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Bacteriological Study on Rawmilk Collected from Hawassa Smallholder Dairy Farms

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Abstract: A study conducted to determine bacterial load and isolation and identification of bacteria in raw milk samples from November 2011 to April 2012 collected from small holder dairy farms in Hawassa town, Ethiopia. A total of 89 raw milk pooled samples were used for analysis. Total Aerobic Plate Count (TAPC) used to determine level of contamination and selective media and biochemical tests conducted to isolate and identify bacteria in milk. The mean TAPC of raw milk samples were $4x10^5$, $6x10^5$, $2.2x10^6$CFU/ml from metal can, plastic bucket and jerry can. The total aerobic bacterial count of the samples from jerry can was significantly higher than the samples from metal can and plastic bucket ($P<0.05$). The milk samples collected from owners that were using borehole water for cleaning teats and equipment's had significantly higher total aerobic plate count than those were using pipe water ($P<0.05$). The proportion of 63.6, 81.5 and 92.3% of the samples were from metal can, plastic bucket and jerry can, respectively; had a total aerobic plate count above acceptable limit. On the other hand, a proportion of 83.6 and 59.6% of the raw milk samples of borehole and pipe water user have had a total aerobic plate count above the acceptable limit of $1x10^5$CFU/ml, respectively. In this study 159 bacterial isolates belongs to nine genera were identified and these include *Staphylococcus* species (35.2%), *Streptococcus* species (15.7%), *Bacillus* species (15.1%), *Enterococcus* species (10.1%), *Escherichia coli* (8.8%), *Klebsiella pneumoniae* (5%), *Corynebacterium* species (4.4%), *Enterobacter aerogenes* (3.8%) and *Citrobacter diversus* (2.5%). The high level of aerobic plate counts and bacterial isolates in this study indicates poor quality and public health risk to consumers, which suggests the need for improved hygienic practice at farm level.

Key words: Total Aerobic Plate Count · Bacterial Isolation · Raw Milk · Small Holders · Hawassa Ethiopia

INTRODUCTION

Milk is used throughout the world as a human food at least in one form or another. 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 [1]. Food products of animal origin play an important role in sufficient and balanced nutrition of human beings. Milk and milk products are among the most important food products of animal origin. Milk is described as a complete food because it contains protein, sugar, fat, vitamins and minerals [2]. Since milk is a major component in the human diet all over the world but it also serves as a good medium for the growth of many microorganisms especially pathogenic bacteria [3].

Milk may contain both pathogenic and non-pathogenic organisms. Pathogenic organisms, which may come directly from the cow's udder, are species of *Staphylococcus*, *Streptococcus*, *Mycobacterium*, *Brucella*, *Escherichia coli*, *Corynebacterium* etc. Various other pathogenic causing diseases like cholera and typhoid may find access in the milk from various other sources, which may come directly from the udder and may also enter in the milk from milkers' hands, utensils, cow barn, water, etc. [4].

Milk from sub-clinically mastitic cows commonly contains the etiological agents, while milk from non mastitic cows is known to be often contaminated from extraneous dirt or unclean processing water [5]. While in non-mastitic cows, milk secreted sterile into the alveoli of the udder. Microbial contamination occurs mainly during

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and after milking. Microorganisms in bulk tank milk originate from the interior of teats, the farm environment and surface of the milking equipment [6]. Milk contamination sources include the internal and external source of the udder. External sources include skin, milking equipment, milker, contaminated water and milk transportation tankers. Increasing different bacterial population will also change milk components and results in unfavorable odor and flavor, increased rate of spoilage and decrease in its maintenance. It also increases the risk of transmission of zoonotic diseases [8]. Raw milk can be a large source of diseases. Some of the most obvious are the animal disease to which humans are susceptible and which may occur in milk of cows are Brucellosis, Tuberculosis, Listeriosis, Salmonellosis, Q fever, Campylobacteriosis, Enterohemorrhagic colitis and *Staphylococcus* food spoiling microorganisms [9].

In Ethiopia raw milk and milk products are frequently consumed in different establishments and individuals home. Hygienic quality control of milk and milk products in Ethiopia is not usually conducted on routine basis. Apart from this, door-to-door raw milk delivery in the urban and peri-urban areas is commonly practiced virtually with no quality control at all levels [10].Although milk and milk products represent an important place in the nutrition of consumers as well as nutrition and income of producers, there is limited information regarding bacterial load of raw milk produced by small holders in Hawassa town. The objective of this study is:-

To evaluate bacterial load of raw milk produced by small holder dairy farms in Hawassa town and isolate major bacterial contaminants of raw milk produced by small holder dairy farms.

MATERIALS AND METHODS

Study Area: The study was conducted from November 2011 to May 2012 in Hawassa town. Hawassa is located in the Sidama Zone 275 Km south of Addis Ababa at a direction 21° south. Hawassa is a capital of the Southern Nations, Nationalities and Peoples Regional State (SNNPRS). The town lies on the latitude and longitude of 7°3'N 38°28'E, respectively; and an elevation of 1708 m.a.s.l. The town has a total population of 258,808, of whom 133,123 are men and 125,685 women; with an area of 157.21 square kilometers [11]. The livestock census data revealed that 1470 households own dairy farms in the town, out of which 636 households owned cross breed and the remaining 834 were local breeds [12]. There are two type of farming system among small holder dairy farms. Almost all farmers with local breeds were using extensive farming system and those which have cross breeds were using intensive farming system.

Study Population: Study population was cross breed lactating cows of small holder dairy farms in Hawassa town were included. These groups of small dairy farms are the one which have experience of selling or distributing to the customer. Therefore these groups of animals were a target of this study. sample were selected from cross breed dairy farms, where the sample represents 14% of the total cross breed farm.

Study Methodology

Study Design and Sampling Procedure: The study type used was cross-sectional study, which was conducted from November 2011 to May 2012. Eighty nine dairy farm that were selected during study period has been distributed into four sub cities proportionally, where high sample size were allocated to high population size.

Sample Collection: Milk samples were collected from small holder dairy farms in Hawassa city. Ten mL of milk samples were collected aseptically from each milk container and poured into sterile sampling bottle. The samples were collected from pooled milk that was placed in milk container (metal can, plastic bucket and jerry can), before the milk was transported to milk shopping. Information whether they have been using borehole or tape water for their sanitation purpose, At time of teat and container cleaning, whether they were using worm or cold water and Floor type, number of parity were gathered. After collection, the samples were placed in an ice box and transported to Hawassa University Veterinary Microbiology Laboratory for analysis.

Total Aerobic Plate Count (TAPC): Milk contamination level was determined by the TAPC method. For each samples of milk, serial ten-fold dilutions (10^{-1} to 10^{-5}) of the samples were made using sterile ringer solution. An inoculums of 1ml of each dilution was mixed thoroughly with melted plate count agar cooled to 50°C (Oxoid, Hampshire, England) by pour plate method, two plates were inoculated from each dilution and control plates were prepared from media and reagent without milk dilutions in order to exclude the environmental contaminants. The inoculated agar was allowed to settle at room temperature and then incubated at 37°C for 48 hours. Plates that

yielded between 25 to 250 colonies per plate were counted. Colonies were counted visually using tally methods by marker pen. Total bacterial count of the samples was calculated according to Yousef *et al*. [13] by using the formula of:

$$CFU/mL = \frac{\text{average number of colonies from duplicate plates}}{\text{Dilution factor x volume plated}}$$

A standard plate count of ≤ $1x10^5$ colony-forming units (CFU) per mL has been globally accepted for good-quality raw milk [14, 15]. Therefore, the final total bacterial count of less than 10^5CFU/mL was considered good; milk with a total plate count of greater than 10^5CFU/mL indicate gross contamination.

Bacterial Isolation and Identification: Bacterial isolation and identification was done according to Quinn *et al*., [16]. One standard loop of milk was streaked on 7% sheep blood agar (Oxoid, Hampshire, England). The inoculated plates were incubated aerobically at 37°C. The plates were checked for growth after 24 hours and if there was no growth, incubated for additional 24 hours. The plates were examined for growth, morphologic features such as colony size, shape, texture (Rough, mucoid, smooth) and hemolytic characteristics. Among colonies with different growth characteristics, two or three representative colonies were selected and further sub-cultured on Brain heart infusion agar (CDH, Ambala, India) and MacConkey agar (Oxoid, Hampshire, England) and incubated at 37°C for 24 hours. On MacConkey agar; presence and absence of bacterial growth, colony characteristics (size, colour) and presence or absence of lactose fermentation were noted and recorded. The pure isolates were streaked on Brain heart infusion (BHI) slant and incubated at 37°C for 24 hours and kept at 4°C for further biochemical characterization.

For primary identification of bacteria, once pure culture is obtained, [17]. A Gram stain procedure were made to establish the Gram's reaction (Gram-positive or Gram-negative) and cellular morphology (Coccus or Rod). Gram-positive cocci bacteria that were identified, further characterized using catalase test, culture on MacConkey agar and growth on Mannitol salt agar. Those catalase negative and grown on MacConkey agar were categorized as *Enterococcus* species and those catalase negative and unable to grow on MacConkey agar were catogorized as *Streptococcus* species. Catalase positive and Gram-positive cocci bacteria which were grown on Mannitol salt agar were categorized as *Staphylococcus* species. *Staphylococcus* species with golden-yellow colony, those ferment mannitol and coagulase positive bacteria were categorized as *Staphylococcus aureus* [17].

Secondary biochemical tests were done for Gram-negative rod shaped bacteria. Biochemical tests such as indole, methyl-red, Voges-Proskauer, citrate utilization (IMViC) and carbohydrate fermentation were carried out [17].

Data Management and Analysis: Data generated from this study were entered into excel spread sheet and summarized using descriptive statistical methods. Comparison between above and below the accepted limit of CFU/mL count were analyzed within and between variables using Epicalc-2000 software. Significant associations among variables were considered as significant when the value of chi-square is above 3.84 and *p* is less than 0.05.

RESULTS

Level of Bacterial Contamination: Level of bacterial contamination was determined using TAPC colony forming unit (CFU/mL) findings, where categorized as below and above the accepted limit of standard ($1x10^5$CFU/mL). Association with various assumed risk factor were compared and level of significant difference above the accepted limit standard considered as high level of contamination. Each assumed risk factors were compared within and between variables separately. Hence, milk storage, source of water, floor type, number of lactating cow and type of water used to clean udder showed significant difference ($p<0.05$). Out of the total sample observed; plastic bucket, Jerry can, borehole water, graveled floor, lactating cow above three parity and the use of cold water for cleaning udder and hand had significantly higher CFU/mL above the limit of standard. On the other hand, comparison were made between risk factors, there were no significant difference ($p>0.05$) except water source ($p<0.05$) where borehole higher than pipe water as shown in Table 1.

Out of the total 89 milk samples collected 65 (73%), 11 (12.4%) and 13 (14.6%) samples were collected from plastic bucket, metal can and Jerry can, respectively. Statistical analysis showed that the total bacterial count of Jerry can had high count and statistically significant different ($P<0.05$) with mean of $2.2x10^6$CFU/mL as compared to plastic bucket and metallic can as shown in Table 2. Water source also showed significant difference ($p<0.05$) but other variables did not show significant difference ($p>0.05$).

Table 1: Comparison of CFU/mL above and below the accepted limit of standard in different assumed risk factors

Assumed risk factors		No of samples	No (%) of sample (%)<1x10^{5}CFU/mL	No (%) of sample (%)>1x10^{5}CFU/mL	X^2 (P-value) within Variable	X^2 (P-value) between variable >1x10^{5}CFU/mL
Milk storage	Metalic	11	4(36.4)	7(63.6)	0.76(o.38)	
	Plastic	65	12(18.5)	53(81.5)	18.5(0.00)	
	Jerry can	13	1(7.7)	12(92.3)	5.42(0.02)	2.42(0.29)
Water source	Pipe	37	15(40.5)	22(59.5)	1.3(0.26)	
	Bore	52	8(15.4)	44(84.6)	16.8(0.02)	5.10(0.02)
Floor type	Graveled	28	5(17.9)	23(82.1)	8.2(0.00)	
	Soiled	20	7(35)	13(65)	1.7(0.19)	
	Paved	41	14(34.1)	27(65.9)	3.77(0.05)	1.96(0.37)
Water used for cleaning	Warm	23	7(30.4)	16(69.6)	3.07(0.08)	
	Cold	66	17(25.8)	49(74.2)	12.5(0.00)	0.13(0.72
No of parity	1 and 2	27	9(33.3)	18(66.6)	2.69(0.10)	
	3 and 4	44	14(31.8)	30(68.2)	5.2(0.02)	
	≥5	18	4(22.8)	14(77.8)	4.17(0.04)	0.55(0.75)

Table 2: Number and proportions of variables, and mean bacterial count as compared with assumed risk factors

Assumed risk factors		No of samples	%	Mean± SD	P-value
Milk storage	Metallic	11	12.4	$4x10^5 \pm 7.9x10^5$	1
	Plastic	65	73	$6x10^5 \pm 3.8x10^5$	0.78
	Jerry can	13	14.6	$2.2x10^6 \pm 3x10^6$	0.001
Water source	Pipe	37	41.6	$5.5x10^5 \pm 7.5x10^4$	1
	Bore	52	58.4	$1.2x10^6 \pm 1.9x10^6$	0.014
Floor type	Graveled	28	31.5	$1.1x10^6 \pm 2.2x10^6$	1
	Soiled	20	22.4	$5.9x10^5 \pm 8.8x10^5$	0.42
	Paved	41	46.4	$7.3x10^5 \pm 7.9x10^5$	0.18
Water used for cleaning	Warm	23	25.8	$6.5x10^5 \pm 7.8x10^5$	1
	Cold	66	74.2	$8.5x10^5 \pm 1.6x10^6$	0.27
No of lactating cow	1 and 2	27	30.3	$7.5x10^5 \pm 8.3x10^5$	1
	3 and 4	44	49.4	$8.9x10^5 \pm 1.8x10^6$	0.70
	≥5	18	20.2	$6.9x10^5 \pm 7.4x10^5$	0.91

Table 3: Proportion of different bacterial isolates recovered from raw milk of Hawassa city smallholder dairy farms

Gram stain	Isolates	No of positive	Relative percentage
Gram positive (128, 80.5%)	*Bacillus* spp.	24	15.1
	Staphylococcus aureus	14	8.8
	Other *Staphylococcus* spp.	42	26.4
	Corynebacterium spp.	7	4.4
	Streptococcus spp.	25	15.7
	Enterococcus spp.	16	10.1
Gram negative (31, 19.5%)	*E. coli*	13	8.2
	Klebsiella pneumonia	8	5
	Citrobacter diversus	4	2.5
	Enterobacter aerogenes	6	3.8
Total		159	100

Table 4: Isolation and characterization of Gram positive bacteria

Growth on blood agar	growth MacConkey	Gram stain	Arrangement on staining	Catalase	Mannitol salt agar	Lactose fermentation on MacConkey	coagulase	Bacteriological result
+	-	+ rod	large rod	+	-	-	-	*Bacillus* spp.
+	-	+ cocci	Cluster	+	-	-	-	*Staphylococcus* spp.
+	-	+ cocci	Cluster	+	golden yellow	-	+	*Staphylococcus aureus*
+	-	+ rod	Irregular	+	-	-	-	*Corynebacterium* spp.
+	-	+ cocci	Chain	-	-	-	-	*Streptococcus* spp.
+	Pink	+ cocci	Chain	-	-	+	-	*Enterococcus* spp.

Table 5: Gram negative isolates and their biochemical characteristics

Growth MacConkey	Gram stain /KoH	Catalase	lactose on MacConkey	Indole	Methyl red	Voges-Proskuer	citrate	urease	TSI slant/but	H_2S	Motility	Bacteriological result
Pink	- Rod	+	-	-	-	+	+	+	y/y	-	+	*Enterobacter* spp.
Pink	- rod	-	+	-	-	+	+	+	y/y	-	-	*Klebsiella*
Pink	- rod	+	+	+	+	-	-	-	y/y	-	+	*E. coli*
Pale	- rod	+	-	+	+	-	+	+	R/Y	-	+	*Citrobacter* spp.

Bacterial Isolate: Samples were cultured and examined, bacteria were grown from all samples cultured and of these samples 159 bacterial isolates were identified. The most predominant bacteria isolated were Gram positive 128 (80.5%) and followed by Gram negative 31 (19.5%).

Out of the 159 isolates represented different bacterial species, *Staphylococcus* species 35.2% of which 25% were *Staphylococcus aureus* followed by *Streptococcus* species 15.7%, *Bacillus* species 15.1%, *Enterococcus* species 10.1%, *E. coli* 8.2%, *Klebsiella pneumoniae* 5%, *Corynebacterium* species 4.4%, *Enterobacter aerogenes* 3.8% and *Citrobacter diversus* 2.5% shown in Table 3, 4 and 5.

DISCUSSION

Level of Contamination: In this study, raw cow milk samples were analyzed for bacterial contamination at the farm level by TAPC method. Raw milk sampled was considered as having unacceptable hygienic quality when TAPC exceeds $1x10^5$CFU/mL [14,15]. The study observed the contamination level of milk against various assumed risk factors. Accordingly, the TAPC of raw milk in different type of milk storage showed that the mean count of $4x10^5$, $6x10^5$ and $2.2x10^6$CFU/mL in metal can, plastic buckets and jerry can, respectively. This finding agrees with Kivaria *et al.* [15] from Tanzania who reported $7.1x10^6$, $7.8x10^6$ and $8.5x10^6$CFU/mL for metal can, plastic bucket and jerry can, respectively; but the contamination level in this study is slightly lower than Kivaria *et al*. [15] report. Proportion of samples those scored above the limits was 63.6, 81.5 and 92.3% in metallic can, plastic bucket and jerry can, accordingly. The mean TAPC of the samples from the metal can was lower than the plastic bucket and jerry can. This might be due to the metal can is easy to clean and as plastic bucket could scratch easily that could hinder proper cleaning. TAPC of milk samples were significantly higher in jerry can than the metal can and plastic bucket. Milking began in plastic or metallic bucket then it transferred to the jerry can. Subsequently, steps of transfer from one container to another might give for the high level of contamination. On the other hand, narrow mouthed jerry can could contribute for high level contamination since it hinders proper cleaning.

In all small holders sampled, for this study, have practice washing teats before milking using either borehole water 58.4% or pipe water 41.9%. The bacterial load of samples that were collected from those farms that were using pipe water for cleaning udder and equipment's had lower TAPC than those used borehole water. Therefore, the source of microorganisms in this study might be due to the contamination of water by dust, animals, plants, people and other inanimate objects moreover, bore water is not chlorinated and the material used for pulling borehole water usually remain outside, this could also has contribution for contamination. The finding agrees with different scholars where they have mentioned that the presence of microorganisms in milk were influenced by unclean water, unhealthy cow, unclean utensils, inappropriate bactericidal treatment of utensils, insufficient cooling of milk [16,17].

Bacterial Isolates: In the course of the study, bacteria belongs to the nine genera were isolated. The most predominant were *Staphylococcus* species (35.2%) followed by *Streptococcus* species (15.7%), *Bacillus* species (15.1%), *Enterococcus* species (10%), *E. coli* (8.2%), *Klebsiella pneumoniae* (5%), *Corynebacterium* species (4.4%), *Enterobacter aerogenes* (3.8%) and *Citrobacter diversus*(2.5%).

The type and number of bacteria present in milk indicate the hygienic quality of milk. Those isolates of *Bacillus* species, *Staphylococcus* species, *Micrococcus* species, *Streptococcus* species and coliform microorganisms can cause spoilage of the milk when present in raw and pasteurized milk [19]. In this study 8.8% of *Staphylococcus aureus* isolates were identified and this could be a concern of human health as some strains of *Staphylococcus aureus* are capable of producing heat stable enterotoxin [6]. Although *E. coli* is a frequent organism in milk and its products, the prevalence of *E. coli* itself in milk and milk products as a possible cause of disease is insufficient because *E. coli* is normally ubiquitous organism [4]. However, the occurrence of a specific verocytotoxigenic strains may cause hemmorhagic colitis; the most important one is the enterohemmorrhagic type *E. coli* O157: H7 [20] that cause

of food borne illness and is now considered as important human pathogens [21]. Transmission of coliform organisms potentially including *E. coli* O157: H7 occurs through ingestion of raw milk [15]. In this study 8.2% of *E. coli* was isolated and this is also a source of concern as verocytotoxigenic *E. coli* could present that produce toxin that can cause illness to consumers of milk in the study area.

Bacillus cereus produces two different forms of food poisoning; the diarrheal syndrome caused by heat stable enterotoxin and emetic syndrome involving a very heat stable enterotoxin [15]. In this study 15.1% of *Bacillus* species were isolated from the milk samples and this need a concern as *Bacillus cereus* could cause a public health hazard. The bacteria of the genus *Enterococcus* species also known as *Enterococci* are considered to be important in food as indicator of spoilage or potential pathogenic organisms. In dairy products both *E. faecalis* and *E. faecium* species are relatively heat resistant as well. Most enterococci are also relatively resistant to freezing. Higher levels of *Enterococci* in milk are considered to be the result of contamination during the collection or processing of milk [22]. In this study 10.1% of *Enterococci* were isolated. The presence of *Enterococci* implies a risk that other enteric pathogens may be present in the milk. *Enterococci* are therefore of particular importance in food and public health microbiology. *E. faecalis* is as a causative agent of gastro enteritis [14].

CONCLUSION AND RECOMMENDATION

Milk intended for human consumption must be pathogens free and if conditions not permit should contain few bacteria. This study showed that majority of milk produced in the farm was poor in quality. Therefore, 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. Based on this study the following recommendations are made:

Awareness should be created among dairy cow owners about the importance of adequate udder preparation, hygienic milking technique, use of clean dairy equipment, washing utensils and milkers' hands using properly treated water to improve the milk hygienic quality and its shelf life.

Raw milk intended for consumption should be subjected to heat treatment at least equivalent to pasteurization.

Potable water should be available for effective cleaning and sanitizing of milk equipment's and udder preparation, otherwise well heat treated water should be used for such purpose

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