Isolation, Identification And Antimicrobial Susceptibility Profiles Of *E. Coli* O157: H7 From Raw Cow Milk In And Around Modjo Town, Ethiopia

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Abstract: A cross sectional study was conducted from December 2017 to April 2018 in and around Modjo town, Ethiopia with the objectives of isolating and identifying *Escherchia coli* O157:H7 from raw cow milk, assessing the sources of milk contamination and determining the antimicrobial susceptibility patterns of the isolates. A total of 214 milk and swab samples were collected for laboratory detection of *Escherichia coli* O157:H7. Samples were cultured in selective media to detect the presence of non-sorbitol fermenting (NSF) *Escherichia coli*. Biochemical and serological tests were employed to confirm *E. coli* O157:H7 strain. The overall isolation rate of *E. coli* and *E. coli* O157:H7 were found to be 46.26% and 4.2%, respectively. The highest prevalence was recorded in samples obtained from cafeterias (11.76%) compared with samples from farms (2.78%) and milk collection centers (2.63%). There was statistically significant difference ($p<0.05$) of *E. coli* O157:H7 between sample sources, sample types and sampling points except between farm scale and types of materials (plastic and stainless steel) used to contain milk. All isolated *E. coli* O157:H7 strains were subjected to 10 selected antibiotics using the agar disc diffusion method for their susceptibility patterns. Even though all the isolates were susceptible to ciprofloxacine (100%), most of the isolates showed resistance to more drugs with 100% resistance of all the isolates to ampicillin and cefoxitin. It was concluded that unhygienic practices of milking and post-harvest handling along the dairy value chain contribute to the microbial contamination of milk. The detection of *E. coli* O157:H7 in milk show public health significance due to its zoonotic potential. These findings stress the need for an integrated control of *E. coli* O157:H7 from production to consumption of foods of animal origin, particularly milk which was the focus of this study. So that there should be awareness creation and public education in milk supply chain from production to consumption to limit the milk-borne pathogens resulting from contamination. [Corresponding Author: Negesse Welde, Fufa Abunna, Bihonegn Wodajnew: Isolation, Identification And Antimicrobial Susceptibility Profiles Of *E. Coli* O157: H7 From Raw Cow Milk In And Around Modjo Town, Ethiopia]

Key words: Antimicrobial susceptibility test, Ethiopia, Escherichia coli O157:H7, Isolation, Milk, Modjo

1. Introduction

Foodborne diseases are widespread diseases with a great public health and well-being concerns of individuals and countries of the modern world. Most significantly developing countries are largely affected by foodborne infections (Carbas *et al.,* 2012). Among the major infectious agents, *Escherichia coli* O157:H7 has frequently been associated with foodborne illness. Particularly, over the past decade, *E. coli* O157:H7 has been reported increasingly from all parts of the world as one of the most serious foodborne pathogens leading to severe illnesses and high mortality rates in humans (Blanco *et al.,* 2003; Jo *et al.,* 2004). This is in fact due to the small infectious dose fewer than 40 cells of *E. coli* O157:H7 can have the ability to cause illness in some people (Strachan *et al.,* 2005). These pathogenic shiga toxin producing *Escherichia coli* (STEC) strains are widely associated with both outbreaks and sporadic cases of foodborne disease in humans, ranging from complicated diarrhoea to haemorrhagic colitis, and haemolytic uraemic syndrome (Bach *et al.,* 2002; Blanco *et al.,* 2003).

This strain results in outbreaks of disease in many parts of the world. For example, 9, 652 human

illness was reported from Europe in 2004 due to infection with entero-hemorrhagic *Escherichia coli* (Stein, 2005). In southern Africa and Swaziland in 1992 an outbreak of *E. coli* O157:H7 affecting thousands was happened due to contamination of surface water with cattle dung and animal carcasses. Report from United State of America indicated that an estimated 74,000 cases and 61 deaths annually are attributable to *E. coli* O157:H7 infection. In the 1980s, most outbreaks due to *E. coli* O157:H7 were occurred from inadequately cooked hamburgers and raw milk. Later, outbreaks were traced to other dairy products such as yogurt and cheese (Doyle *et al*., 2006; Mora *et al*., 2007). More recently, in 2016 outbreak of *E. coli* O157:H7, in slaughtered animals were the main sources of infection and cause illness to eleven people in United State of America (CDC, 2016).

Escherichia coli O157:H7 has been found in the intestinal tract of asymptomatic ruminants. However, cattle have been identified as a major reservoir of *E. coli* O157:H7 and zoonotic transmission to humans usually occurs through consumption of contaminated foods of bovine origin such as beef and dairy products (Acha and Szyfress, 2001; IFT (Institute of Food Technology), 2003; Perelle *et al*., 2007). Fecal contamination of other food products or direct contact with infected animals can also lead to human infection. Milk and milk products are among the most common sources of STEC O157:H7 infection mainly due to fecal contamination (Armstrong *et al.,* 1996).

The consumption of raw milk and its derivatives is common in Ethiopia, which is not safe from a consumer health point of view as it may lead to the transmission of various diseases (Shunda *et al.,* 2013). The ability of raw milk to support the growth of several pathogenic microorganisms can lead to spoilage of the product, which cause infections in consumers. There are many commonly known bacterial pathogens still of concern today in raw milk and other dairy products including *Escherichia coli* (Yilma and Faye, 2006; Abera, 2008; Oliver *et al.,* 2009) and *E. coli* O157:H7 serotype was the focus of this study.

Different studies conducted in Ethiopia revealed fragmented substantial prevalence of *E. coli* O157:H7. Particularly, the current study is the first of its kind since no single study was conducted in the present study area pertaining to *E. coli* O157:H7 from milk at farm levels, milk collection centers and cafeterias. Moreover, studies on isolation, identification and antimicrobial susceptibility tests were not conducted in the current study site. However, the screening of milk for pathogenic organisms will play a vital role in restricting human infection. So that, investigation on the isolation, identification and antimicrobial resistance patterns of *E. coli* O157:H7 from cattle have

paramount importance to design methods that minimize the possible transmission of the agent between humans and cattle. Moreover, it is also important in minimizing the risk of increasing the emergence of antibiotic resistant *E. coli* O157:H7 serotype.

Therefore, the objectives of the present study were:

➢ To isolate and identify *E. coli* O157:H7 from raw cow milk in selected dairy farms, milk collection centers and cafeterias at Modjo town,

➢ To assess the source of milk contamination and to determine to what extent the milking activities and milk equipment act as a source of contamination, and

➢ To determine the antimicrobial susceptibility patterns of *E. coli* O175:H7.

2. Materials And Methods
2.1. Description of the study area
The study was conducted at Modjo town located in East Shoa Zone of the Oromia Region about 66km south east of Addis Ababa. It lies at latitude 8°35'N and longitude 39°7'E at an elevation of 1790 meters above sea level. The area gains rainfall twice a year those known as long and short rainy seasons. The main rainy season extends from June to September. The average annual rainfall, temperature, and mean relative humidity are 776mm, 19.4°C and 59.9%, respectively (CSA, 2005).

2.2. Study population
The study populations were apparently healthy lactating cows in selected dairy farms in and around Modjo town. Even though the main sample was milk from dairy cows at farm level, the investigation included different study units such as milk collection centers, cafeterias and swabs from milkers' hand and milk equipment along the dairy value chain of the town. The samples were bulk milk from farms, milk collection centers and cafeterias. Swabs from milkers' hand, buckets and milk tank were integrated in the study. Types of milk storage containers were also considered in the investigation. The inclusion criteria of the study were based on the availability of milk during the time of sample collection and willingness of the appointed body.

2.3. Study design
A cross sectional study was conducted from December 2017 to April 2018 to isolate and identify *E. coli* O157:H7 from raw cow milk in the dairy value supply chain, to assess the source of contamination and to determine the antimicrobial resistance profiles of *E. coli* O157:H7. First, baseline information was obtained from Modjo livestock and fishery resource development to identify the total number of farms and farm size, milk collection centers and cafeterias in and

around the town. According to the documented information obtained from Modjo livestock and fishery resource development there was more than 100 small holder (<5 cows), 37 medium scale (5 to 30 cows) and 8 large scale (>30 cows) were identified. Survey was also done for two days with the help of animal health assistant professional to familiarize the study area and each study units.

2.4. Sample size determination

The sample size was calculated with an expected prevalence of 83.3% from a previously published research work by Alehegne (2004) on prevalence of *E. coli* from milk at Bishoftu. The desired sample size for the study was determined by using the formula described by Thrusfield (2005) as follows:

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

$$n = \frac{(1.96)^2 \, 0.833(1 - 0.833)}{0.05^2} = 213.76$$

Approximately, n=214

Where; n is the sample size, Z (1.96) is the statistic corresponding to level of confidence 95%, P is the expected prevalence and d is precision which was taken as 5%. Therefore, a total of 214 samples were collected.

2.5. Method of data collection

2.5.1. Sampling methods and procedures

Based on the information obtained, sampling strategy was done as follow. First the farms were stratified into stratum according to their scale (small, medium and large) based on herd size. Then the farms were randomly selected from the groups for the study. A total of 25 farms (12 small, 8 medium and 5 large), 3 milk collection centers and many cafeterias were identified as major sources of sample since they are the main supplier of milk for consumers in the town. A simple random sampling technique was applied to collect raw milk and swab samples from each selected dairy farm. A purposive sampling approach was used during selection of sampling units (milk collection center and cafeteria). This sampling approach was to find people who are willing to provide sample. Accordingly, in the present study a total of 214 samples were collected aseptically using sterile test tubes (144 from farms, 38 from milk collection centers and 32 from cafeterias).

2.5.2. Sample collection and transportation

Raw cow milk samples were collected from farm, milk collection centers and cafeterias for isolation and identification of *E. coli* O157:H7. At farm level bucket and milkers' hand swab also included in the sampling procedure. Samples were properly coded based on sample source, farm scale, sample type, sampling point and type of materials. Types of samples collected in quantity were 169 raw milk and 45 swabs (swabs from milkers' hand, bucket, milk collection center and cafeterias). Upon arrival, samples were processed separately by pre-enriching in buffered peptone water. Then further processes were followed after samples were incubated for 24 hours. The samples were collected aseptically in sterilized bottles and kept in icebox with ice packs. Then samples were transported from the study area to microbiology laboratory of Addi Ababa University College of Veterinary Medicine within four hours of collection.

Bacterial isolation and identification were conducted as described by (APHA, 1992) and it had kept in refrigerator at 4°C until the time of analysis and culturing was conducted within 24 hours as described by (Quinn *et al.,* 004). Approximately, 20ml of milk was collected into sterile sampling bottles while for swab samples, sterile cotton swabs with breakable stick were used by immersing it in a tube containing 10ml of dilution fluid, buffered peptone water (Annex 1), for sampling and transporting the swab samples (ISO, 18593:2004).

2.6. Study methodologies and laboratory analysis

2.6.1. Isolation and identification of Escherichia coli and Escherichia coli O157:H7

Laboratory analyses were carried out in microbiology laboratory at Addis Ababa University College of veterinary medicine. Two kinds of laboratory analyses were performed. The first was analysis for isolation and identification of *E. coli* O157:H7 both in raw milk and milk associated equipment samples. The second was determination of antimicrobial susceptibility pattern of *E. coli* O157:H7 isolates to most commonly used antimicrobials.

Isolation was done as described below. One milliliter of each raw milk sample was enriched using 9ml buffered peptone water (Sisco Research Laboratories Pvt. Ltd., Mumbai, India) and incubated at 37°C for 18-24 hours after proper homogenization for 1 minute. Similarly swab sample in 10ml buffered peptone water was incubated at 37°C for 18-24 hours as described by (Quinn *et al.,* 2004). This is because non selective pre-enrichment is necessary for the effective recovery of low levels of stressed *E. coli* and *E. coli* O157 strains. After that the enrichment broths were pre-warmed to prevent cold shocking of the organisms and slowing their initial growth as described by (Clifton-Hadley, 2000).

A loop full of milk aseptically taken from all of the sample bottles and the swabs from their respective enrichment bottles were inoculated on MacConkey agar (Oxoid Ltd., Hampshire, England) for primary

isolation of *E. coli* and incubated aerobically at 37°C for 24 hours. The plates were observed for the growth of *E. coli* (pink colony or lactose fermenter). A single isolated colony was picked and subcultured on Eosin Methylene Blue (EMB) agar plates (Oxoid Ltd., Hampshire, England) for the formation of metallic sheen (Annex 6, figure 1). Simultaneously another single colony with similar characteristics was picked and stained with Gram's stain (Annex 2). The isolate was examined for stain and morphological characteristics using microscope. Suspected colonies of *E. coli* (pinkish color appearance on MacConkey agar and metallic sheen on Eosin Methylene Blue agar were then subcultured onto nutrient agar (Huanki, Ltd., Guangdong, China). Brain heart infusion agar medium (Sisco Research Laboratories Pvt. Ltd., Mumbai 93 India, BM 018, Lot 12053944) was used to preserve the isolates.

The major biochemical tests performed during the analyses were indole production, methyl red (MR), voges proskaur (VP), simmon's citrate and oxidase test on peptone water, methyl red-voges proskaur medium and simon citrate agar, respectively as described by (ISO, 2003) except oxidase test which is done on oxidase strip reagent paper. Then the bacterium that was confirmed as *E. coli* was subcultured onto sorbitol macConkey agar (Oxoid Ltd., Hampshire, England) from nutrient agar (Huanki, Ltd., Guangdong, China) and plates were incubated at 35°C for 20 to 22 hours. *E. coli* O157:H7 does not ferment sorbitol and, therefore, produces colorless colonies (Annex 6, figure 2). In contrast, most other *E. coli* strains ferment sorbitol and form pink colonies (Soomro *et al.*, 2002). Later on the confirmed pure cultures considered as both *E. coli* and *E. coli* O157:H7 were transferred to nutrient agar to be used for additional confirmatory biochemical tests (Quinn *et al.*, 2002) and non-sorbitol fermenting (colorless) colonies from sorbitol subjected to serological test (Annex 3).

2.6.2. Serological test

All non-sorbitol fermenting colonies from the sorbitol macConkey agar were serologically confirmed by using *E. coli* O157:H7 latex agglutination assay containing latex particles (beads) coated with antibodies specific for the *E. coli* O157 and the *E. coli* H7 antigen on the test organisms, forming a visible antigen antibody precipitate as described by (DeBoer and Heuvelink, 2000). Identification of *E. coli* O157:H7 was carried out following the manufacturer's instructions (Annex 3); hence colonies that agglutinated were considered to be *E. coli* O157:H7 (Annex 6, figure 4). These non-sorbitol fermenting colonies were assayed with a dryspot *E. coli* O157 latex test kit (Oxoid), *E. coli* H antiserum H7 (BD Difco, Beckton, Dickinson, USA), and agglutination

test. Colonies that displayed agglutination in the dryspot *E. coli* O157 latex test kit were considered to be *E. coli* O157, while colonies that displayed agglutination with *E. coli* H antiserum H7 were considered to be *E. coli* O157:H7 as described by (March and Ratnam, 1989).

2.6.3. Antimicrobial susceptibility test of Escherichia coli O157:H7

The antimicrobial susceptibility test was performed according to the Kirby-Bauer (1966) disc diffusion method using 10 commercially available antimicrobial disks (Annex 4). Mueller Hinton (MHI) agar media (Oxoid, Ltd., Hampshire, England) and sterile saline solution were used as a culture media and for inoculum preparation, respectively. All the isolated *E. coli* O157:H7 strains were tested for sensitivity to most commonly used antimicrobials including, gentamicin (GEN) (10μg), ampicillin (AMP) (25μg) (Himedia, India), cefoxitin (FOX) (30μg) (Oxoid, UK), ciprofloxacin (CIP) (5μg), sulfamethoxazole (RL) (100μg) (Oxoid, UK), trimethoprim (TR) (25μg) (Himedia, India), streptomycin (S) (10μg) (Oxoid, UK), doxycycline (DO) (30μg) (Oxoid, UK), tetracycline (TE), (10μg) (Oxoid, UK) and nalidixic acid (NA) (30μg). The inoculums were uniformly streaked on each respective agar plates. Then antimicrobial discs were applied and plates were incubated at 35°C for 18-24 hours. Thereafter, results were interpreted according to Clinical and Laboratory Standards Institute (CSLI) (2012) 'interpretive criteria for Enterobacteriaceae (Annex 5).

2.7. Data management and analysis

The data obtained at the time of study were classified, entered, filtered and coded using Microsoft Excel® 2010 spreadsheet. Then the data subjected to chi-square and fisher's exact test in order to assess the association between comparable variables by making use of STATA version 12 (Stata Corp. Texas, USA) for appropriate statistical analysis. Before subjected to statistical analysis, the data were thoroughly screened for errors and improper coding. For antimicrobial susceptibility test the results will be interpreted according to Clinical and Laboratory Standards Institute (CLSI, 2012), interpretive criteria for Enterobacteriaceae.

3. Results

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

In the present study, the overall prevalence of *E. coli* and *E. coli* O157:H7 out of 214 examined samples were found to be 99(46.26%) and 9 (4.2%), respectively (Table 1). Among the sample sources, the highest isolation of *E. coli* was from cafeterias (71.88%), followed by milk collection centers (65.79%) and farms (35.42%). The isolation rate of *E.*

coli based on sample types were 55.56% and 43.79% for swab and raw milk sample, respectively. The distributions of *E. coli* at farm level were 33.33%, 38.10% and 34.72% in small, medium and large scale, respectively. The type of materials that used to contain milk was also been considered and the prevalence were found 57.80% in plastic materials and 18.33% in stainless steels. With regard to, sampling point *E. coli* isolates were found 43.79%, 60%, 58.33%, 45.45% and

57.14% for bulk milk, and swabs from milkers' hand, milking bucket, cafeteria and milk collection center equipment, respectively (Table 2). The highest isolation was from milkers' hand swab and bucket swab followed by swab from milk collection center equipment. The isolation rate of *E. coli* associated with sample source and type of container used was found statistical significant (p<0.05) (Table 2).

Table 1: The overall prevalence of *E. coli* and *E. coli* O157:H7

Isolates	Number of samples examined	Positive	Prevalence (%)
Escherichia coli	214	99	46.26
Escherichia coli O157:H7	214	9	4.2

Table 2: Isolation rate of *E. coli* in different study categories and sampling units in the study area

Variables	No of samples examined	No of *E. coli* Positives (%)	χ2/fisher's exact test	p-value
Sample source				
Dairy farms	144	51 (35.42)		
Milk collection centers	38	25 (65.79)	21.0861	0.000
Cafeterias	32	23 (71.88)		
Sample type				
Raw milk	169	74 (43.79)		
Swab	45	25 (55.56)	1.9798	0.159
Sampling point				
Bulk milk	169	74 (43.79)		
Milkers' hand swab	15	9 (60.00)		
Milking bucket swab	12	7 (58.33)	-	0.626^F
Cafeteria swab	11	5 (45.45)		
Collection center swab	7	4 (57.14)		
Farm scale				
Small	30	10 (33.33)		
Medium	42	16 (38.10)	0.2038	0.903
Large	72	25 (34.72)		
Type of container				
Plastic	109	63 (57.80)		
Stainless steel	60	11 (18.33)	24.4867	0.000
Total	**214**	**99 (46.26)**		

0.626^F indicate the value from fisher's exact test

Out of 214 samples, 9(4.2%) were contaminated by *E. coli* O157:H7. The isolation rate of this pathogenic strain among the sample sources were 11.76% from cafeterias, 2.63% from milk collection centers and 2.78% from dairy farms. Their isolation rates based on sample types were found to be 11.11% and 2.37% for swab and milk sample, respectively. The distribution of *E. coli* O157:H7 at farm level were 0%, 4.76% and 2.78% in small, medium and large scale, respectively. During the study time the type of materials that used to contain milk also considered and

the prevalence were found to be 3.67% and 0% in plastic and stainless steel, respectively. With regard to sampling point, *E. coli* O157:H7 isolates were found 2.37%, 6.67%, 16.67%, 8.18% and 0% for bulk milk, and swabs from milkers' hand, milking bucket, cafeteria and milk collection center equipment, respectively (Table 3). The isolation rate of *E. coli* O157:H7 associated with sample source, sample type and sampling point was found statistical significant in fisher's exact test (p<0.05) (Table 3).

Table 3: Isolation rate of *E. coli* O157:H7 in different study categories and sampling units in the study area.

Variables	No of samples examined	No *E. coli* O157:H7 Positives (%)	Fisher's exact test
Sample source			
Dairy farms	144	4 (2.78)	
Milk collection centers	38	1 (2.63)	0.060
Cafeterias	32	4 (11.76)	
Sample type			
Raw milk	169	4 (2.37)	
Swab	45	5 (11.11)	0.022
Sampling point			
Bulk milk	169	4 (2.37)	
Milkers' hand swab	15	1 (6.67)	
Bucket swab	12	2 (16.67)	0.020
Cafeteria swab	11	2 (18.18)	
Collection center swab	7	0 (0)	
Farm scale			
Small	30	0 (0)	
Medium	42	2 (4.76)	0.667
Large	72	2 (2.78)	
Type of container			
Plastic	109	4 (3.67)	
Stainless steel	60	0 (0)	0.298
Total	**214**	**9 (4.2)**	

3.2. Antimicrobial susceptibility pattern of *E. coli* O157:H7

The study on antimicrobial sensitivity of *E. coli* O157:H7 revealed a varying degree of susceptibility to antimicrobial agents used. The isolates were found highly susceptible to ciprofloxacine, trimithoprim and nalidixic acid whereas highly resistance to ampicillin and cefoxitin followed by tetracycline, streptomycine and doxycycline.

Table 4: Antimicrobial susceptibility profiles of *E. coli* O157:H7

Type of drugs	Number (%) of *E. coli* O157:H7		
	Susceptible	Intermediate	Resistant
Ampicillin	0 (0%)	0 (0%)	9 (100%)
Sulphamethoxazole	4 (44.5%)	3 (33.3%)	2 (22.2%)
Cefoxitin	0 (0%)	0 (0%)	9 (100%)
Gentamycin	5 (55.6%)	2 (22.2%)	2 (22.2%)
Ciprofloxacin	9 (100%)	0 (0%)	0 (0%)
Streptomycin	0 (0%)	2 (33.33%)	7 (77.7%)
Doxicycline	0 (0%)	3 (33.3%)	6 (66.7%)
Trimethoprim	8 (88.9%)	0 (0%)	1 (11.1%)
Tetracycline	0 (0%)	2 (22.2%)	7 (77.8%)
Nalidixic acid	7 (77.8%)	2 (22.2%)	0 (0%)

4. Discussion

The presence of *E. coli* in milk is an indicator of fecal contamination of milk and other milk associated equipment. Contamination of milk with *E. coli* could be from direct fecal contact, contaminated water, equipment and milkers' hand. The overall prevalence of *E. coli* in this study was found to be 46.26%, with 43.79% from raw milk sample only. This is an indicator of the likelihood of the occurrence of the pathogenic strain, *E. coli* O157:H7 in milk and other samples. In the current study both pathogenic strain and non-pathogenic *E. coli* were isolated. However, non-pathogenic *E. coli* was used only as an indicator of milk contamination with this pathogen. So that for the none pathogenic *E. coli* only the prevalence was stated with respect to the associated risk factors and categories, but *E. coli* O157:H7 was discussed in detail as follow since it was the focus of the current study as a result of its great public health concern.

In the current study, the overall isolation rate of *E. coli* O157:H7 was found to be 9(4.2%) suggesting that there is higher contamination of milk. Similarly, *E. coli* O157:H7 isolated from raw milk was 4(2.37%) out of 169 milk samples. The remaining 5 isolates were from swab samples. According to the results indicated in (Table 3), a greater prevalence was observed in samples collected from cafeterias (11.76%) compared to those collected from the milk collection centers (2.63%) and farms (2.78%), which were in line to the report of (Nigatu *et al*., 2017) with the highest prevalence was recorded in samples obtained from vendors (5%) than samples collected from farmers (0.6%) in traditionally marketed raw cow milk in and around Asosa town, Western Ethiopia. This could be due to the presence of somewhat similar hygienic activities from production to distribution. The highest prevalence of the pathogen in cafeterias could be due to the need of longer time for milk to reach cafeterias at ambient temperature under poor hygienic conditions which support the growth of the bacteria in the milk samples and use of inadequate type of equipment that increase the chance of the pathogen recovery in milk.

The prevalence of *E. coli* O157:H7 was also higher in swab samples (11.11%) compared to those from raw milk samples (2.37%) meaning that the contamination had originated from unhygienic practices, which endangers the health of raw milk consumers. This high isolation rate of *E. coli* O157:H7 in milk and swab sample poses a threat to milk consumers. Although the isolation rate of *E. coli* O157:H7 showed significant difference with regard to sample sources, types of sample and sampling points in fisher's exact test (p<0.05), it didn't reflect any significant difference with regard to farm scale and types of milk storage equipment (plastic and stainless) (p>0.05). This could be due to similar management and hygienic practices irrespective to farm scale.

Even though the pathogenic strain was not isolated from stainless steel, its prevalence in milk samples collected and stored in plastic containers was found to be 3.67% (Table 3) which was in agreement with the report of (Nigatu *et al*., 2017) who indicated expected prevalence of 3.5% in plastic containers from raw cow milk. This indicated that higher rate of contamination was detected in the samples collected from milk held in plastic containers than stainless steel. In contrast to 1.6% prevalence of *E. coli* O157:H7 in milk sample collected from stainless steel materials by (Nigatu *et al*., 2017), the current study couldn't find such pathogen in milk sample collected from this type of materials. This could be due to the smaller samples of milk kept in stainless steel, the variation in cleaning practices of milk storage materials and the difference in bacterial contamination of water by animal feces in these two different study areas.

Many reports from various regions of the word provided different prevalence rates of *E. coli* O157:H7 from raw milk sample. Some of the reports of the world on the prevalence of these pathogenic strains were in line with the current study results whereas others showed smaller or higher expected prevalence. The current study finding from raw milk (2.37%) was in agreement with the research work of different countries reported as 2.6% from Egypt (Arafa and Soliman, 2013), 2.0% again from Egypt (Abdel Khalek *et al*., 2001), and 2.0% from Ogun State, Nigeria (Ivbade *et al*., 2014) from raw milk. These could be due to having somewhat similar milking and milk handling practices along the dairy value chain. The report of (Nigatu *et al., 2*017) as 2.9% expected prevalence from traditionally marketed raw cow milk in and around Asosa town was also a little bit analogous to the current result. These similar findings could be due to the presence of somewhat related milk production, milk handling, storage, distribution and hygienic practices irrespective to the regions.

However, lower isolation of *E. coli* O157:H7 was reported as 1% (Omore *et al*., 2001) isolated in milk samples from marketing survey in the Kenyan highlands and 1% again in Egypt (Amer and Soliman, 2004) from raw milk. In contrast to the current finding, higher isolation of *E. coli* O157:H7 from raw milk was reported as 8% in Ethiopia Hiko *et al*. (2008), 12% in Bishoftu (Bedasa *et al*., 2018) from raw milk, 6.9% in Holeta (Aylate *et al*., 2013) from the study on the prevalence of subclinical mastitis in lactating cows, 8.3% from Iran (Hashemi *et al.,* 2010), 9.6% again from Iran (Tahamtan *et al.,* 2006), 3% from Austria (Allerberger *et al.,* 2001), 33.5% from Malaysia (Chye *et al.,* 2004) in raw milk samples, 27.08% from Basrah City-Iraq (Abbas *et al.,* 2012), 3.9% from Germany (Klie *et al.,*1997) and 6% from Egypt (Abdul-Raouf *et al.,*1996) from raw milk. These higher isolation rates may be because of higher risk of contamination during production, greater chance of contact with fecal materials, unhygienic activities, the use of contaminated water and improperly cleaned milk containers, transporting and storing milk with temperature that is suitable for bacterial recovery and improper handling of milk after milking.

Interestingly, in contrast to this study findings and the prevalence of *E. coli* O157:H7 in different parts of the world, some previous studies also reported as negative result for this pathogenic strain. For example, (Swai and Scotchman, 2011) did not isolate *E. coli* O157:H7 in milk. In the study by (Addo *et al*., 2011) in Ghana reported negative results in all 250 milk samples tested. *E. coli* O157:H7 also was not isolated by (Can *et al*., 2015) from raw milk samples. This could be as a result of the presence of strict sanitary measures or policy in these regions. The

actors in milk production, collection and distribution may also have awareness on the hygienic practices that must be performed in milk production.

Generally, there is great variation in the prevalence of *E. coli* O157:H7 in dairy value chain, across the regions, and in different studies. These differences may arise from various reasons. The variation in the prevalence of *E. coli* O157:H7 in the dairy value chain might be due to the time taken and mishandling during transportation. However, the variation in animal production and management, hygienic activities, milking system and milk handling practices might be the sources for the difference in frequency of isolates across the regions. The variation that was seen in prevalence 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 as stated by (Soomro *et al*., 2002). The presence of *E. coli* may not necessarily indicate a direct fecal contamination of milk, but it is an indicator of poor hygiene and unsanitary practices during milking and further handling of milk (Arafa and Soliman, 2013).

In this study, the methods of production, transportation, handling, and sale of milk were found prone to contamination. Hence, milk can be easily contaminated from different sources including the contaminated udder, milk handlers with poor personal hygiene, water of poor quality, and inappropriately cleaned or sanitized containers, all of which contribute to milk contamination as described by (Chye, 2004; Ali, 2011; Arafa and Soliman, 2013).

The use of antibiotics in the treatment of *E. coli* O157:H7 infection is controversial, since antimicrobial therapy may increase the risk of development of hemolytic uremic syndrome (Molbak *et al*., 2002). Although some studies do not advise antibiotic treatment for infections caused by such bacteria, others suggest that disease progression may be prevented by administering antibiotics during the early stage of infection (Schroeder *et al*., 2002). Thus, for the better response, an antimicrobial susceptibility test is necessary (Quinn *et al*., 2011). Hence, on the basis of this necessity, antimicrobial susceptibility testing was conducted on the isolates recovered from all the samples.

Drug susceptibility test was done on each *E. coli* O157:H7 isolates against ten commonly used antimicrobials and the isolates were characterized as susceptible, intermediate and resistant based on the size of zone of inhibition (National Committee for Clinical Laboratory Standards (NCCLS), 2004). In this study, all the isolates of *E. coli* O157:H7 were highly susceptible to ciprofloxacin and trimethoprim followed by nalidixic acid and gentamicin. According

to the test results most of the *E. coli* O157:H7 (≥50%) isolates were resistant to ampicillin and cefoxitin (100%), streptomycin and tetracycline (77.7%) and doxycycline (66.7%).

All isolate of the current study was resistance to ampicillin (100%) that was somewhat in line with the report of (Bedasa *et al*., 2018) from Bishoftu. Similar findings were reported by (Mora *et al.,* 2005; Srinivasan *et al.,* 2007; Taye *et al*., 2013). All *E. coli* O157:H7 isolates were also found resistance to cefoxitin which was in slight agreement with the report of (Nigatu *et al*., 2017) from Asosa town. This resistance nature of isolates might be due to the use of inappropriate antibiotics for the treatment of diseases (Sharada *et al*., 2010) and excessive use of antimicrobials for therapeutic and prophylactic treatment (Majalija *et al*., 2010). In the current study most of the isolates were resistance to tetracycline (no single susceptible isolate were found) which was in line with the report of (Nigatu *et al*., 2017). Comparable to this, various authors also reported that *E. coli* is resistant to tetracycline (Hiko *et al*., 2008; Bekele, 2012; Mude *et al*., 2017). In contradict to this (Bedasa *et al*., 2017) from Asosa and (Mohammed *et al*., 2014) from Dire Dawa reported that most of the isolates were susceptible to tetracycline.

In comparable to the present study with none of the isolates were found susceptible to streptomycin, (Nigatu *et al*., 2017) and (Bedasa *et al*., 2018) have reported high resistance rate of 81.8% and 85.7%, respectively to this drug. All of the isolates of this study were highly susceptible to ciprofloxacin which was comparable to the report of (Bedasa *et al*., 2018) with 6 out of 7 isolates were susceptible to this antibiotic. In contrast to the present study (Bedasa *et al.,* 2018) reported that none of the isolates were resistance to gentamycine. According to the report of (Nigatu *et al*., 2017) 44.44% of the isolate were resistance to this drug which was somewhat higher than the current finding (22.22%). This variation probably attributed to the expression of resistant gene code by the pathogen which associated with emerging and re-emerging aspects of the isolates with regards of different agro-ecology (Reuben and Owuna, 2013).

As described above in the present study, most isolates have multiple drug resistance. This finding potentially implies the presence of a serious drug resistance problem. This multidrug resistance occurred might be due to administration of different antibiotics for prophylaxis or infection, indiscriminate use of antibiotics in the farms and another possibility is that cows are being treated with antibiotics for other conditions, thereby selecting for resistant populations of *E. coli* O157:H7. Such multidrug resistance may apparently be occurred which may ultimately replace the drug sensitive microorganisms from antibiotic

saturated environment (Van De Bogaard and Stobberingh, 2000). In addition to these, wrong dosage, wrong routes of administration, arbitrary drug combinations, imprudent use of drugs, and poor awareness on antimicrobial residues in animal products and drug withdrawal period might lead antimicrobial resistance development such comparable justifications were also declared by (Sharma *et al.*, 2012).

The prevalence of antimicrobial resistant bacteria has increased worldwide. Resistance rates vary across countries because of variation in antimicrobial agent usage and programs for the prevention of antimicrobial resistant bacteria and level of awareness in dairy producers (CDC, 2013). Evidence has also been indicated that resistant strains of pathogens can be transmitted to humans through food (Khachatourians, 1998). Therefore, raw milk apart from the potential source of food borne bacterial pathogens, it can also cause severe health risk to consumers due to antimicrobial residues.

6. Conclusion And Recommendations

Milk produced, distributed, and supplied to consumers in the study area contains a potential source of milkborne pathogens which is hazardous to public health. Much of the milk supplied to consumer in the town was managed under poor hygiene. The presence of *E. coli* O157:H7 in foods of animal origin may arise from unhygienic conditions during processing, handling and distribution. The results obtained in this study showed that the raw milk produced and marketed at Modjo town has high bacterial contamination. The isolation rate was also increased through the milk value chain. The sources of this pathogen in the raw cow milk may be from poor hygienic and sanitation practices during milking, contaminated udders, water and containers, lack of cooling facilities and milk handlers themselves. In addition to these, milk storage equipment contributes for the quality of milk, by which stainless steel was preferred for storage than plastic containers. The presence of *E. coli* O157:H7 in raw cow milk samples showed that the produced milk is of poor quality and have public health risk to the consumer. In general, *E. coli* O157:H7 was found as major contaminants of milk in the study area.

Moreover, the current study also indicated that *E. coli* O157:H7 isolates were resistant to most of the antimicrobials which may aggravate the infections by this pathogenic strain in the future. The higher prevalence of multidrug resistant *E. coli* O157:H7 isolates in dairy products is especially alarming. This resistance nature of the isolates may cause risk for animal health, public health, production and food safety. So that this study findings indicated that the

milk production activities in the dairy value chain starting from production at farm level to the consumption needs strict hygienic practices and the need of rational use of antimicrobials to reduce risk of multidrug resistance which is the big problem of the modern world.

Therefore, based on the above conclusion the following recommendations are forwarded:

➢ There is a need to improve the quality and safety of milk that produced and marketed in the study area through awareness creation on hygienic practices and handling of raw milk.

➢ Provision of training and awareness creation for producers, milk collectors, milk retailers and consumers about milk handling practices and the risk of milk borne diseases.

➢ Adequate sanitary measures should be taken at all stages of milk supply chain from production to consumption to provide wholesome dairy products to the society.

➢ Raw milk should be boiled before consumption.

➢ There should be a comprehensive educational program about the potential hazards associated with *E. coli* O157:H7 infection and antibiotic resistance in foods of animal origin.

➢ There is a need to reduce the source of contamination at any stages from production to consumption and utilize the proper type of milk storage materials to minimize the risk of contamination.

➢ Monitoring the use of antibiotics in animal husbandry and human therapy is necessary to minimize the development of antibiotic resistance and to keep these valuable drugs functional.

➢ Dairy producers should also adopt and be awakened on proper drug withdrawal period for milk to be consumed.

➢ Preventing fecal material from contaminating milk is an important to reduce the prevalence of *E. coli* O157:H7 in raw milk.

7 List Of Annexes

Annex1: Media preparation, use and storage

1) Buffered peptone water

The medium (Sisco research laboratories Pvt. Ltd. 26, Navketan Indi., Andheri (E), Mumbai 93, India, BM 020) is composed of pancreatic digest of casein (10 g/l), sodium chloride (5 g/l), disodium phosphate (3.5 g/l) and dihydrogen potassium phosphate (1.5 g/l) and final of pH 7.2 ± 0.2 at 25°C. It was prepared according to manufacturer's instructions by which 20 g of the powdered medium was suspended in one liter of distilled water. It was mixed well and dissolved by heating with frequent agitation and boiled for one minute until completely

dissolved. The medium was dispended into the conical flask with aluminum foil stopper and then sterilized by autoclaving at 121°C for 15 minutes and cooled to 25°C before use.

1ml of each raw milk sample was enriched using 9ml buffered peptone water and incubated at 37°C for 18-24 hours after proper homogenization for 1 minute. Similarly swab sample in 10ml buffered peptone water was incubated at 37°C for 18-24 hours. This is because non selective pre-enrichment is necessary for the effective recovery of low levels of stressed *E. coli* and *E. coli* O157 strains. After that the enrichment broths were pre-warmed to prevent cold shocking of the organisms and slowing their initial growth. All the unused prepared media were stored under refrigeration temperature.

2) MacConkey agar

The medium (Sisco research laboratories Pvt. Ltd. Plant Site 1: D-88/2, MIDC, Turbhe-400705, New Mumbai. India, MM 011) is composed of peptone (17 g/l), agar (13.5 g/l), lactose (10 g/l), bile salts (1.5 g/l), sodium chloride (5 g/l), neutral red (0.03 g/l), agar (12 g/l), crystal violet (0.001) and final of pH 7.4 ± 0.2 at 25°C. The medium was prepared according to the manufacturer's instructions by which 50.03 g of the powdered medium was suspended into one liter of distilled water. It was boiled to dissolve completely and followed by sterilization by autoclaving at 121°C for 15 minutes, and cooled to below 45°C-50°C and poured onto separate of sterile Petri dishes. The medium was allowed to solidify in plates for at least 30 minutes prior to use and then put upside down in the refrigerator temperature until used.

1. Eosin Methylene Blue (EMB) agar

Eosin Methylene Blue (EMB) agar (Levine) (Oxoid®, Ltd., Basingstoke, Hampshire, England, CM0069, Lot 1043112) is composed of peptone (10 g/l), lactose (10 g/l), dipotassium hydrogen phosphate (2 g/l), eosin Y (0.4 g/l), methylene blue (0.06 g/l), agar (15 g/l) and final pH of 6.8 ± 0.2 at 25°C. It was prepared by Suspending 37.5 g in one liter of distilled water. Then it was brought to boil to dissolve completely and sterilized by autoclaving at 121°C for 15 minutes. Furthermore the medium was made cool to 60°C and it was shaken the in order to oxidize the methylene blue, to restore its blue colour, and to suspend the precipitate which was an essential part of the medium. Thereafter, the medium was poured into plates and allowed to solidify at room temperature, and stored upside down at 4 to 8°C until subsequent conventional use.

2. Sorbitol MacConkey (SMA) agar

Sorbitol MacConkey (SMA) agar (Oxoid®, Ltd., Basingstoke, Hampshire, England, CM0813, Lot 1116827) is composed of peptone (20 g/l), sorbitol (10 g/l), bile salts No.3 (1.5 g/l), sodium chloride (5g/l),

neutral red (0.03 g/l), crystal violet (0.001 g/l), agar (15 g/l) and final pH of 7.1 ± 0.2 at 25°C. It was prepared according to manufacturer's instruction, whereby 51.5g powder medium was suspended in one liter of distilled water and brought to the boil to dissolve completely. Then it was sterilized by autoclaving at 121°C for 15 minutes. Thereafter allowed to cool to 50°C and Poured into sterile Petridishes, and lastly allowed to solidify at room temperature, and stored upside down at 4 to 8°C, refrigerator until used.

3. Nutrient agar

The medium NA (Huanki®, Ltd., Guangdong, China, CM022020, and Lot 3104856) is composed of peptone (5 g/l), beef extract (3 g/l), sodium chloride (5 g/l), agar (15 g/l) and final pH of 7.3 ± 0.2 at 25°C. The medium was prepared according to the manufacturer's instructions whereby 33 g of the powdered medium was suspended in 1 liter of distilled water in flasks, heated with frequent agitation and boiled for 1 minute to completely dissolve the powder. Then the mixed solution was sterilized by autoclaving at 121°C for 15 minute an allowed to cool to 45°C-50°C. Subsequently, the medium was dispensed into plates. The agar medium was left to solidify and petri dishes were stored upside down in refrigerator.

4. Methyl Red and Voges-Proskauer (MR-VP) medium

MR-VP Medium (Himedia Laboratories Pvt., Ltd., Mumbai-400086, India, M070, Lot 0000219697) is composed of buffered peptone (7g/l), dextrose (5 g/l), dipotassium phosphate (5g/l) and final pH 6.9 ± 0.2 at 25°C. It was prepared according to manufacturers' instruction by which 17 grams of powder was suspended in one liter of distilled water. The medium was heated (if necessary) to dissolve it completely and then sterilized by autoclaving 121°C for 15 minutes. Then it was allowed to cool and two sets of test tubes were dispensed by MR-VP broth. The tubes were placed in refrigerator until the inoculum is prepared.

5. Simmon's citrate agar

The medium (Sisco research laboratories Pvt. Ltd. Plant Site 1: D-88/2, MIDC, Turbhe-400705, New Mumbai. India, SM 017) is composed of magnesium sulfate (heptahydrate) (0.2 g/l), ammonium dihydrogen phosphate (1 g/l), dipotassium phosphate (1 g/l), sodium citrate (dehydrate) (2 g/l), sodium chloride (NaCl) (5 g/l), bromothymol blue (0.08 g/l) and agar (15 g/l) adjusted at final pH of 6.8 ± 0.2 at 25°C. It was prepared according to manufacturer's instruction by which 24.28 gram of powder was suspended in one liter of distilled water. The medium was heated to boil to dissolve the medium completely and then sterilized by autoclaving 121°C for 15 minutes. Then after cooled to 45-50°C

and poured in to sterile test tubes, in so doing cooled in slanted position and finally stored in a refrigerator.

6. Brain Heart Infusion (BHI) agar

The medium (Sisco Research Laboratories Pvt. Ltd., Mumbai 93 India, BM 018, Lot12053944) is composed of calf brain infusion form (200 g/l), beef heart infusion form (250 g/l), proteose peptone (10g/l,) sodium chloride (5 g/l), dextrose (2g/l), disodium phosphate (2.5 g/l), agar (15 g/l) and finally adjusted at pH of 7.4 ± 0.2 at 25°C. The medium was prepared according to manufacturer's directions, thus 38 g of the powdered medium was added into one liter of distilled water, mixed well, gently heated and brought to boil to dissolve the medium completely. Then, it was sterilized by autoclaving at 121°C for 15 minutes, cooled to 45°C to 50°C and poured into sterile Petri dishes. The plates were left at room temperature till the medium had solidified then put upside down in the refrigerator for subsequent uses.

7. Mueller Hinton (MH) agar

Mueller-Hinton (MH) agar (Oxoid®, Ltd., Basingstoke, Hampshire, England, CM 0337, Lot 1308390) is composed of beef, dehydrated infusion (300 g/l), casein hydrolysate (17.5 g/l), starch (1.5 g/l), agar (17 g/l) and final pH of 7.3 ± 0.1 at 25°C. It was prepared by suspending 38 g of the medium in one liter of distilled water. Then the suspension was mixed thoroughly and heated with frequent agitation and brought to boil for 1 minute to dissolve the medium completely. Then, the medium was sterilized by autoclaving at 121°C for 15 minutes, cooled to 45°C and poured into sterile petri dishes at estimated depth of 4mm, approximately 25ml of liquid agar for 100mm diameter plates and 60ml of liquid agar for 150mm diameter plates, The plates were allowed to solidify at room temperature and stored upside down at 4 to 8°C until used.

Annex 2: Gram staining technique

Principle: Gram staining is used to categorize bacteria as Gram positive or Gram negative, based on their cell wall structure. The reagents used are crystal violet, gram's iodine (mordant), decolourizer, ethanol 95 % and dilute carbol fuchsin (counter stain).

Procedure: The slide was placed on the staining rack over a sink. Crystal violet solution poured on to the smear for 1 minutes. Rinsed with tap water gently. Iodine solution was poured onto the smear for 1 minutes. Rinsed with water gently. The smear was washed with decolourizer (95 % ethanol) for 20 seconds. Rinse with tap water gently. Basic fuchsin or safranin was poured for 1 minutes & Rinsed with tap water. The smear was air dried and examined under oil immersion. During interpretation gram-positive bacteria retain the iodine-bound crystal violet and stain

purple whereas gram-negative bacteria are stained with safranin dye and appear pink.

Annex 3: Biochemical and serological tests

1. Indole production test

Principle: Indole positive bacteria possess an enzyme, tryptophanase, which converts tryptophan (an amino acid) to indole. When indole reacts with para-dimethylaminobenzaldehye (Kovac's reagent) a pink coloured complex is produced.

Procedure: Peptone water broth was prepared and about 5ml was dispensed in test tubes using a sterile pipette. Then, fresh sterile loops were used to pick a well isolated colony of bacteria and inoculated into test tubes, thereafter; the tubes were incubated at 37ºC for 48 hours. After incubation period, 0.5ml of Kovac's indole reagent was added to the inoculated test tubes. The tubes were subjected to gentle shaking and examined for red colour in the surface layer. The development of a bright red colour at the interface of the reagent and the broth within seconds after adding the reagent is an indicator of the presence of indole and it is a positive test.

2. Triple sugar iron (TSI) reaction

Principle: Triple sugar iron (TSI) slant agar is used to determine whether an organism ferments glucose, lactose and sucrose. A TSI slant begins as an orange or red coloured agar at an alkaline PH. If any of the carbohydrates are fermented an acid PH will result and either the butt or the slant and butt will turn yellow.

Procedure: TSI agar slant was prepared. The top of the isolated colony touched with the tip of the inoculating wire loop. The butts of the slant were stabbed with the tip of the inoculating wire loop and carefully with draw it up and the surface was streaked of the slant in S-shape. very loosely capped and incubated overnight at 37°C. During interpretation alkaline (red) slant and acid (yellow) butt indicate glucose fermentation only, acid (yellow) slant and acid (yellow) butt indicate lactose and / or sucrose attacked as well as glucose and blackening of the medium indicate hydrogen sulphide production (H_2S+).

3. Methyl Red and Voges-Proskauer test

Principle: MR-VP broth (Oxoid, UK) was a set of two tests. Methyl red test is a quantitative test for acid production; requiring positive organisms to produce strong acids (Lactic, acetic, formic) form glucose through the mixed fermentation pathway. The MR reaction determines the acid producing ability of an organism in a buffered glucose broth and Voges proskauer (VP) test done to identify organisms that produce acetoin as the chief end product of glucose metabolism and form less quantity of mixed acids. In the presence of atmospheric oxygen and 40% KOH

acetoin is converted to diacetyl and 5% alpha naphtol serves as a catalyst to bring out a red color complex.

Procedure: A loopful of bacterial culture from pure colonies on subcultured nutrient agar was inoculated into the 2.5ml broth then the tubes were incubated for 24/48 hours. One ml of the incubated broth was used for Methyl Red test. For MR test 5-6 drops of MR reagents, were added. Thus MR positive and MR negative samples were indicated by the appearance of red color and yellow color broth medium respectively. The remaining incubated MR-VP broth media were used for VP test. In so doing the culture was made highly alkaline by adding 2 drops of 40% KOH (VP reagents). Subsequently the tubes were shaken well and then 0.5ml alpha naphthol 5% in absolute alcohol was added. Likewise tubes were shaken and kept at 37°C for 10 minutes. A positive reaction was indicated by eosin pink color developing from top which later on darkens to crimson red (Tiwari *et al.*, 2009).

4. Simmon's citrate (citrate utilization) test

Principle: Citrate utilization test was done by Simmon citrate agar media which were prepared in slant. If an organism can use citrate as its only source of carbon the citrate in the media will be metabolized. Bromthymol blue is incorporated into the media as an indicator. Under alkaline conditions this indicator turns from green to blue. The utilization of citrate in the media releases alkaline bicarbonate ions that cause the media pH to increase above 7.4 causes the media blue.

Procedure: After solidification of the prepared simmon's citrate agar, the tubes were inoculated slightly on the slant with the straight wire, and then tubes were incubated at 37°C for 24-48 hours (Tiwari *et al.*, 2009). When the bacterium has citrate lyase enzyme, citrate is used as source of carbon and ammonia as its only source of nitrogen, accordingly the bacterial growth was enhanced and the media were turned into blue color due to alkalinity of the media. In absence of enzyme, for *E. coli*, citrate negative, no growth was better and the media remained deep green in color.

5. Oxidase test

Principle: To detect the presence of cytochrome oxidase, an enzyme which is able to oxidize the substrate tetramethyl-p-phenylenediamine dihydrochloride, forming a coloured end product, indophenol.

Procedure: A piece of filter paper is moistened in a Petri dish with a 1% tetramethyl-p-phenylenediamine dihydrochloride fresh reagent. The test bacterium was streaked firmly across the filter paper with a glass rod. A dark purple color along the streak line within 10 seconds indicates a positive reaction, but *E. coli* is negative so that there

was no development of dark purple color along the streak line.

6. *E. coli* O157:H7 latex agglutination test

Principle: *E. coli* O157:H7 latex test (Remel, RIM ™) includes 3 latex reagents. The particles in each reagent are coated with a different antibody: one against serotype *E. coli* O157, another against *E. coli* serotype H7 and the third with normal rabbit globulin, to serve as the control latex. When test latex particles are mixed with fresh colonies of O157 and H7 strains of *E. coli* an immunochemical reaction occurs, resulting in agglutination. No agglutination indicates the test isolate is not *E. coli* O157:H7. The control latex reagent identifies non-specific agglutination.

Procedure: Bring all reagents and samples to room temperature. Make sure the latex suspensions and control are well mixed by vigorous shaking. Using one of the provided transfer pipettes (green), place one drop of latex onto one circle on the test card. If more than one sample is being tested use additional circles on the test card. Using one of the sample mixing sticks (or an inoculating loop), pick a portion of a suspect colony from the agar plate and thoroughly emulsify in the drop of latex of one of the circles. Using one of the transfer pipettes (red), add one drop of the Positive Control to a second circle. Using another transfer pipette (blue), add on drop of the Negative Control to a third circle. Dispense one free falling drop (with vial held vertically) of the *E. coli* O157 Latex-Antibody bead reagent onto each circle (Positive, Negative, and sample (s)). Rotate the test card using a complete circular motion (through 3 planes) for up to one minute or until agglutination is evident, whichever occurs first. Record the results. If agglutination with the test reagent does occur, it is necessary to test a further portion of the colony with the Control Latex Reagent to ensure that the isolate is not an auto-agglutinating strain. If the agglutination occur with the *E. coli* O157:H7 test latex and control latex is negative, streak the isolate to a blood agar plate, incubate overnight and emulsify a sweep of growth from the blood agar plate in a drop of *E. coli* H7 test latex. The control latex is omitted in this step. Agglutination indicates the presence of *E. coli* H7.

Annex 4: Antimicrobial susceptibility test, the Kirby-Bauer disc diffusion method

Three to five well isolated colonies of the same morphological type were selected from nutrient agar medium (nonselective medium), from 18 to 24 hour agar plate, was touched with the loop, and transferred into a tube containing 4 to 5ml in sterile saline solution. The inoculum was prepared by making direct colony suspension and was adjusted to match the 0.5 McFarland turbidity standard. Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the

adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The dried surface of a Müeller Hinton agar plate, already prepared media, was inoculated by streaking the swab over the entire sterile agar surface. The procedure was repeated by streaking two or more times by rotating the plate approximately 60° each time to ensure an even distribution of inoculum.

Finally, the rim of the agar was swabbed. The lid was left ajar for 3 to 5 minutes to allow for any excess surface moisture to be absorbed before applying antimicrobial discs. Antimicrobial discs were placed onto the surface of the inoculated agar plate by using sterile forceps, no closer than 24 mm from center to center. The discs were pressed gently down to ensure complete contact with the agar surface. The plates were inverted and incubated at 35°C for 18 hours. After incubation, each plate was examined and the diameters of the zones of complete inhibition were measured, using sliding calipers on the back of the inverted petridish including the diameter of the disc against dark back ground. The sizes of the zones of inhibition, to the nearest whole millimeter, were interpreted according to CLSI (2012) criteria as described below (Annex 5).

Annex 5: (CLSI (clinical and laboratory standards institute), 2012) interpretive standards during antimicrobial susceptibility test for Enterobacteriaceae

No	Antibiotic disks	Disc code	Concentration	Diameter of zone of inhibition in millimeter (mm)		
----	------------------	-----------	---------------	Resistant ≤	Intermediate	Susceptible ≥
				Resistant ≤	**Intermediate**	**Susceptible ≥**
1	Gentamicin	GEN	10µg	12	13-14	15
2	Ampicillin	AMP	25µg	13	14-16	17
3	Cefoxitin	FOX	30µg	14	15-17	18
4	Ciprofloxacin	CIP	5µg	15	16-20	21
5	Sulfamethoxazole	RL	100µg	14	15-17	18
6	Trimethoprim	TR	25µg	10	11-15	16
7	Streptomycin	S	10µg	11	12-14	15
8	Doxycycline	DO	30µg	12	13-15	16
9	Tetracycline	TE	10µg	11	12-14	15
10	Nalidixic acid	NA	30µg	13	14-18	19

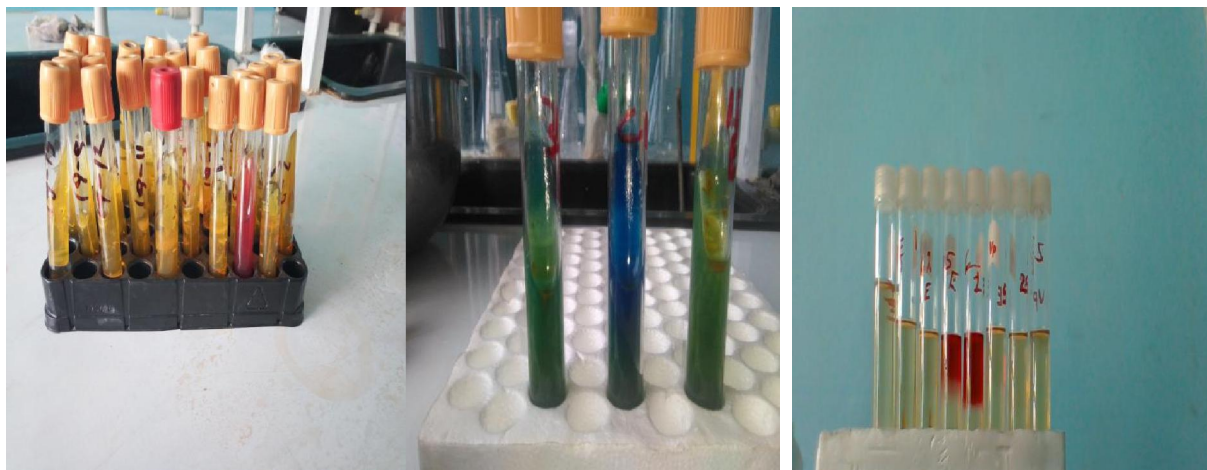**Source:** (CLSI (Clinical and Laboratory Standards Institute), 2012).

Annex 6: Characteristic growth appearance of *E. coli* and *E. coli* O157:H7 on bacteriological media
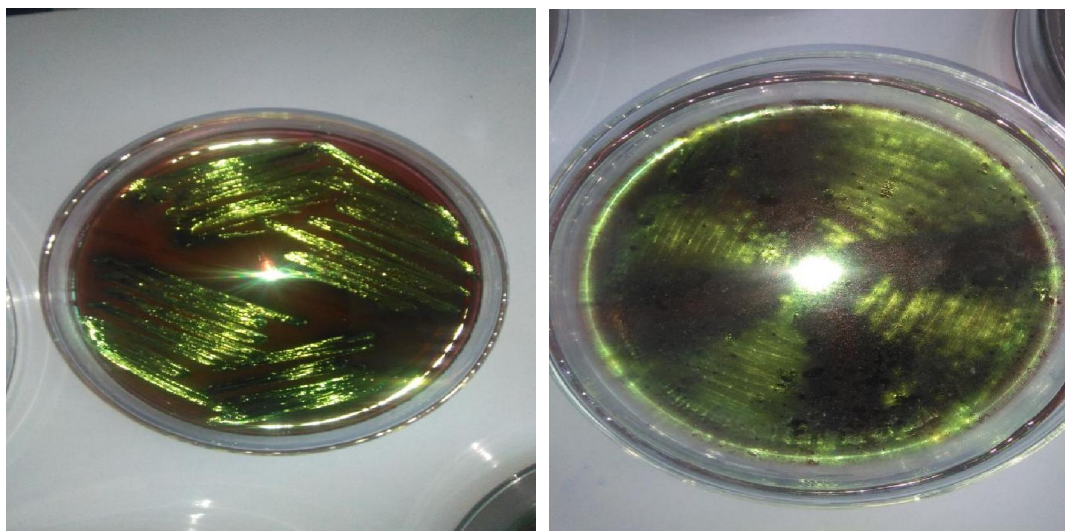
Figure 1: Colonies of *E. coli on* Eosin Methylene Blue agar (green metallic sheen appearance)

Figure 2: Colonies of *E. coli* O157:H7 organisms on sorbitol macConkey (SMA) agar media
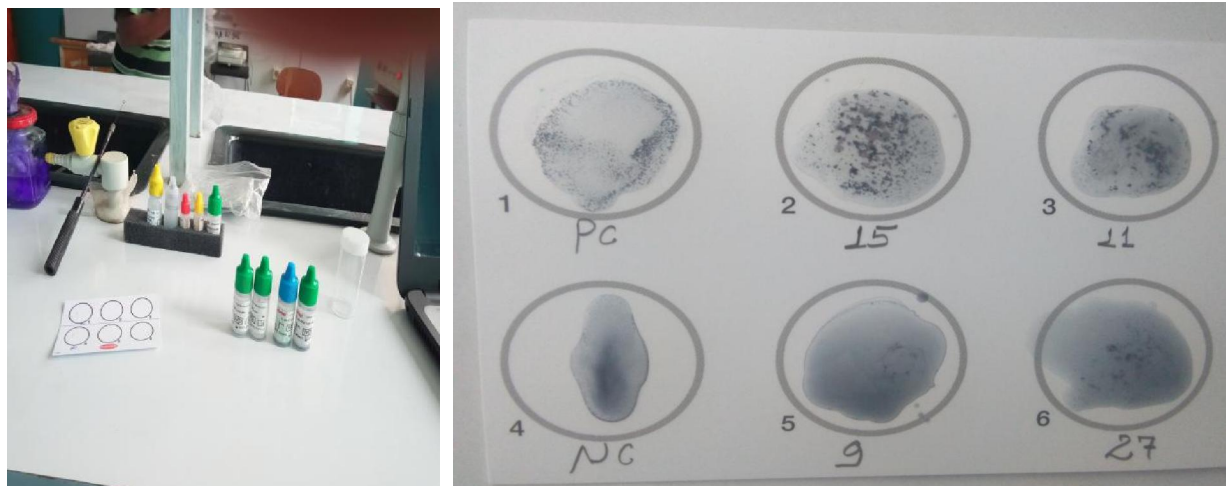

Triple sugar iron test   Citrate utilization test   Voges-Proskauer (VP) test
(Yellow indicate positive) (Green indicate negative) (Red ring indicate positive)


Methyl Red (MR) test (all positive) Indole production test (red ring indicate positive)
Figure 3: Secondary biochemical (IMVIC) test characteristics of *E. coli and E. coli* O157:H7

Latex agglutination kit Latex agglutination test (No 11, 15 and 27 are positive)
(PC is positive control and NC is negative control)
Figure 4: Latex agglutination kit and agglutination test result
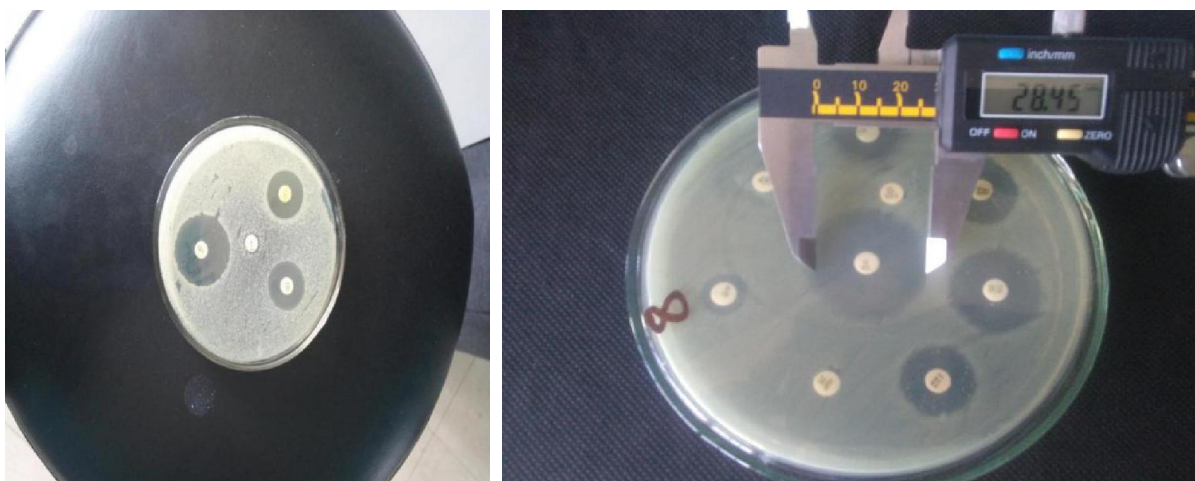
Figure 5: Antimicrobial susceptibility pattern of *E. coli* O157:H7 and zone of inhibition, by vernier caliper.

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6/20/2020