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Research Article

Isolation of *Salmonella* and *E. coli* (*E. coli* O157:H7) and its Antimicrobial Resistance Pattern from Bulk Tank Raw Milk in Sebeta Town, Ethiopia

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Abstract

The present study assessed the occurrence and antimicrobial susceptibility patterns of *Escherichia coli, E. coli* O157:H7 *and Salmonella* species from raw milk collected from dairy cattle farms and collector's bulk tank in Sebeta town, Ethiopia. A total of 142 milk samples were collected for bacterial isolation and identification by using conventional bacteriological techniques and BIOLOG identification system. The identified *E. coli* isolates were tested for antimicrobial susceptibility pattern using four different types of antibiotics by disc diffusion method. The prevalence of *E. coli* was 14/142 (9.9%, 95% CI = 4.9%-14.8%), all the samples were negative for *E. coli* O157:H7 and *Salmonella enteric* was isolated from one milk sample 1/142 (0.7%). The frequency of *E. coli* isolation was higher in those milk samples collected from milk that was stored and transported in plastic containers (12.16%) than samples from those containers made of stainless steel (7.35%), however the difference was not statistically significant (P>0.05). While comparing the prevalence of *E. coli* between samples collected directly from individual dairy farms and milk collectors, statistically significant difference (P=0.028) was observed, higher *E. coli* prevalence was recorded in samples obtained from milk collectors (15.3%) as compared with samples collected from individual dairy farms bulk tank milk (4.3%). All *E. coli* isolates were found to be 100% susceptible to gentamicin followed by amoxicillin (92.9%), sulphamethoxazole-trimethoprim (92.9%) and tetracycline (85.7%). Two of the isolates showed multiple drug resistance to two drugs. These findings showed that raw milk from dairy cattle farms and collector's bulk tank in Sebeta town was contaminated with public health important bacterial species like *E. coli* and *Salmonella* species and the observed resistant to certain antimicrobial drugs also needs attention. To ensure the quality of raw milk, stakeholders engaged in milk and dairy production chain should be trained on hygienic practices.

Keywords: Antimicrobial susceptibility; *Escherichia coli*; *Escherichia coli* O157:H7; Milk; *Salmonella enteric*; Sebeta

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Introduction

Milk and milk products have important role in feeding the rural and urban population owing to its high nutritional value. It is the most perfect single balanced food of high biological value in nature as it contains almost all ingredients of food in right proportion and in any easily digestible form [1].

Milk is virtually a sterile fluid when secreted into alveoli of udder. Microbial quality of milk refers to the cleanness of milk. This is defined by a number of bacteria present in milk. The high bacteria count as well as the presence of pathogenic bacteria in milk not only degrades the milk quality and shelf-life of milk or milk related products but also poses a serious health threat to consumers [2]. Bacteria, yeasts and moulds are the common contaminants of milk. Their rapid growth of microorganisms, particularly at high ambient temperature can cause marked deterioration in quality of the milk and dairy products manufactured from it [3]. Microbial contamination might generally occur from three main sources: within the udder, exterior to the udder and from the surface of milk handling and storage equipment, but the surrounding air, feed, soil, faeces and grass are also possible sources of contamination [4].

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 [5]. The safety of dairy products with respect to food-borne diseases is of great concern around the world. Raw milk can harbor dangerous microorganisms which may pose serious health risks to humans. Over 200 known diseases are transmitted through eating food contaminated by a variety of agents including bacteria, parasite, viruses, and fungi [6]. Some of the bacteria involved in causing food borne diseases due to the consumption of raw milk include *Escherichia coli, Listeria monocytogenes, Salmonella, Campylobacter, Brucella abortus, Staphylococcus aureus, Bacillus cereus, Mycobacterium* spp. and

Clostridium botulinum. If these pathogenic bacteria are present in raw milk, it is a major public health concern, especially for those individuals who drink raw milk frequently [7].

Over the last 20 years, the emergence of major food borne pathogens such as *Salmonella* and *Escherichia coli* have persisted as a major public health concerns and provide clear examples of the persistence of food borne pathogens despite considerable efforts aimed at prevention and control [8]. For this reason, the basic steps in the control of safety and quality of food include analysis of food products for presence of pathogenic microorganisms that cause the majority of alimentary human diseases. Among them, *Salmonella* and *E. coli* O157:H7 are the major ones. These food borne pathogens have frequently been linked to a number of cases of human illness [9].

The increasing use of antibiotics in veterinary practice is suspected to contribute to acceleration of antibiotic resistance in microorganisms [10]. The irrational use of antibiotics in food producing animals could result into antibiotic residues in edible tissues and products [11]. It has been reported that, antibiotics used for treatment of human bacterial infections are used for prophylactic, therapeutic and growth promotion in animals too [12]. Bacteria that have been exposed to low doses of these antibiotics in tissues and products from these animals may be less susceptible to drugs, and when such bacteria enter the human body through consumption of contaminated foods, they may cause infections that are resistant to many antibiotics [13].

Gastroenteritis due to food-borne disease is one of the most common illnesses in Ethiopia, and it is a leading cause of death among people of all ages in the country [14]. The lack of surveillance of food-borne pathogens, poor hygienic conditions and the wide spread cultural practice of raw milk consumption are all major factors contributing to the high risk of exposure of Ethiopians to food-borne pathogens such as *E. coli, E. coli* O157:H7 and *Salmonella* species. In spite of the high risk of exposure to *E. coli, E. coli* O157:H7 and *Salmonella* from animal origin food sources like milk in the country, there is lack of well-organized report indicating the contamination level of animal origin food sources like milk by these zoonotic organisms. Moreover, in the country both veterinary and medical drugs are often misused, creating ideal conditions for the development of resistant strains, thus better understanding of the antimicrobial susceptibility/resistance/patterns of pathogens isolated from animal source foods like milk is needed. Therefore the objectives of this study were:-

To isolate *E. coli* with emphasis on *E. coli* O157:H7 and *Salmonella* species from raw milk obtained from dairy cattle farms and milk collectors in Sebeta town, Ethiopia.
To determine antimicrobial susceptibility and resistance pattern of *E. coli* isolates from milk samples.

Materials and Methods

Study AREA

The study was conducted in Sebeta town South West Showa, from November 2017 to March 2018. The mean annual temperature and rainfall ranges between 15°C to 21°C and 800 mm to 1199 mm respectively and it is located 25 km west of Addis Ababa. Intensive and semi-intensive cattle dairy farms with exotic and cross breeds are managed by the community at the area.

Study design

A cross-sectional study was conducted to determine the prevalence of *E. coli* with emphasis on *E. coli* O157:H7 and *Salmonella* species. Milk samples were collected from individual dairy cattle farms and milk collectors from Sebeta town.

Sample size determination

The approximate sample size required for the study was determined based on the expected prevalence of *E. coli* and the desired absolute precision using the formula stated on Thrusfield [15].

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

Where: n=required sample size
Pexp =Expected prevalence
d = desired absolute precision

The previous study made in Holeta and Burayu by Yohannes [16] showed the prevalence of *E. coli* was 7.1% in cow milk. Therefore, by using this 7.1% expected prevalence, at a confidence level of 95% and required absolute precision of 5%, the calculated minimum sample size was 128 and 142 bulk tank milk samples were collected to increase the precision.

Study Methodology

Sampling methods and procedures

General information was collected from Sebeta town livestock and fisher office to identify the total number of farms, farm size, farming system, and the status and number of milk collectors in Sebeta town. According to the result the majority of the farms were at the household/smallholder level, with farm size not more than 5 cows per farm. Milk collecting site at the main road were identified as main sources of milk for consumers and processing center and included in the study. Simple random sampling technique was applied to collect raw milk samples from each group of collecting site and farms bulk tank. Milk samples were aseptically taken in morning time. The reason for collecting morning milk samples was, most of dairy farms submit their milk to milk collectors in the morning time soon after milking. During collection, approximately about 4-5 ml raw milk samples were aseptically collected from bulk tank milk container of collectors and dairy farms, then placed in sterile universal bottle by using sterile graduated pipette for each samples. Subsequently these samples were labeled and immediately transported to National Animal Health Diagnostic Center (NAHDIC) to isolate *E. coli*, *E. coli* O157:H7 and *Salmonella* species from raw cow milk.

Isolation and identification of *E. coli* and *E. coli* O157:H7

Each raw cow milk samples were inoculated on MacConkey agar, and then incubated at 37^{0}C for 24 hours. Typical colonies on MacConkey agar (pink, due to their ability to ferment lactose) were stained using gram stain and observed for their staining and morphological characteristics and transferred to Eosin Methylene- Blue (EMB) agar. The colonies with green metallic sheen on EMB agar which is typical feature of *E. coli* were transferred to sorbitol MacConkey agar to check the presence of *E. coli* O157:H7 phenotype (inability to ferment sorbitol). Then the *E. coli* suspected colonies were transferred to

nutrient agar to be used for secondary biochemical tests (IMViC tests) [17]. A standard reference strain of *E. coli* (ATCC 25922) was used as a quality control.

Based on Primary and secondary biochemical tests *E. coli* suspected colonies were confirmed by BIOLOG bacterial identification system. Then, BIOLOG system (fully automated coated microplate based bacterial identification system) using GEN III micro plate (Lot number 3003241, BIOLOG, USA) with protocol A method was used to further confirm the species of suspected colonies. A single colony grown on Biolog Universal Growth (BUG) agar medium was selected and emulsified into 'Inoculating Fluid A' (IF A). According to the manufacturer's instructions, cell density of the bacterial inoculum was measured and adjusted for a specified transmittance (90 to 98%) using a turbidimeter. For each isolate, 100 µl of the bacterial cell suspension was inoculated in to each of the 96 well coated micro plates, using automatic multichannel pipette and incubated aerobically at 33^{0}C for 22 hr. BIOLOG microstation reader was used to read the incubated microplate and provides species/sub-species Identification (ID), and then the results were printed out [18].

Isolation of *salmonella* species

Salmonella species isolation was taken based on NAHDIC's test method for *Salmonella* species identification which was adapted from ISO 6579-1:2017 [19]. Briefly, homogenized raw milk sample of 1 ml was added to 9ml of sterilized buffered peptone water and incubated overnight at 37°C. Then for selective enrichment 0.1ml of pre-enrichment was transferred to 10 ml of Rappaport Vassiliadis Soya broth (RVS broth) then were incubated at 41.5 ºC for 24 hrs. Each selective enrichment broth bottle was well shaken and then a loop full from each was streaked onto plates of Xylose Lysine Deoxycholate (XLD) agar and all plates were then aerobically incubated at 37°C for 24 hrs. Pink colonies with or without black centers were transferred to nutrient agar for further test. Then secondary biochemical tests (IMViC tests), TSI, urea and lysine were conducted. A standard reference strain of *Salmonella Typhimurium* ATCC14028 was used as a quality control. Finally *Salmonella* species suspected colonies were confirmed by using GEN III microplate, BIOLOG system.

Antimicrobial susceptibility testing for *E. coli*

The antimicrobial susceptibility test was performed following the standard agar disk diffusion method using commercial antimicrobial disks. The selection criteria of the antibiotics depended on the regular use of the antimicrobials in the animal and human treatments. Mueller-Hinton agar media was used for susceptibility testing. The isolated *E. coli* strains were tested for sensitivity to commonly used antimicrobials in veterinary medicine in the country including Gentamicin (GCN) (10μg), Trimethoprim-sulfamethoxazole (SXT) (25μg), Tetracycline (TE) (30μg) and Amoxicillin 25µg. A standard reference strain of *E. coli* (ATCC 25922) was used as a quality control. Interpretation of results was made according to CLSI Guideline [20].

Data Management and Analysis

Microsoft excel spread sheet was employed for raw data entry and SPSS version 20.0 software was used for descriptive statistics. For all analysis, 95% CI and *P*-value<0.05 was set for statistical significance of an estimate.

Results

Prevalence of ***E. coli***, ***E. coli*** O157:H7 and *Salmonella* species from bovine raw bulk tank milk

A total of 142 milk samples were collected from dairy cattle farms and milk collectors' bulk tank for isolation and identification of *E. coli, E. coli* O157:H7 and *Salmonella* species. Prevalence of *E. coli* and *Salmonella* species are summarized in table 1. *E. coli* and *Salmonella* species were detected in 14/142 (9.9%) and 1/142 (0.7%) raw milk samples, respectively. Based on the other finding *E. coli* O157:H7 was not isolated from bulk tank milk samples collected from the area.

Species of Bacteria	Positive milk samples (%)	95% Confidence Interval
E. coli	14/142 (9.9%)	4.9-14.8%
Salmonella enterica	1/142 (0.7%)	.0-2.1%

Table 1: Prevalence of *E. coli* and *Salmonella* species.

E. coli prevalence in raw milk value chain was evaluated and a higher rate of contamination was detected in the samples collected from collectors (15.3%) than from dairy farms (4.3%) the difference was statistically significant (P<0.05%) (Table 2). The container in which milk was collected was also evaluated and a higher frequency of contamination was detected in the milk samples collected from plastic containers (12.16%) than stainless steel (7.35%). The containers made of plastic were identified to be more prone to be contaminated by *E. coli* than stainless steel, but the difference was not statistically significant (P>0.05%).

Antimicrobial susceptibility/resistance pattern of *E. coli* isolated from bovine bulk tank milk

A total of 14 *E. coli* isolates were tested against using 4 antimicrobial discs following CLSI guidelines. All *E. coli* isolates were found to be 100% susceptible to gentamicin followed by amoxicillin (92%) and sulphamethoxazole-trimthoprim (92%) and then tetracycline (85%), figure 1. One isolate showed resistance for amoxicillin and another for sulphamethoxazole-trimethoprim and two isolates were resistant for tetracycline. Two of the isolated *E. coli* species showed multiple resistances to two drugs (one isolate for Amoxicillin-Tetracycline and the other for Tetracycline-Sulpha-Trimethoprim).

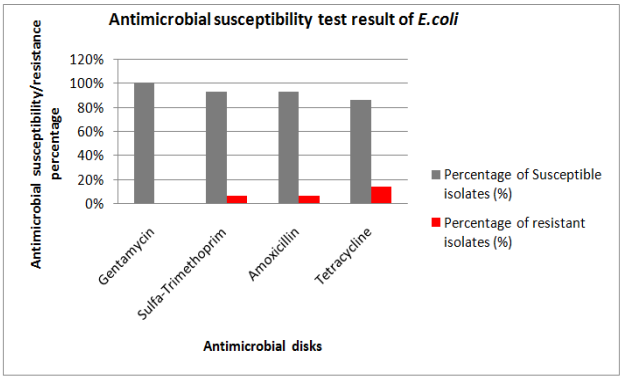

Figure 1: Antimicrobial susceptibility test result of *E. coli*.

Source of milk sample	Number of samples tested	Positive samples	Percentage of E. coli isolation from milk samples	Chi square	P-value
From farm	70	3	4.3	4.825	0.028
From collectors	72	11	15.3		
Type of container					
Plastic container	74	9	12.16	0.922	0.337
Metal/Stainless steel/ milk can	68	5	7.35		

Table 2: *E. coli* prevalence in raw milk value chain.

Discussion

Milk, a perishable complete nutritious food is considered to be a good medium of growth for many of the microorganisms [21]. *E. coli* is a normal inhabitant of the intestines of animals and humans. However, its recovery from food may be of public health concern due to the possible presence of enter- pathogenic and/or toxigenic strains like *E. coli* O157:H7 which can lead to sever gastro intestinal disturbances [22] and other life threatening syndromes on the consumer [23].

Escherichia coli and salmonella are not only regarded as an indicator of fecal contamination but more likely as an indicator of poor hygiene and sanitary practices during milking and further handling. In this study, a total of 142 raw milk samples were studied and from these 14(9.9%) milk samples were contaminated with *E. coli,* other finding *E. coli* O157:H7 was not isolated (0%) and *Salmonella enterica* was identified from 1(0.7%) sample. The isolated percentage of *E. coli* is in agreement with the report by [16,24] who reported 11.6% and 7.1% respectively. On the other hand, the present study was relatively lower as compared to the studies by [25-28] previously reported as 26.57%, 27.91%, 25% and 33.9% respectively. The other finding of the present study is that *Escherichia coli* O157:H7 was not isolated (0%) from bulk tank milk samples. Many other studies on the prevalence of *E. coli* O157:H7 from raw milk reported that the isolation rate was low and varied between 0 % and 10% [29]. It has been suggested that the microorganisms are not actually excreted in the milk but probably result from faecal contamination of milk during the milking process [30].

Salmonella species cause enteric infection characterized mainly by gastroenteritis on humans and other animals worldwide, and sometimes in severe cases it can result in systemic infection and even death. In general, *Salmonella* prevalence observed in study was 1/142 (0.7%). Other studies [31-33] reported prevalence of 0%. Moreover study at Jigjiga City of Somali Regional State also reported 1 (3.3%), lower percentage of *Salmonella* species [34]. Moreover, several studies recorded that *Salmonella* was not detected in milk samples [35-38]. On the other side relatively higher percentages of 8.7% in Nigeria [39] and 20% in Ethiopia was reported [40].

The variation that was seen in prevalence of *E. coli* and *Salmonella* species in different studies may be due to difference in sample size, farming system, farm size, milking equipment, milking technique, geography, ecology, duration of milk transportation, and hygienic conditions [22]. The presence of *E. coli* may not necessarily indicate a direct fecal contamination of milk but is an indicator of poor hygiene and unsanitary practices during milking and further handling of milk and presents a potential hazard for people consuming such products [41]. Moreover, bacterial identification techniques used by different researches may also be one factor where using only primary and secondary biochemical tests by most of the authors and other limited studies further confirm the isolates by techniques like BIOLOG, this reduces the number of positive samples.

Raw milk in value chain is commonly distributed locally to consumers with no controlled measures to maintain the safety and quality before it reaches consumers in Sebeta. The prevalence of *E. coli* was different at the raw milk chain. Difference in prevalence of *E. coli* was observed on the source of raw milk samples, from farm and collectors. Higher prevalence was recorded in collector's raw bulk tank milk (15.3%) as compared to milk samples from individual dairy farms (4.3%) raw bulk tank milk. The observed high prevalence of *E. coli* from milk collectors was higher than those previous reports from Holeta and Sululta farmers 3.84% and 11.53% [31]. The observed differences might be due to the longer time for transporting milk to the collectors at ambient temperature under poor hygienic conditions which support the growth of the bacteria in the milk samples taken from the collectors. Moreover the collectors receive milk from several individual dairy farms and there is no well organized checking system for quality of milk during collection.

The container in which milk was collected was also evaluated and a higher rate of contamination was detected in the samples collected from milk held in plastic containers (12.16%) than stainless steel (7.35%). The containers made of plastic were identified to be more prone to be contaminated by *E. coli* than stainless steel, but the difference was not statistically significant ($p>0.05$). Similar findings were also reported before in the country [28].

The development of antimicrobial resistance among the pathogenic bacteria poses a problem of high concern. The present study showed that *E. coli* isolates were highly sensitive to gentamicin followed by amoxicillin and sulphamethoxazole-trimthoprim (92%) and then tetracycline (85%). Similarly other studies from Mekele town [26] revealed that the susceptible to some antibiotics like gentamicin (100%) and tetracycline (60%). Moreover, other study [27] has also reported all the isolates were found to be 100% susceptible to gentamicin and sulphamethoxazole-trimthoprim (76%). Multiple drug resistance patterns were also analysed, accordingly, 2(14.28%) of the *E. coli* isolates have shown resistance to two of the antimicrobials tested. In particular, these bacterial isolates have primarily shown multidrug resistance to Amoxicillin-Tetracycline and the other for Tetracycline-Sulpha-Trimethoprim. Previous studies have also reported the development of multiple drug resistance by *E. coli* [42,43]. In general, the development of antimicrobial resistance could possibly be due to overuse and misuse of the antimicrobials by herd owners and animal health professionals. Furthermore, even the undergone

bacterial mutations may be the cause for the development of antimicrobial resistance. Drugs like tetracycline are commonly used in the country for treatment and disease prevention in animal health sector of the country. Some studies describe the direct correlation of antimicrobial use to antimicrobial resistance in veterinary medicine at a supranational level, based on publicly available data sources [44]. Thus, the observed proportion of resistance of *E. coli* isolates to tetracycline may be related with the widespread usage of the drug in the country.

Conclusion

The findings obtained in this study revealed that raw cow's milk were found to be contaminated with *E. coli* and *salmonella* species. The sources of *E. coli* and *salmonella* in the raw cow milk may be from contaminated udders, contaminated water, poor sanitation practices, contaminated containers, and milk handlers themselves. Since the milk is transported and managed at an ambient temperature, high microbial populations can be reached within short period of time. Based on the antimicrobial susceptibility pattern, most of the *E. coli* isolates were found to be susceptible to gentamicin, amoxicillin, sulphamethoxazole-trimethoprim and tetracycline and few isolates have also showed resistance. Accordingly, to ensure the quality of raw milk, every actor engaged in milk and dairy production chain should be trained for hygienic practices. Proper antibiotics usage in animal production also needs attention.

Competing Interests

The authors declare that they have no competing interests.

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