Prevalence and antimicrobial susceptibility of *Escherichia coli* O157:H7 in raw cow's milk in Gojo and Shukute towns, central Ethiopia

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Abstract

Escherichia coli O157:H7 is one of the most important emerging foodborne pathogens and cause life-threatening disease condition in consumers worldwide. A cross-sectional study was carried out from February 2020 to August 2020 in Gojo and Shukute towns, Jeldu district, West Shewa Zone, Oromia region, Ethiopia to isolate and identify *E. coli* O157:H7 from raw cow's milk samples and determine antimicrobial susceptibility of the isolates. A total of 262 milk samples (127 from Gojo and 135 from Shukute towns) were collected from dairy cows (126 from udder, 115 from milking bucket) and 21 from collection tanks and examined bacteriologically. The isolates were tested with a series of biochemical tests followed by a latex agglutination test for identification and confirmation of *E. coli* O157:H7. The antimicrobial susceptibility profile of the isolated *E. coli* O157:H7 was performed using the Kirby-Bauer disk diffusion method. The study revealed 1.5% (95% Confidence interval [CI]: 0.4–3.8%) of the collected raw milk was contaminated with *E. coli* O157:H7. The isolates showed 100% susceptibility to azithromycin, norfloxacin, nitrofurantoin, amikacin, chloramphenicol, tetracycline, and trimethoprim-sulfamethoxazole. In contrast, the isolates showed 75% and 100% resistance to ampicillin and cefotaxime, respectively. In conclusion, the consumption of raw milk may constitute a public health hazard due to contamination with *E. coli* O157:H7.

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Farmers and farmworkers should be trained on milk hygiene and proper milk handling practices.

Introduction

Nutritionally, milk has been defined as the nearly perfect food, and it is considered as one of the most important diet items of society (Olatunji *et al.*, 2012). However, the fluid nature of milk and its chemical composition renders it as one of the ideal media for microbial growth and multiplication (Claeys *et al.*, 2013). Microbes may gain entry into raw milk directly from the feces of dairy cows, from the farm environment particularly contaminated water sources and utensils used for the storage of milk on-farm or during transportation(Eberhart, 1977). *E. coli* serotype O157:H7, known variously as enterohemorrhagic, verocytotoxin-producing, or Shiga toxin-producing organism, is one of the most commonly known bacterial pathogens in raw milk and other dairy products (Rahimi *et al.*, 2012). It is transmitted to humans by consumption of contaminated foods derived from cattle, especially meat and raw milk, and person-to-person spread, particularly in family groups within households (Bavaro, 2012). Many studies have been conducted in Ethiopia to identify E. coli O157:H7 and the antimicrobial susceptibility profile of the isolates from raw milk (Disassa et al., 2017; Gemechu et al., 2017; Bedasa et al., 2018; Kanenuset al., 2018), cattle carcasses (Taye et al., 2013), sheep and goat meat (Mersha et al., 2010), feces and skin of sheep and goats (Mersha et al., 2010), yogurt and cheese (Bedasa et al., 2018). However, there is no information on the prevalence and antimicrobial susceptibility profile of E. coli O157:H7 in raw milk of cows in Gojo and Shukute towns where a large population of cattle is reared for milk. Therefore, this study was conducted to assess the prevalence of E. coli O157:H7 in raw cow's milk in Gojo and Shukute towns, and determine the antimicrobial susceptibility profile of the isolates.

Materials and methods

Study area

The study was conducted in Gojo and Shukute towns of Jeldu district, West Shewa Zone, Oromia region, Ethiopia (Figure 1). Jeldu district is found at a distance of 120 km West of Addis Ababa. It is located between latitudes of 9°02' 47" to 9°15' 00" N and longitudes of 38°05' 00" to 38°12' 16" E, and the altitude of the district ranges from 1800 to 2550 meters above sea level (m.a.s.l). Jeldu district receives an average annual rainfall of 2500 mm, and the annual mean daily temperature ranges between 17ºC and 25 ºC. The human population of Jeldu district is 202,655 of which102,796 are females and the remaining 99,859 males. The district has total cattle, sheep, goat, and equine population of 78,300, 50,450, 25,560, and 45,210,respectively (CSA, 2015).
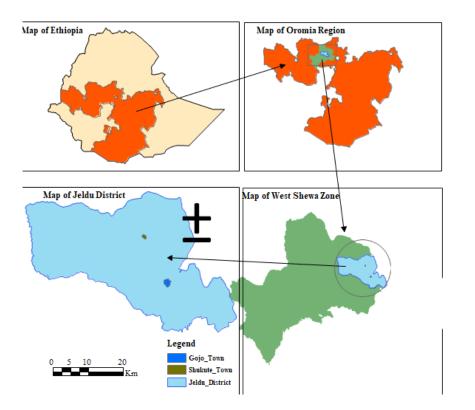

Figure 1. Map showing the study area where the samples were collected.

Study design and sample size determination

A cross-sectional study was conducted on lactating dairy cows in Gojo and Shukute towns to estimate the prevalence, associated risk factors, and antimicrobial susceptibility of *E. coli*O157:H7. The sample size was determined according to Thrusfield (2007) with an expected prevalence of 12% (Bedasa *et al*. 2018), 95% confidence level, and desired absolute precision of 5% using the following formula.

$$N = \frac{(Za/_2)^{2} * P(1-P)}{d^{2}}$$

WhereN is the required total sample size, *Z a/2 is the critical value at 95% certainty (1.96),* P is the expected prevalence of the organism, and d, stands for marginal error/ the desired absolute precision of 5%. Accordingly, the calculated sample size was 162; however, to increase the precision of the study the sample size was increased and set to 262.

Study population

The study population for this study includes all lactating dairy cows found in Gojo and Shukute towns. The dairy animals are managed under extensive, intensive, and semi-intensive management systems. A total of 15 dairy farms managed under intensive and semi-intensive systems and 10 herds managed under extensive management were selected from the list of all dairy farms in Gojo and Shukute towns using the simple random sampling method.

Sample population

The sample population/study unit comprises 262 lactating cows, of which, 153 were selected from the extensively managed herd, and 109were selected from the intensive and semi-intensively managed farms. The sample population was selected from the study population using the simple random sampling method (lottery technique) after the construction of a sampling frame.

Sample collection and transportation

About 15ml of raw milk sample was collected carefully into a separate sterile test tube from the teat of lactating cows, the milking buckets, and milk storage

tanks of the study dairy farms during the morning (7 AM) and evening (6 PM) milking sessions. The samples were then labeled, kept in an icebox containing ice, and transported to Ambo University zoonosis and food safety research laboratory. Upon arrival to the laboratory, samples were processed immediately or stored at a temperature of 4°Cuntil processed.

Isolation

The refrigerated samples were thawed at room temperature for 4 to 6 hours before processing the specimen. One milliliter of each milk sample was added into nine milliliters (1: 9 ratio) of buffered peptone water (HiMedia Laboratories, India) in a test tube, homogenized for 2 minutes, and incubated at 35-37°C for 20-24 hrs for enrichment to increase recovery of the organisms (OIE, 2016). The culture was then inoculated onto MacConkey agar (Blulux laboratories, USA) for isolation of *E. coli* and incubated aerobically at 37 °C for 24 hrs. The plates were observed for the growth of suspect *E. coli* colonies (smooth, circular, pink colonies). Specimens from the isolated colonies were picked and sub-cultured on Eosin Methylene Blue (EMB) agar (HiMedia Laboratories, India) and incubated at 37°C for 24 hrs. Bacterial colonies which show the typical characteristic greenish metallic sheen were assumed as *E.coli* (Dantebo *et al.*, 2019).

Simultaneously, a specimen from the same colony was picked, stained with Gram's stain, and examined for stain and morphological characteristics using bright-field microscopy. Suspected colonies of *E. coli* (pinkish color appearance on MacConkey agar, greenish metallic sheen on EMB, and Gram-negative rods) were then inoculated on nutrient agar and incubated aerobically at 37 °C for 24 hrs for the biochemical test.

Biochemical tests

Pure colonies from the nutrient agar culture were picked and inoculated into triple sugar iron (TSI) agar (Difco, MI, USA) slant by stabbing the but and streaking the slant, and after 48 hours of incubation at 37°C, yellow slant with yellow butt, presence of gas bubbles, and absence of black precipitate in the butt was considered as indicative of *E. coli* (Radu *et al.*, 1998; Dantebo *et al.,* 2019). These isolates were then subjected to indole production, methyl-red,

Voges-Proskauer, and citrate utilization (IMViC) biochemical tests (Heuvelink *et al.*, 1998; Radu *et al.*, 1998; Feng and Monday, 2000; Müller *et al.*, 2003). Isolates that were positive for indole and methyl red tests but negative for Voges-Proskauer and Citrate utilization tests were assumed as *E. coli* (Holt *et al.*, 1994).

The identified *E. coli* isolates were further sub-cultured onto MacConkey agar with sorbitol (CRITERION, USA) and incubated at 37ºC overnight. Unlike other *E. coli* strains, isolates of serotype O157:H7 do not ferment D-sorbitol, hence appearing as smooth, circular, and colorless to gray colonies with the smoky center. In contrast, most other *E. coli* strains ferment sorbitol and form pink colonies (Müller *et al.*, 2003; Amenu *et al.*, 2019). However, many organisms other than *E. coli* O157:H7, especially other serogroups of *E. coli* and *Proteus* spp., may not ferment sorbitol and thus may be confused with *E. coli* O157:H7 (Benard, 2008).

Serological test

All non-sorbitol fermenting colonies of *E. coli* from the McConkey-sorbitol agar were subjected to a slide agglutination test using *E. coli* O157:H7 latex test kit (Oxoid CM0813, Hampshire, UK). The latex kit consists of four components: latex test reagent, latex control reagent, and positive and negative control suspensions. The test latex consists of blue latex particles sensitized with a specific rabbit antibody against O157 somatic antigen and the control latex consists of blue latex particles sensitized with pre-immune rabbit globulin. The positive and negative controls are suspension of inactivated *E. coli* O157:H7 cells and *E. coli* O116 cells in buffer, respectively. The latex beads are coated with antibodies that bind to O157 antigens on the test organisms, forming a visible antigen-antibody precipitate.

The latex kit was checked for its performance, by using the control suspensions and reaction of the positive control showing positive result, and then run according to the manufacturer's instructions. Briefly, a drop of latex test reagent and 0.085% sterile saline water were dispensed into the reaction card separately and a few presumptive colonies (4-5 colonies) of *E. coli* O157:H7 were taken and emulsified into the saline water on the latex card, then slowly mixed with the test latex reagent and checked by rocking in a circular motion for agglutination within one minute. Isolate showing visible agglutination by reacting with the test latex solution was further tested with the control latex

reagent to ensure that the isolate is not an auto-agglutinating strain. Finally, colonies giving a precipitation reaction within one minute, but didn't react with control latex were confirmed as *E. coli* O157:H7 positive.

Antimicrobial susceptibility test

The antimicrobial susceptibility test was performed according to the National Committee for Clinical Laboratory Standards (CLSI, 2018) using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (HI Media laboratories, Mumbai, India) plates prepared according to the manufacturer's recommendation. The bacterial suspension was prepared by adding 2–4 colonies from pure fresh nutrient agar plate to a 5 ml tube containing buffered peptone water and incubated aerobically at 37 °C for 6 hrs. The turbidity of the broth culture was adjusted using sterile saline solution to McFarland 0.5 turbidity standard to obtain desired bacterial concentration (Wayne, 2009). A sterile cotton-tipped swab was immersed into the culture, rotated against the side of the tube above the fluid level to remove the excess fluid, and then swabbed in three directions uniformly on the surface of Mueller-Hinton agar plates. After the plates dried, antibiotic disks were placed on the inoculated plates using sterile forceps. The standard reference strain of *E. coli* ATCC 25922 was used as the control strain.

The antibiotic disks (ampicillin 10µg, gentamycin 10µg, azithromycin30µg, tetracycline30µg, norfloxacin 10µg, chloramphenicol30µg, nitrofurantoin 300µg, trimethoprim-sulfamethoxazole 25µg, cefotaxime 30µg, amikacin 30µg, and nalidixic acid 30µg)were selected based on their potential public health importance, availability of the antimicrobial disks during the research work and the recommendations of the guideline of antimicrobial susceptibility testing (CLSI, 2018). The antibiotic disks were gently pressed onto the Mueller-Hinton agar plates to ensure firm contact with the agar surface and incubated at 37 °C for 16–18 hrs. Finally, the zone of inhibition around the discs was measured to the nearest millimeter using a ruler, and the isolates were classified as susceptible (S), intermediate (I), or resistant (R) based on the critical points recommended by CLSI (CLSI, 2018).

The isolates showing resistance to three or more antimicrobial subclasses were considered as multiple drug-resistant (MDR)

Ethical consideration

The ethical clearance for the study was obtained from the Research Ethical Committee of the College of Agriculture and Veterinary Science of Ambo University, Reference number ASREC/015/Oct.20/2019 Issued on October 20/ 2019.

Data management and analysis

All data generated in this study were entered into the Windows Microsoft Excel spreadsheet and exported to STATA Version 11.0 software (STATA corp. College Station, Texas, USA) for analysis. Descriptive statistics (frequency tables) were used to summarize the prevalence of *E. coli* O157 and the findings of the antimicrobial sensitivity test.

Results

Prevalence of *E. coli* O157:H7 in raw milk

Out of the 262 raw milk samples examined, 65 (24.8%) were positive for *E. coli*. Of the *E. coli* positive samples, four samples tested positive for *E. coli* O157:H7 resulting in an overall prevalence of 1.5% (95% CI: 0.4– 3.8%). Based on the source of samples examined the prevalence of *E. coli* O157:H7 in milk sampled from collection tanks was 9.52% (95% CI: -4.17-23.22) and the prevalence in milk sampled from buckets was1.74% (95% CI: -0.69-4.16), whereas milk sampled directly from the udder was found to be free of *E. coli* O157:H7.

Antimicrobial susceptibility

An antimicrobial susceptibility test was carried out on four *E. coli* O157:H7 isolates using eleven antibiotic drugs. Results of the study showed that all of the four isolates (100%) were highly susceptible to azithromycin, norfloxacin, nitrofurantoin, amikacin, chloramphenicol, tetracycline, nalidixic acid, and trimethoprim-sulfamethoxazole, while, all of them (100%) were seen to be intermediately susceptible to gentamycin. Seventy-five percent (3/4) of the *E. coli* O157:H7 isolates were seen to be highly resistant against ampicillin, similarly, all (100%) of the four isolates demonstrated a high level of resistance(100%) against cefotaxime. Out of the 4 *E. coli* O157:H7 isolates examined, none were resistant to three or more antimicrobials (Table 1).

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Table 1. Antimicrobial susceptibility test results of *E. coli* O157:H7 isolated from raw milk in the study area

Antimicrobial agent and their concentration	Susceptible	Intermediate	Resistant
Amikacin 30 µg	4 (100%)	0 (0%)	0 (0%)
Ampicillin 10 µg	0 (0%)	1 (25%)	3 (75%)
Azithromycin 30 µg	4 (100%)	0 (0%)	0 (0%)
Cefotaxime30 µg	0 (0%)	0 (0%)	4 (100%)
Chloramphenicol 30 µg	4 (100%)	0 (0%)	0 (0%)
Gentamycin 10 µg	0 (0%)	4 (100%)	0 (0%)
Nalidixic acid 30 µg	4 (100%)	0 (0%)	0 (0%)
Nitrofurantoin 300 µg	4 (100%)	0 (0%)	0 (0%)
Norfloxacin10 µg	4 (100%)	0 (0%)	0 (0%)
Tetracycline 30 µg	4 (100%)	0 (0%)	0 (0%)
Trim-sulfamethoxazole 25µg	4 (100%)	0 (0%)	0 (0%)

Discussion

This study was conducted to estimate the magnitude of *E. coli* O157:H7 in raw milk of cows and to determine the susceptibility of the isolates to eleven antimicrobials in Gojo and Shekute towns. The findings of the study showed an overall *E. coli* O157:H7 prevalence of 1.5% which was slightly lower than the prevalence report of 2.9% by Disassa *et al.* (2017) and the 2.4% prevalence stated by Amenu *et al.* (2019). The current prevalence, 1.5%, was by far much lower than the report of 10.4% prevalence by Mekuria and Beyene (2014), 12% by Bedasa *et al.* (2018), and 43.8% prevalence by Ranjbar *et al.* (2018). The lower prevalence observed in this study could be due to better hygienic practices in the farm and milking environment acquired through experience and/or training by the community of the current study area. It could also be due to the low prevalence of the bacteria in the animals. In general, the observed differences in the prevalence of *E. coli* O157:H7 between this and the other studies as well as among the different studies cited above might be attributed to different factors contributing to the contamination of raw milk, such as herd size, management, and husbandry situations, hygienic condition of the dairy farms and milking utensils, hygienic practices before and after milking, hygiene of the feed and water, disinfection customs of farms and bacterial load of the farm environment. (Ashenafi *et al.,* 2020).

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Results of the current study showed that all of the. *coli* O157:H7 isolates were highly susceptible to azithromycin, norfloxacin, nitrofurantoin, amikacin, tetracycline, trimethoprim-sulfamethoxazole, and chloramphenicol. These results were in agreement with previous reports by Bekele *et al.* (2014) and Beyi *et al.* (2017) from Ethiopia. The high susceptibility observed for these drugs might be due to their infrequent veterinary use, implying that such antibiotics can potentially be engaged to treat *E. coli* O157:H7 infections in humans in the study area. On contrary, a study from Asosa town, Ethiopia, reported 81.8%resistant strains to tetracycline, 54.5% to norfloxacin, and 27.3% resistant strains to trimethoprim-sulfamethoxazole (Disassa *et al.,* 2017). Similarly, a report from Saudi Arabia showed resistant *E. coli* O157: H7 strains to nalidixic acid (Naser and Wabel, 2007).

All of the *E. coli* O157: H7 isolates in the present study were found to be resistant to cefotaxime which was in agreement with the report of Naser and Wabel (2007) from Saudi Arabia. This study also showed that 75% of the isolates were resistant to ampicillin, which is in agreement with the findings of Atnafie *et al.* (2017) from Ethiopia. Aynadis and Aweke (2020) reported a higher resistance rate (100%) to ampicillin from Ethiopia. This high level of resistance to ampicillin might be due to its frequent use in the study area in the public health sector. The high prevalence of antimicrobial resistance of E. *coli* O157:H7 strains might be due to inappropriate use of the drugs, under dosage, or usage of the same type of antibiotics for a long time, for either therapeutic or prophylactic or both purposes, in an area.

The use of antibiotics in the treatment of *E. coli* O157:H7 infection is controversial, since antimicrobial therapy may increase the risk of the development of hemolytic uremic syndrome (Molbak *et al.*, 2002). However, an antimicrobial susceptibility test is necessary for better response (Quinn *et al.*, 2011).

Conclusions

The current study finding demonstrateda1.5% prevalence of *E. coli* O157: H7 in raw milk of dairy cattle in Gojo and Shukute towns, Jeldu district of West Shewa Zone, Ethiopia. Given the low infective dose of *E. coli* O157: H7, and the deep-rooted tradition of consuming raw milk by the local community, even the current low prevalence should be considered as an important public health hazard. The resistant *E. coli* O157: H7 strains against ampicillin and cefo-

taxime could pose a serious threat to public health in the study area. Proper hygienic handling of milk and consumption of boiled or pasteurized milk are recommended to reduce the public health risk of *E. coli* O157: H7 in the study area.

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