answered to my satisfaction. I am aware that I have the right to withdraw from the giving the sample at any step without affecting my social wellbeing or medical care I deserve. I agreed to participate in this study. It is because the finding of this research will be disseminated to policy makers and medical professionals that will be useful to design a strategy on controlling bacteriological quality of milk. I know that there will be not any incentive for being given the sample once for few minutes.

Signed :________________________(not named)

Date :__________________________(E.C)

Annex III

Flow chart for isolation and identification of bacteria from milk sample

Milk sample

↓

Inoculation to **BHI** → or Nutrient agar

↓

Incubation at 37^0C for 24 hours

↓

Inoculation of blood agar

↓

Incubation at 37^0C for 24 hours

↓

Observation of colony characteristics and undertaking Gram's staining

↓

Sub Culturing on blood agar and MacConkey agar

↓

Incubation 37^0C for 24 hours

↓

Primary identification

- Growth on MacConkey agar
- Catalase test
- Oxidase test
- O-F test
- Motility test

↓

Secondary biochemical tests

- Carbohydrate fermentation
- Citrate Utilization
- Urease activity test

↓

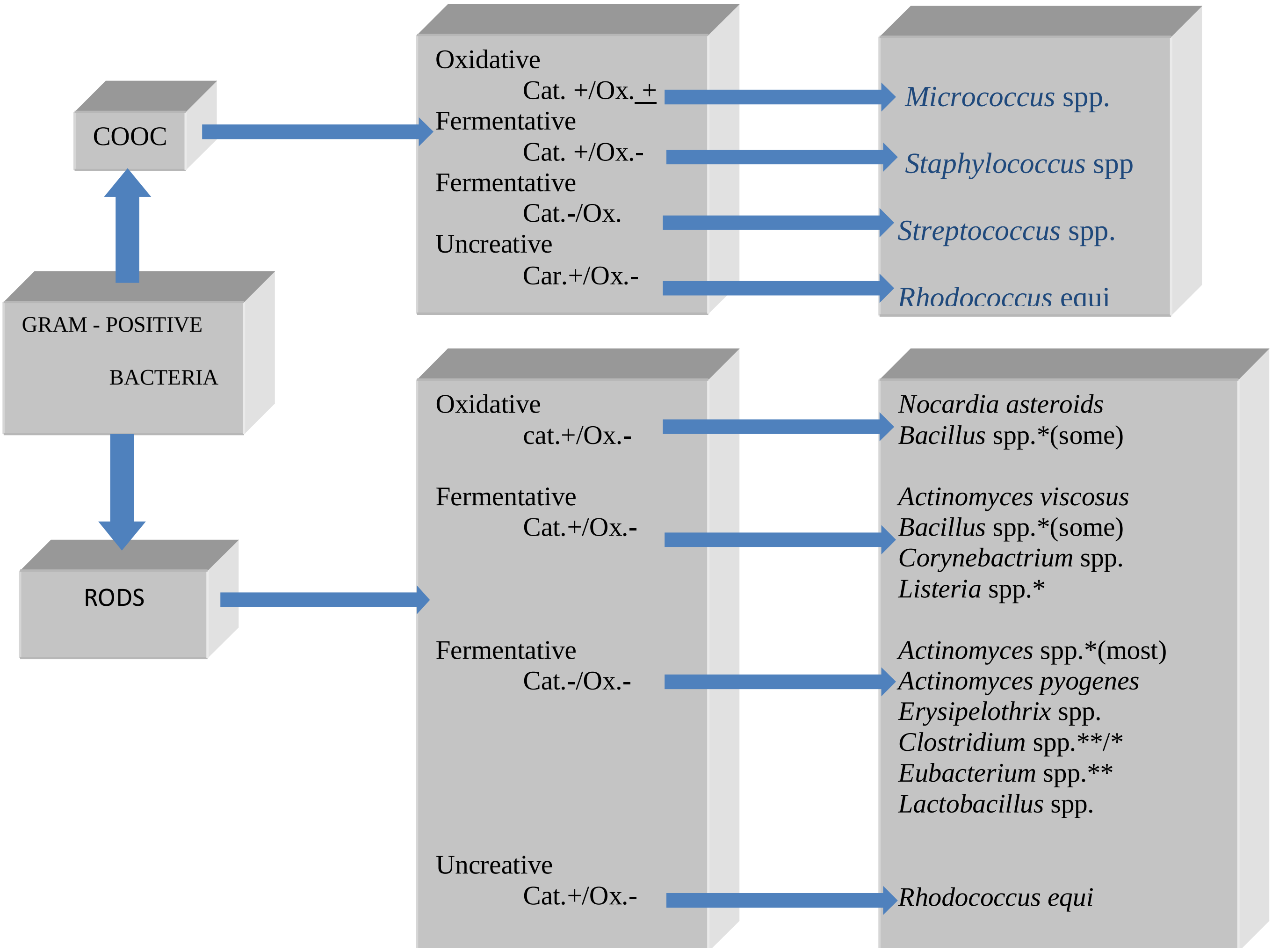
Serotyping

↓

Antimicrobial susceptibility tests

Annex IV

Primary identification of Gram-positive bacteria

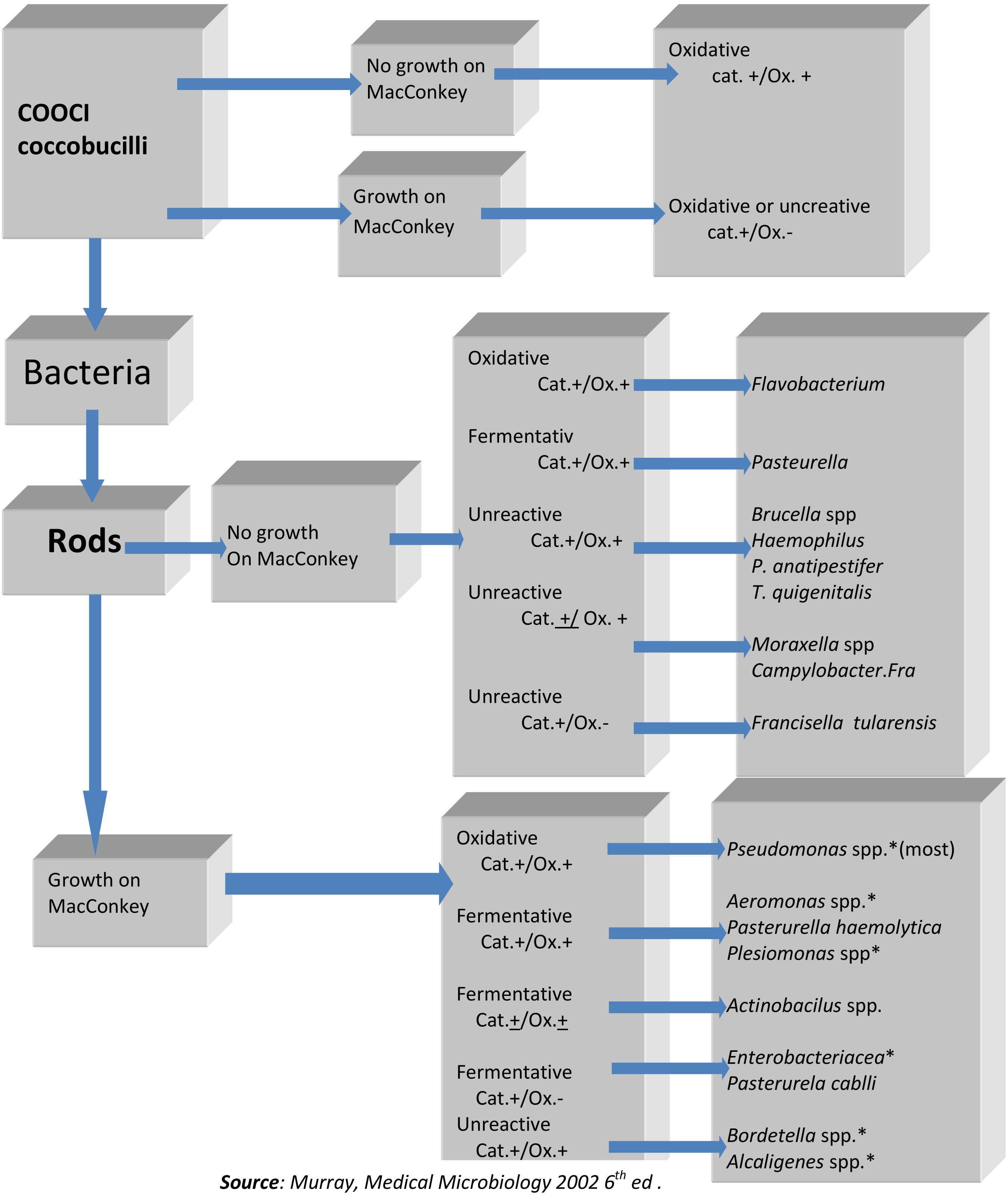


(Cat. = Catalase; Ox. = oxidase; += positive reaction; -=negative reaction; ± = variable; *=motile;**=anaerobic)

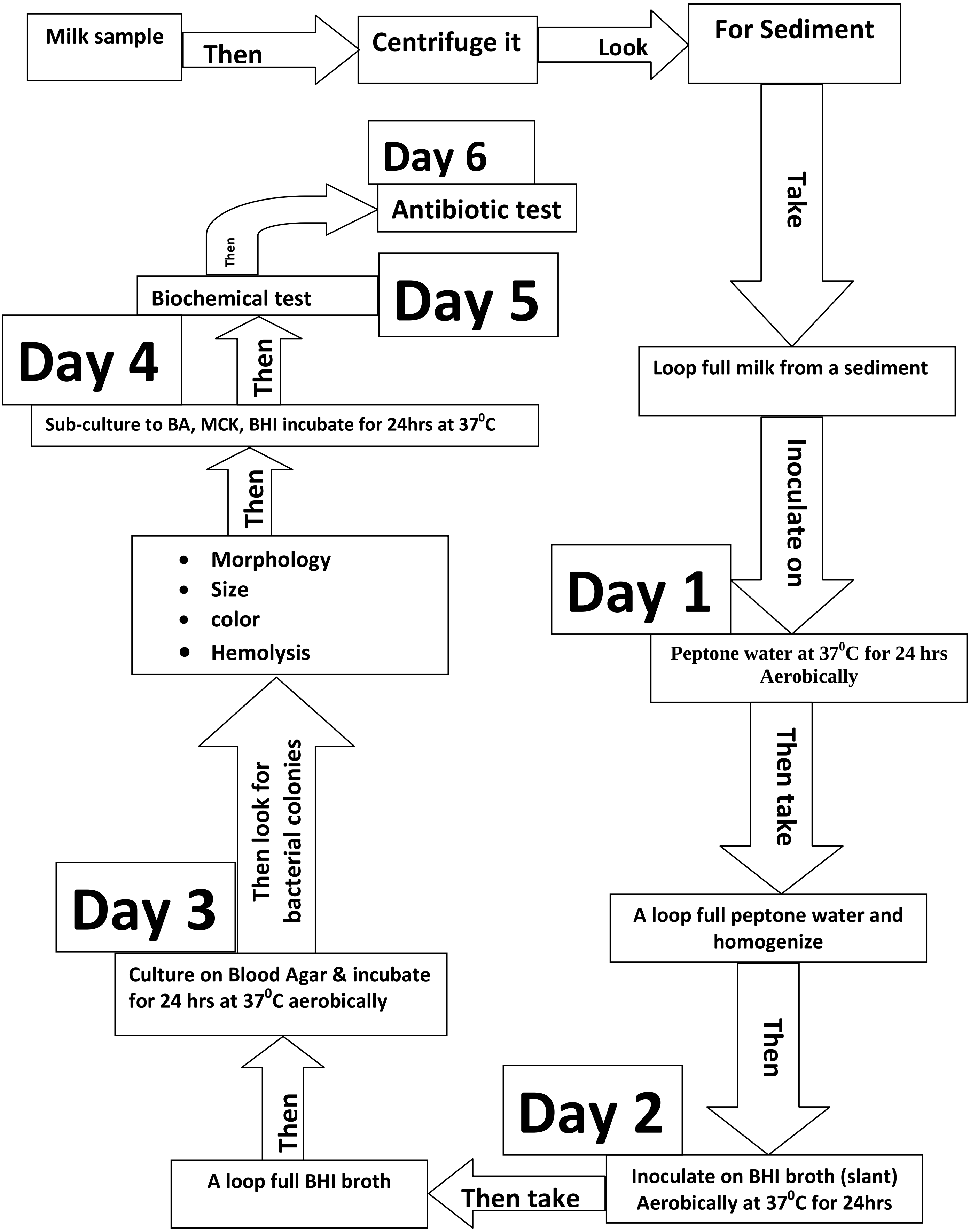
***Source**: Murray, Medical Microbiology 2002 6th ed .*

Annex IV

Primary identification of Gram - Negative bacteria



Source: *Murray, Medical Microbiology 2002 6th ed .*


Milk sample
Then
Centrifuge it
Look
For Sediment
Take
Loop full milk from a sediment
Inoculate on
Day 1
Peptone water at 37⁰C for 24 hrs Aerobically
Then take
A loop full peptone water and homogenize
Then
Day 2
Inoculate on BHI broth (slant) Aerobically at 37⁰C for 24hrs
Then take
A loop full BHI broth
Then
Day 3
Culture on Blood Agar & incubate for 24 hrs at 37⁰C aerobically
Then look for bacterial colonies
Morphology
Size
color
Hemolysis
Then
Sub-culture to BA, MCK, BHI incubate for 24hrs at 37⁰C
Day 4
Then
Biochemical test
Day 5
Then
Day 6
Antibiotic test

Annex V

Assurance

The undersigned agree to accept responsibility the scientific ethical and technical conduct of the research project and for the provision of required reports as per terms and condition of the research publications office in the time of grant is forwarded as the result of this publication.

Title of the study: Bacteriological analysis of cow milk in Hawassa town, SNNPRS Ethiopia

Principal investigator:

Name: Deresse Daka

Address: Hawassa University P. O. box 1560

Date ___________________________

Signature _______________________

Co-investigator:

Name: Dr. Solomon G/Selassie

Address: Addis Ababa University FOM

Date ___________________________

Signature _______________________

Name: Dr. Kassaye Aragaw

Address: Hawassa University Faculty of Veterinary Medicine

Date ___________________________

Signature _______________________

Name: Dawit Yihedego

Address: Hawassa University faculty of Medicine

Date ___________________________

Signature _______________________