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Isolation, Identification and Antimicrobial Susceptibility Testing of Escherichia Coli from Intensive Dairy Farms in Modjo and Adama, Central Ethiopia

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Abstract

A cross-sectional study was conducted from February2019 to June2019in intensive dairy farms located in Modjo and Adama, central Ethiopia with the objective of isolating and identifying Escherichia coli from raw cow's milk sample, bulk milk sample, hand swap sample, floor sample and faces and determine its antimicrobial susceptibility pattern. Of the total 166 samples E. coli was isolated from 21/166 (12.65%). Of the 21 E. coli isolates, 14/56 (25%) lactating cow's milk, 0 (0%) bulk milk, 0 (0%) hand swap, 1/8 (12.5%) floor sample and 6/84(7.1%) faces were found to be positive for E. coli organisms by culture methods. Samples were propagated on nutrient broth and nutrient agar media followed by culture on selective media–Eosin Methylene Blue Agar, MacConkey agar. All the isolates were found to have the specific morphological, cultural characteristics and were selected for biochemical test and microscopic examination after staining. Biochemical properties of the isolates were studied and reaction in TSI agar slant was also observed. Antimicrobial susceptibility tests were also performed. Accordingly, all E. coli isolates were susceptible to gentamicin 100%, followed by cefoxitin (87.7%), ciprofloxacin (76.2%), nitrofurantoin (71.4%), nalidixic acid (62%), cefuroxime (61.9%) and streptomycin (52.4%). Furthermore, resistance of 76.2%, 66.7%, and 57.1% was recorded to tetracycline, methicillin, and erythromycin respectively. In conclusion, E. coli was isolated from the dairy farms where the majority of isolates showed resistance to one or more antimicrobials tested. Hence, improving the hygienic practices at the farm level is very important to safeguard the community from infections with antimicrobial resistant foodborne bacterial that could pose public health and therapeutic problems to consumers.

Key Words: Adama; Antimicrobial Resistance; Escherichia coli; Identification; Isolation; Modjo; intensive dairy farms:

Introduction

Food borne diseases and poisoning are the widespread and great public health concerns of the modern world. Both developed and developing countries are largely affected by food borne infections (Ali and Anil, 2013). Contaminants largely bacteria, constitute the major cause of food

borne diseases. Escherichia coli are one of the leading causes of human gastroenteritis. There exist many factors that contribute to this development. Among these are the adaptive ability of the pathogen itself, the changing characteristics of the population, the increasing globalization of the food trade, and changes in industrial structure and in consumer behavior (Legesse G et al., 2015) Escherichia coli are a Gram-negative, rod-shaped, coli form bacterium of the genus that iscommonly found in the lower intestine of most mammalian species (Tenaillon et al., 2010). Most E. coli are commensal and harmless, but small proportions are an important cause of disease worldwide (Caprioli et al., 2005; Fairbrother and Nadeau, 2006; Pennington, 2010). Zoonotic STEC include E. coliO157:H7 strains and these strains are increasing being reported globally (Fairbrother and Nadeau, 2006; Preussel et al., 2013). Thus, this study aimed to investigate the isolation and identification of E. coli from intensive dairy farms. Milk-borne pathogens cause human diseases ranging from gastrointestinal disturbances characterized by diarrhea and vomiting to other, generalized, and even life-threatening food borne illnesses (Oliver et al., 2005). They have not only of public health importance but also economic importance. In addition to causing serious economic losses in dairy cattle production, they pose a major barrier for trade of animals and animal products, and this could seriously impair socio-economic progress especially in developing countries like Africa. E.coli is one of the most significant food-borne Pathogens that have gained increased attention in recent years According to Pal (2007). Many countries have milk quality regulations, including limits on the total number of bacteria in raw milk, to ensure the quality and safety of the final product. However, hygienic quality control of milk and milk products in Ethiopia is not usually conducted on routine basis. There is little information on the microbial quality of raw milk (Zelalem and Faye, 2006) especially in the pastoral and agro-pastoral area of southern Ethiopia, where milk consumption plays a significant role in the diet of the community (Workuetal., 2012).E. coliis one of the important bacteria of gut flora (Eckburg et al., 2005). Among the pathogenic E. coli, Shiga toxigenic E. coli (STEC) strains have been reported mostly in Latin America, India, Bangladesh and many other developing countries (Kaddu-Mulindw et al., 2001; Rehman et al., 2014). Some pathogenic strains have been variously described as verotoxigenic E. coli (VTEC) or Shiga-like toxin-producing E. coli (SLTEC). Most recently, the designation has been simplified to Shiga toxin-producing E. coli (STEC) in recognition of the similarities of the toxins produced by E. coli and Shigella dysenteriae (Fischer Walker et al., 2012; Murray et al., 2007). Such potent toxins can cause severe damage to the intestinal lining, even in healthy individuals. E. coli-produced toxins are responsible for symptoms such as hemorrhagic colitis. Hemorrhagic colitis is associated with bloody diarrhea and hemolytic uremic syndrome (HUS), which is seen in the very young and can cause renal failure and hemolytic anemia. Both illnesses can be harmful and, in very severe cases, can lead to death (Murray et al. 2007; FDA 2015). Generally, E. coli can survive at both low and high temperatures ranging from 7°C (44.6°F) to 50°C (122°F). It can also survive under acidic conditions (at pH levels around 4.4), making it able to survive in mildly acidic food (WHO, 2017). Escherichia coli are a normal inhabitant of the intestines of animals and humans; its recovery from food may be of public health concern due to the possible presence of enteropathogenic and/or toxigenic strains which lead to sever gastrointestinal disturbance. It is among many pathogenic microorganisms which can get access to milk and dairy products and is considered as a reliable indicator of contamination by manure, soil, and contaminated water (Oliver et al.,2009; WHO, 2004). Foodborne diseases are common in developing countries, including Ethiopia, because of the prevailing poor food handling and sanitation practices, inadequate food safety laws, weak regulatory systems, lack of financial resources to invest in safer equipment, and lack of education for food-handlers. The National Hygiene and Sanitation Strategy program reported that about 60% of the disease burden was related to poor hygiene and sanitation in Ethiopia. Unsafe sources, contaminated raw food items, improper food storage, poor personal hygiene during food preparation, inadequate cooling and reheating of food items, and a prolonged time lapse between preparing and consuming food items have been identified as contributing factors for outbreaks of food borne diseases (FAO and WHO, 2004; Oliver et al., 2005). 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 (Shundaet al., 2013). The ability of raw or processed milk to support the growth of several spoilage or pathogenic microorganisms can lead to spoilage of the product or infections and intoxications in consumers.

Objectives of the study:

- To isolate and identify E. coli from lactating raw cow`s milk, bulk sample, hand swab sample,

floor sample and faces samples taken from intensive dairy farms in Modjo and Adama central, Ethiopia.

- To determine the antimicrobial susceptibility pattern of E. coli isolates.
- To select the best antimicrobial drug treatment against E. coli infection.

Materials and Methods

Description of the Study Area:

Study was conducted on intensive dairy farms in Modjo and Adama, central Ethiopia. Mojo (also transliterated as Modjo) is a town in central Ethiopia, named after the nearby Modjo River. Located in the Misraq Shewa Zone of the Oromia Region, it has a latitude and longitude of 8°39′N 39°5′E with an elevation between 1788 and 1825 meters above sea level. It is the administrative center of Lome woreda (Modjo-Awassa Road, 2013).Adama (Oromo: Adaamaa or Hadaamaa;) is a city in central Ethiopia and the previous capital of the Oromia Region. Adama forms a Special Zone of Oromia and is surrounded by Misraq Shewa Zone. It is located at 8.54°N 39.27°E at an elevation of 1712 meters, 99 km southeast of Addis Ababa. The city sits between the base of an escarpment to the west, and the Great Rift Valley to the east (Workineh Kelbessa, 2001).

Description of the Study population:

A cross-sectional study was conducted to isolate and identify E. coli from different samples. The study population comprised apparently health cattle.

Description of the Study design and sample size:

A cross sectional study was conducted from February 2019 to June 2019 to isolate E. coli, selected intensive dairy farms and to determine antimicrobial susceptibility for the isolates. The samples were collected systematically from selected intensive dairy farms in mojo and Adama central, Ethiopia. Totally166 samples were collected from both sites.

Description of the Sample collection Methods, Procedures and transportation:

The study was conducted from February2019 to June 2019 and raw milk; bulk milk sample, flour samples, hand swap samples and fecal samples were collected and processed completely. About ≈15g fecal samples were collected through rectal palpation from dairy animals immediately before stunning and milk samples were collected directly from teats. The udder and teats were thoroughly cleaned and dried before sampling; each teat was rubbed gently with cotton swabs moisturized with 70% ethyl alcohol. The first 3–4 streams of milk were discarded, and approximately 30ml of milk was collected aseptically by sterile screw topped universal bottle and the sample were transported using an ice box (4oC) for further processing and microbiological analysis. Isolation and identification of E. coli from milk samples were passed on the basis of colony morphology in different media, staining characteristics and biochemical properties (ISO 18593, 2004). Each sample will be collected aseptically and placed in a sterile tube. The samples were collected carefully and placed in sterile bottle using disposable gloves to avoid contamination. After labeling the bottle to identify species, household and date of collection, it was kept in cooler with ice. Finally, the samples were transported to the Veterinary Microbiology laboratory, College of Veterinary Medicine and Agriculture of the Addis Ababa University (AAU-CVMA) using ice box in cold chain for Microbiological analysis. Up on arrival, the samples were stored in refrigerator at 4°C for 24 hrs until being processed for isolation as described by (Quinn et al., 2004; Oyeleke et al., 2008).

Laboratory Work:

Pre-enrichment of the samples: Before processing, the samples were kept in +40C refrigerator. After this, pre-enrichment of sample was carried out by putting 10 g of faces or 10 ml of milk in a Whirl-Pak filter bag and 90 ml of buffered peptone water (BPW) was added, respectively. After that the mixture in the bag was homogenized by using lab blender. This pre-enrichment broth was incubated at 37 0C for 24 hr to increase recovery of the organisms (OIE, 2016), then processed the next day for isolation of E. coli.

Enrichment of samples: Immediately after collection of the samples were enriched in nutrient broth and incubated at 370C for overnight.

Culturing of extracted enrichment: Laboratory procedures were conducted according to the general guidelines of (ISO, 2001). The method is based on enrichment in a selective broth medium (Modified Tryptone Soya Broth supplemented with Novobiocin) and plating on a MacConkey and then the application of a rapid screening test for confirmation. All enriched samples were cultured on sterilized MacConkey agar (Oxoid) and incubated for 24 hours at 37°C for clarification. Then the confirmed pure cultures considered as E. coli positive was transferred to nutrient agar to be used for additional biochemical and serological confirmation (Quinn et al., 2004).

Isolation and identification of E. coli:

E. coli is catalase positive, oxidase negative, Gram-negative, short rods or cocco bacilli and reduces nitrates. There are many other biochemical tests to indicate the presence of E. coli. For instance, Voges and Proskauer found a test to detect acetone and 2, 3-butanediol produced when Klebsiella and Enterobacter ferment glucose (Mac Fadden et al., 2000). Isolation and identification of E. coli were performed by standard bacteriological methods. The samples were incubated at 37°C for 24 hrs on the same day upon arrival at the laboratory on MacConkey agar (Oxoid Ltd., Cambridge, UK) which is selective and differential medium for E. coli. A pink colony was picked and sub-cultured on Eosin Methylene Blue (EMB) agar (Oxoid Ltd., Cambridge, UK) to obtain pure colony. Colonies with metallic green sheen on EMB (characteristic of E. coli) were later characterized microscopically using Gram's stain according to the method described by Merchant and Packer. After isolation of the organism on the selective media, differential screening media, triple sugar iron (TSI) agar (Difco, MI, USA) was used for further characterization. Yellow slant, yellow butt, presence of gas bubbles, and absence of black precipitate in the butt was observed which indicates E. coli. Then the isolates were subjected to different biochemical tests such as sugar fermentation test and indole production test, methyl-red, Voges-Proskauer, and citrate utilization (IMViC) test (Quinn P. J. et al., 2002; Nazir et al. 2005).

Cultural and Colonial examination

- On Nutrient Agar: Small, regular, circular, translucent colonies
- On Mac conkey Agar: Small, regular, smooth, circular pink colonies with spreading growth, lactose fermenting colonies
- On Eosin-methylene-blue agar: Metallic sheen colony to growth.

Antimicrobial Susceptibility Profile of E. coli:

Principle: The pathogenic organism is grown on Mueller-Hinton agar in the presence of various antimicrobial impregnated filter paper disks. The presence or absence of growth around the disks is an indirect measure of the ability of that compound to inhibit that organism (Quinn et al., 2002). Antimicrobial susceptibility tests were performed by standard disc diffusion technique using commercially available antimicrobial disks (Annex 8at Figure8.) and recommended from the guideline of antimicrobial susceptibility testing from CLSI (2015).Each isolated bacterial colony from pure fresh culture was transferred into a test tube of 5 ml Tryptone Soya Broth (TSB) (Oxoid, England) and incubated at 37°C for 24 hrs. The turbidity of the culture broth was adjusted using sterile saline solution and by adding more isolated colonies to obtain turbidity usually comparable with that of 0.5 McFarland standards (approximately 3x108 CFU per ml). In brief, a 0.5 Mac-Farland standardized suspension of the bacteria was prepared in 0.85% sterile normal saline solution Mueller-Hinton agar (Bacton Dickinson and Company, Cockeysville USA) plates were prepared according to the manufacturer guidelines. A sterile cotton swab was immersed into the suspension and rotated against the side of the tube 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 antibiotic disks were gently pressed onto the agar to ensure firm contact with the agar surface, and incubated in an inverted position at 37°C for 24 hrs. Following this the diameter of inhibition zone formed around each disk was measured using a black surface, reflected light and transparent ruler by lying it over the plates (CLSIFAD, 2012; Jan H. Kirby-Bauer, 2013). After incubation for 24 hours, clear zones of inhibition were produced by the bacterial growth and diffusion of the antibiotics and these were measured in millimeter using a caliper and interpreted as susceptible, intermediate and resistant according to the standardized table supplied by the manufacturer, (CLSIFAD, 2012; Jan H. Kirby-Bauer, 2013),Table 1. Each isolate was tested for a series of ten antimicrobials, cefoxitin (CRX) (30 µg), gentamicin (GN) (10 µg), streptomycin (S) (10 µg), Nalidixic acid (NAL) (30 µg), ciprofloxacin (CIP) (5 µg), tetracycline (TTC) (30 µg), nitrofurantoin (NIT) (30 µg), erythromycin (ERY) (15 µg), cefuroxime (CRX) (30 µg), methicillin (MET) (5 µg) all from Oxoid company, England. Following incubation, the diameters of clear zones produced by antimicrobial inhibition of bacterial growth were measured to the nearest mm for each disc using transparent straight-line ruler and then classified as resistant, intermediate, or susceptible according to published interpretive chart of CLSI.

Table 1: Guidelines for antibiotic discs used for antimicrobial susceptibility test of E. coli with their respective concentrations

Antimicrobial Agent	Disc code	Disk of Potency	Zone of Diameter: (Nearest whole millimetre)		
			S	I	R
Gentamicin	GM	10µg	≥ 15	13–14	≤ 12
Nalidixic acid	NA	30 µg	≥19	14-18	≤13
Tetracycline	TTC	10 µg	≥ 19	15–18	≤ 14
Streptomycin	S	10 µg	≥ 15	12–14	≤ 11
Erythromycin	ERY	15 µg	≥23	14-22	≤13
Ciprofloxacin	CIP	5 µg	≥ 21	16–20	≤ 15
Nitrofurantoin	NIT	300 µg	≥17	15-16	≤14
Cefoxitin	CXT	30 µg	≥18	15-17	≤14
Cefuroxime	CRX	30 µg	≥18	15-17	≤14
Methicillin	MET	5 µg	≥15	12-14	≤11

Source: Clinical Laboratory Institute Standards (CLIS, 2015) (Hint: S=susceptible, I= intermediate, R=Resistance

Data Management and Analysis

The data was entered into excel spread sheet and different statistical models were employed to analyze the data collected using Statistical Package for Social Sciences (SPSS) version 20software. Descriptive statistics was used to describe the frequency and percentage of the results. Chi-square test was used to check the association of potential risk factors with the occurrence of E. coli (X2=0.017; df=4). The association was taken as significant when p-value is less than 0.05 and not significant when p value is greater than 0.05.

Results

Isolation and identification of E. coli organisms were conducted on raw cow's milk and faces samples using conventional culture and biochemical analysis. Escherichia coli 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. This agrees with the report by (Mohanty et al., 2013). E. coli isolated from animal faces and milk gave smooth pink colonies on the solid isolation medium MacConkey agar, indicating lactose fermentation. Lactose is included in this medium as a fermentable carbohydrate together with a pH indicator, usually neutral red. Their confirmed test by streaking on EMB agar produced the dark-centered colonies with metallic green sheen. EMB agar is a popular selective and differential medium. The aniline dyes, eosin and methylene blue, are the selective agents but also serve as an indicator for lactose fermentation by forming a precipitate at low pHs Strong lactose fermenters produce green black colonies with a metallic sheen. Based on biochemical analysis, E. coli isolates were found to be catalase positive, methyl red positive, indole positive and citrate negative. The isolates were also found to be fermentative or facultative anaerobes and having ability to utilize the three sugars (glucose, sucrose and lactose) on oxidation- fermentation test and triple sugar iron slant agar test, respectively.

- Triple sugar –iron (TSI): E. coli showed a yellow butt and a yellow slope to indicate the fermentation of lactose, sucrose and possibly glucose. The bubbles in the medium indicate gas production from glucose fermentation.
- Indole production: E. coli isolates gave a red color in the upper layer of the medium, indicating a positive result for indole production

- Voges Proskauer test: isolates showed a negative reaction towards VP test. No pink color was formed during the test which means that the isolates did not form acetone.

Table 2: The occurrence of E. coli in milk, Bulk milk, Hand swap, Floor sample and feces sample type both in Adama and Modjo

Sites	Type of samples	No of sample Examined	*E. coli* positive	Percent
Modjo	Milk Sample	32	6	18.75
	Bulk milk	4	0	0
	Hand swap	4	0	0
	Floor sample	4	1	25
	Faces sample	43	4	9.3
	Subtotal	87	11	12.6
Adama	Milk Sample	24	8	33.3
	Bulk milk	5	0	0
	Hand swap	5	0	0
	Floor sample	4	0	0
	Faces sample	41	2	4.9
	Subtotal	79	10	12.7
	Total	166	21	12.65

(X2=0.017, Modjo; Adama)

In Modjo study: Totally 87 samples were collected, from those 32 were lactating cow's milk,4bulk milk, 4 were Hand swap, 4 Floor sample and 43 were Faces sample. The occurrence of E. coli in milk sample, Bulk milk, Hand swap, Floor sample and feces sample were 6/32 (18.17%), 0/4 (o%), 0/4 (0%), 1/4 (25%) and 4/43 (9.3%) respectively. The proportion or percentages of E. coli from Modjo were 11/87 (12.6%), (See table 1).

In Adama study: Totally 79 samples were collected, from those 24 were lactating cow's milk, 5 bulk milk, 5 were Hand swap, 4 Floor sample and 41 were Faces sample. The occurrence of E. coli in milk sample, Bulk milk, Hand swap, Floor sample and feces sample were 8/24 (33.3%), 0/5 (0%), 0/5 (0%), 0/4 (0%) and 2/41 (4.9%) respectively. The proportion or percentages of E. coli from Modjo were 10/79 (12.7%), (See table 1).

Table 3: Proportion of E. coli isolated from different sample types in the study sites (Modjo and Adama)

Type of samples	No of sample examined	*E. coli* positive	Percent
Milk Sample	56	14	25
Bulk milk	9	0	0
Hand swap	9	0	0
Floor sample	8	1	12.5
Faces sample	84	6	7.1
Total sample	166	21	12.65

E. coli was isolated from 21/166 (12.65%) of the total samples. Out of the 21E. coli isolates, 14/56 (25%) lactating cow's milk, 0 (0%) bulk milk, 0 (0%) hand swap, 1/8 (12.5%) floor sample and 6/84(7.1%) faces were found to be positive for E. coli organisms by culture methods. The highest isolation of E. coli was from 25% lactating cow's milk, followed by 12.5% flour sample. In the present study, the highest isolation rates of E. coli were from Adama site (12.7%). Therefore, the occurrence of E.coil in Adama were slightly greater than Modjo, means 99% was similar in this study.

Table 4: Antimicrobial susceptibility profile of E. coli isolated from milk, bulk milk, hand swap, floor sample and feces sample type both in Adama and Modjo.

Drugs	Disc code	Susceptible	Intermediate	Resistant
Gentamicin	10μg	21 (100%)	-	-
Nalidixic acid	30 μg	13 (62%)	4 (19%)	4 (19%)
Tetracycline	10 μg	5 (23.8%)	-	16 (76.2%)
Streptomycin	10 μg	11 (52.4%)	4 (19.0%)	6 (28.6%)
Erythromycin	15 μg	5 (23.8%)	4 (19%)	12 (57.2%)
Ciprofloxacin	5 μg	16 (76.2%)	4 (19%)	1 (4.8%)
Nitrofurantoin	300 μg	15 (71.5%)	2 (9.5%)	4 (19%)
Cefoxitin	30 μg	18 (85.7%)	-	3 (14.3%)
Cefuroxime	30 μg	13 (61.9%)	-	8 (38.1%)
Methicillin	5 μg	7 (33.3%)	-	14 (66.7%)

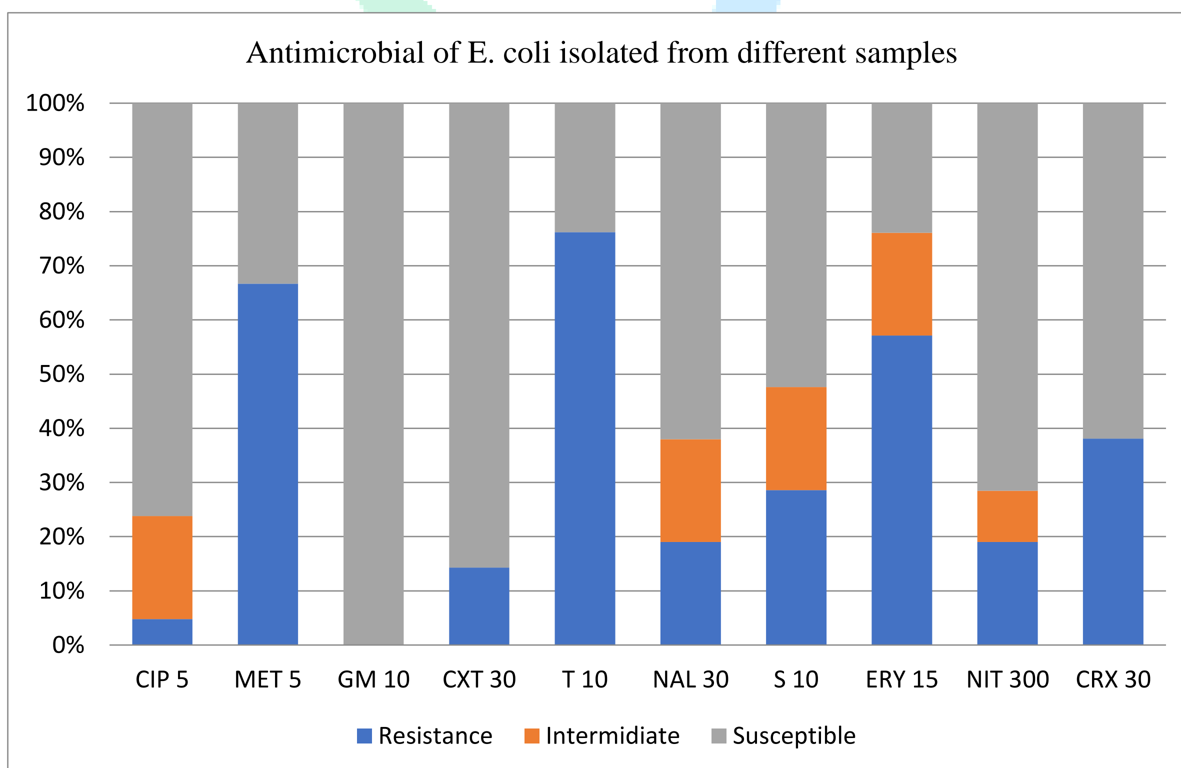

Figure 1: Antimicrobial of E. coli isolated from different samples

Table 5: Multidrug resistance profile of the E. coli isolated from the study area

No of antimicrobials	Types of antimicrobials resisted	No. of resistant isolates (%)
3	3	23.1
4	6	46.1
5	4	30.8
Total	**13**	**100**

Multidrug resistance defined as resistance for two or more antimicrobials (Dominic et al., 2005) was found in different isolates. Accordingly, 76.2% of E. coli isolates showed resistance to two or more antimicrobial agents.

Discussion

Escherichia coli are a normal inhabitant of the intestines of animals and humans; its recovery from food may be of public health concern due to the possible presence of enteropathogenic and/or toxigenic strains which lead to sever gastrointestinal disturbance. It is among many pathogenic microorganisms which can get access to milk and dairy products and is considered as a reliable indicator of contamination by manure, soil, and contaminated water (Oliver et al.,2009; WHO, 2004). 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 (Soomro et al., 2002). The presence of E. coli in pasteurized milk does not reflect the survival of the organism to the appropriate level of pasteurizing temperature. Rather, it might be due to poor hygienic handling after the milk is pasteurized, which contributes to milk contamination (Ali and Abdelgadir, 2011). Furthermore, 25% of lactating cow's milk sample was found to harbor E. coli, which is somewhat in agreement with the report of 26% prevalence reported by Farhan et al. (2014) and 23.3% by Elbagory et al. (2016). However, the prevalence is far lower when compared to the reports of Shunda et al. (2013) from Mekelle town (44%). This might be due to differences in animal management, milking systems, and milk handling practices among different countries. In this study, the methods of production, transportation, handling, and sale of milk are 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 and/or sanitized containers, all of which contribute to milk contamination (Ali, 2011; Chye et al., 2004; Soomroet al., 2002). The use of antibiotics in the treatment of E. coli 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 administrating 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). On the basis of the aforementioned necessity, antimicrobial susceptibility testing was conducted on the isolates recovered from all the samples. The present study showed that E. coli isolates were highly sensitive to gentamicin (100%) and cefoxitin (87.7%). Meanwhile, the majority of the isolates were resistant to tetracycline, methicillin and erythromycin. Various authors reported that E. coli is resistant to tetracycline (Hiko et al. 2008; Mude et al., 2017), which similarly, is to the results of the present study. However, Hiko et al. (2008) and Bekele (2012) from Ethiopia and Magwira et al. (2005) from Botswana revealed that the resistance of E. coli does exist mainly to streptomycin, which is contrary to the results of the present study. But in Dire Dawa, Mohammed et al. (2014) reported that E. coli was susceptible to tetracycline, which disagrees with the present study finding. Different researchers reported antimicrobial resistance of E. coli isolates of raw milk in their previous studies from Ethiopia. Reports from other researchers had also indicated E. coli isolates' resistance tetracycline (88.9%), reported by (Abebe et al., 2014) in Tigray, Ethiopia.The high level of resistance of tetracycline

(76.2%), methicillin (66.7%) and erythromycin (57.1%) obtained in this study might be as a result of suboptimal, prolonged and interrupted use of antimicrobials for prophylaxis and treatment of infection. Therefore, in this study gentamicin and cefoxitin were found to be the most effective drugs against E. coli infection. Relatively similar findings have been reported by (Salehi et al., 2006; Tesfaye et al., 2009; Bagre et al., 2014), who found all E. coli isolates were 100% susceptible to gentamicin.

Conclusion and Recommendations

The present study showed that E. coli was isolated from lactating raw cow`s milk (25%), floor sample (12.5%) and (7.1%) faces samples from Modjo and Adama towns, central Ethiopia. The study also revealed that raw cow's milk was found to be highly contaminated with the E. coli. The presence of this isolates in milk and some of dairy products is considered as a reliable indicator of contamination by manure, soil and contaminated water. The antimicrobial susceptibility testing of the isolates showed that resistance was recorded to antibiotics mainly to tetracycline (76.2%), methicillin (66.7%) and erythromycin (57.1%), however, the isolates were susceptible to some antibiotics like gentamicin (100%), cefoxitin (87.7%).

Based on the above remarks, the following recommendations need to be considered:

- Public awareness about Foodborne pathogens should be given at the community level,
- Rational use of antimicrobial therapy should be encouraged to minimize the risk of antimicrobial resistance development,
- Laboratory diagnostic methods should be done to identify the agents of infection and so, to use the appropriate effective drug for the agents identify.

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APPENDICES

Annex I: Sample collection and laboratory activities work sheet laboratory analysis

Table 6: Sample collection and laboratory activities work sheet for laboratory analysis

Data collection:	Laboratory analysis:
No.	Pre-enrichment
Date	Enrichment
Species	Colony Characteristics
Sample type	Biochemical test
Sample ID.	Antimicrobial Susceptibility Test

Annex II: Secondary biochemical tests

Indole test:

Principle: Tryptophan is an amino acid that can undergo deamination and hydrolysis by bacteria that express tryptophanases enzyme. Indole is generated by reductive deamination from tryptophan via the intermediate molecule indole pyruvic acid. Tryptophanase catalyzes the deamination reaction, during which the amine (-NH2) group of the tryptophan molecule is removed. Final products of the reaction are indole, pyruvic acid, ammonium (NH4+) and energy. Pyridoxal phosphate is required as a coenzyme (Jorgensen et al., 2015; Greenberg et al., 2005).

Procedure: The sub-cultured and purified colonies were tested for hydrogen supplied and indole production using Triple Sugar Iron agar (TSI) slant (Oxoid) and Indole production test. The isolates giving a result of yellow slant and butt with gas but no hydrogen sulfide (Y/Y/ H2S -) production on TSI slant agar after incubation of the media at 37°C for 24 hours were kept with tubes capped loosely to maintain aerobic conditions. Indole test was carried out using one pure colony inoculated into 4 ml of tryptone soya broth (Oxoid) with a straight inoculation wire. Incubation was done for overnight at 37°C. Then few drops of Indole (Kovac's) reagent was added to the tryptone soya broth culture to test for indole production (formation of red ring indicating positive reaction) as stated at Figure6.

Procedure of Indole Test:

- ✓ Take sterilized test tubes containing 4 ml of tryptophan broth.
- ✓ Inoculate the tube aseptically by taking the growth from 18 to 24 hrs culture.
- ✓ Incubate the tube at 37°C for 24-28 hours.
- ✓ Add 0.5 ml of Kovac's reagent to the broth culture.
- ✓ Observe for the presence or absence of ring.

Expected results:

- ✓ Positive: Pink colored rink after addition of appropriate reagent

Negative: No color change even after the addition of appropriate reagent. E.g. Klebsiella pneumonia. Therefore E. coli is indole positive

Citrate utilization test (Simon's Citrate slant):

Principle: Organisms capable of utilizing ammonium dihydrogen phosphate and citrate will grow unrestricted on this medium. If citrate can be used, the microbe will accumulate alkaline/basic by products. Bacteria that can grow on this medium produce an enzyme, citrate-permease, capable of converting citrate to pyruvate. Pyruvate can then enter the organism's metabolic cycle for the production of energy. Growth is indicative of utilization of citrate, an intermediate metabolite in the Krebs cycle. The utilization of citrate depends on the presence of an enzyme citrase produced by the organism, which breaks down the citrate to oxaloacetic acid and acetic acid. These products are later converted to pyruvic acid and carbon dioxide.

Procedure: A loopful of colony was streaked onto a Simmons citrate agar slant, and then incubated for 24 to 48 hrs at 37°C in incubator. Change in medium color to blue-collar indicates a positive result.

Interpretation of Results:

- ✓ A positive reaction is indicated by growth with development of a deep blue color reaction within the medium.
- ✓ A negative reaction is evidenced by no growth or growth with the medium remaining green in color. Therefore E. coli is Simon's Citrate negative

Triple Sugar Iron Agar (TSI) test:

Principle: TSI agar is used to determine whether a Gram-negative rod utilizes glucose and lactose or sucrose fermentative and forms gas production. The formation of CO 2 and H 2 is indicated by the presence of bubbles or cracks in the agar or by separation of the agar from the sides or bottom of the tube. The production of H 2 S requires an acidic environment and is indicated by blackening of the butt of the medium in the tube.

Procedure: With a straight inoculating wire, touch the top of a well isolated colony. Inoculate TSI by first stabbing through center of the medium to the bottom of the tube and then streaking the surface of the agar slant. Leave the cap on loosely and incubate the tube for 18-24 hours at 35oC in an incubator.

Result interpretation:

- ✓ Alkaline slant/no change in the butt (K/NC) = Glucose, lactose and sucrose non-utilize
- ✓ Alkaline slant/acid butt (K/A) = Glucose fermentation only
- ✓ Acid slant/acid butt (A/A), with bubble or cracks = Glucose, sucrose, and/or lactose fermented with gas production (as stated at Figure4).
- ✓ Alkaline slant/acid butt (K/A), black at the butt = Glucose fermentation only and Hydrogen supplied production.

Therefore E. coli is TSI- positive

Methyl-red test:

Principle: To test the ability of the organism to produce and maintain stable acid end products from glucose fermentation and to overcome the buffering capacity of the system.

Procedure: Colony was inoculated in MR-VP broth and incubated at 37°C for 24 hrs. After incubation, 3-4 drops of methyl red reagent was added. Converting media color to red is a positive result.

Result interpretation:

- ✓ Positive result is red (indicating ph below 6).
- ✓ Negative result is yellow (indicating no acid production).

Therefore E. coli is MR- positive

Vogas-Proskauer test:

Principle: To determine the ability of the organisms to produce neutral end product acetyl methyl carbinol (acetoin) from glucose fermentation.

Procedure: Colony was inoculated in MRVP broth and incubated at 37°C for 24 hrs. Then two drops of VP1 and four ofVP2 were added. Appearance of red color after 15 min indicates a positive result.

Result Interpretation:

- ✓ Positive: Pinkish red color at the surface of the medium.
- ✓ Negative: Yellow color at the surface of the (as stated at Figure7).

Therefore E. coli is VP negative

Annex III: Composition and preparation of Medias use for transportation and culture of the bacteria

Table 7: Buffered Peptone Water (BPW) (HIMEDIA, India)

Ingredients	gm/litre
Sodium chloride	5.00gm
Casein enzyme hydrolysed	10.00gm
Disodium hydrogen phosphate	9.00gm
Mono potassium hydrogen phosphate	1.5gm
M Final PH at 25 OC	7.0 ±0.2

Preparations: Add 20 gram to 1 litter of distilled water. Heat if necessary, to dissolve the medium completely. Mix it well and sterilize by autoclaving at 120 OC for 15 minutes.

Annex IV: Medias used for the primary isolation of E. coli

Table 8: MacConkey agar (CMO115, Oxoid)

Formula	**grams per litre**
Peptone	20.0
Lactose	10.0
Bile salts	No.3 1.5
Sodium chloride	5.0
Neutral red	0.03
Crystal violet	0.001
Agar	15.0
PH	7.1 (Approximately)

Preparation:51.5 grams of the powder were suspended in a litre of distilled water brought to boil to dissolve completely and dissolved completely and sterilized at (1210C for 15 minutes).The molten agar was cooled to 500c and approximately 20ml poured into a Petri dish (90mm in diameter) and allowed to cool and solidify at room temperature.

Table 9: Eosin Methylene Blue (EMB) (Oxoid, ® Hampshire, England)

Ingredients	**gm/litter**
Peptone	1 0 .00 g
Lactose	5. 00 g
Dipotassiummono hydrogen phosphate	2. 00 g
Mthylene blue	0. 06 g
Eosin Y	0.04 g
Agar	13.50 g
PH at 25OC	7.1 ± 0.2

Annex V: Medias used for the secondary isolation of E. coli

Table 10: Nutrient Agar (Oxoid, ® Hampshire, England)

Ingredients	**gm/litter**
Peptones digest of animal tissue	5.00 gm
Yeast extracts	1.50 gm
Beef extract	1.50 gm
Sodium chloride	5.00 gm
Agar	15.00 gm
PH at 25 OC	7. 4 ± 0.2

Directions: Powder: suspended 28g in 1liter of distilled water. Boil to dissolve completely. Sterilize by autoclaving at 121 OC for 15 minutes.

Table 11 : MR-VP Medium :

Ingredients	gm/litter
Peptone	7.0gm
Glucose	5.0
Phosphate buffer	5.0

Preparation: Suspend 17g in 1 liter of distilled water. Mix well, distribute in a final container and sterilize by autoclaving at 121 0 C for 15 minutes.

Table 12: Simmons citrate agar:

Ingredients	gm/litter
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium chloride	5.0
Magnesium sulphate	0.2
Agar	15.0
Bromotymol blue	0.08

Preparation: Suspend 24.2g of the powder in 1litre of distilled water and mix well. Heat with frequent agitation and boil to completely dissolve the powder. Sterilize by autoclaving at 121 0 C for 15 minutes, then dispense in tubes in slant form.

Table 13 : SIM Medium :

Ingredients	gm/litter
Pancreatic digest of casein	2.0
Peptic digest of animal tissue	6.1
Ferrous ammonium sulphate	0.2
Agar	3.5

Preparation: suspend 30.00 gram of the powder in 1 litre of distilled water. Mix thoroughly. Heat with frequent agitation and boil for a minute. Sterilize by autoclaving at 121 0 C for 15 minutes.

Table 14 : Tryptone Soya Broth (OXOID CM0129 500G):

Ingredients	gm/litter
Pancreatic digest of casein	17.00
Enzymatic digest of soya bean	3.00
Sodium Chloride	5.00
Dipotassium hydrogen phosphate	2.50
Glucose	2.50
Final PH: at 25ºC	7.3 ± 0.2
Distilled water	1 litter

Preparation: Suspend 30 grams of the medium in one litre of distilled water. Mix well. Heatslightly, until complete dissolution of the medium if necessary. Sterilize by autoclaving at 121°C for 15 minutes, then dispense in tubes.

Table 15 : Triple Sugar Iron Agar (OXOID CM0277 500G):

Ingredients	gm/litter
Peptone Mixture	20.00
Lactose	10.00
Sucrose	10.00
Sodium Chloride	5.00
Beef Extract	3.00
Yeast Extract	3.00
Glucose	1.00
Ferrous Ammonium Citrate	0.30
Sodium Thiosulphate	0.30
Phenol Red	0.024
Bacteriological Agar	12.00
Final pH: at 25°C,	7.4 ± 0.2
Distilled water	1 litter

Preparation: Suspend 65 grams of the medium in one litre of distilled water. Bring to boil todissolve completely. Mix well and Sterilize by autoclaving at 121° C for 15 minutes. Distribute in sterile tubes and cool it in a slanted position, as to obtain butts of 1.5–2 cm depth.

Annex VI: Media and antibiotic discs used during the antimicrobial sensitivity testing with their respective concentration

Table 16: Mueller Hinton agar (OXOID CM0337, 500G):

Ingredients	gm/litter
Beef Infusion	300.00
Casein hydrolysate	17.50
Starch	1.50
Bacteriological Agar	17.00
Final PH: at 25°C	7.4 ± 0.2
Distilled water	1 litter

Preparation: Suspend 38 grams of medium in one litter of distilled water. Bring to boil to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes.

Annex VII: McFarland turbidity standard preparation

Solution 1: 1.175 grams of $BaCl_2 2H_2O$ was measured. 50 ml of distilled water was added and mixed well. Make up to 100 ml with distilled water.

Solution 2: 99 ml of distilled water was put into the flask. 1 ml of concentrated H_2SO_4 were added into the flask contained distilled water. 0.5 McFarland turbidity=99.5 ml "solution 2" (1% H_2SO_4 + 0.5 ml"solution 1" 1.175% $BaCl_2$).

Materials Required:

- Petri plate containing microbial culture (For example, Escherichia coli)
- Inoculation loop
- Bunsen burner
- Saline solution
- McFarland solution
- Cotton swab
- Antibiotic disks (Streptomycin (S), Ciprofloxacin (CIP), Gentamicin (G, etc...
- Incubator

- Caliper…etc.

Procedure:

- Select a pure culture plate of one of the organisms to be tested.
- Aseptically emulsify a colony from the plate in the sterile saline solution. Mix it thoroughly to ensure that no solid material from the colony is visible in the saline solution.
- Repeat until the turbidity of the saline solution visually match that of the standard turbidity.
- Take a sterile swab and dip it into the broth culture of organism.
- Gently squeeze the swab against the inside of the tube in order to remove excess fluid in the swab.
- Take a sterile Mueller-Hinton agar (MHA) plate or a nutrient agar (NA) plate.
- Use the swab with the test organism to streak a MHA plate or a NA plate for a lawn of growth.
- After the streaking is complete, allow the plate to dry for 5 minutes.
- Antibiotic discs can be placed on the surface of the agar using sterilized forceps.
- Gently press the discs onto the surface of the agar using flame sterilized forceps or inoculation loop.
- Carefully invert the inoculated plates and incubate for 24 hours at 37° C.
- After incubation, use a metric ruler to measure the diameter of the zone of inhibition for each antibiotic used.
- Compare the measurement obtained from the individual antibiotics with the standard table to determine the sensitivity zone.
- Compare the measurement obtained from the individual antibiotics to the standard table to determine whether the tested bacterial species is sensitive or resistant to the tested antibiotic.

Annex VIII: Some of the pictures taken during the processing (analysis)

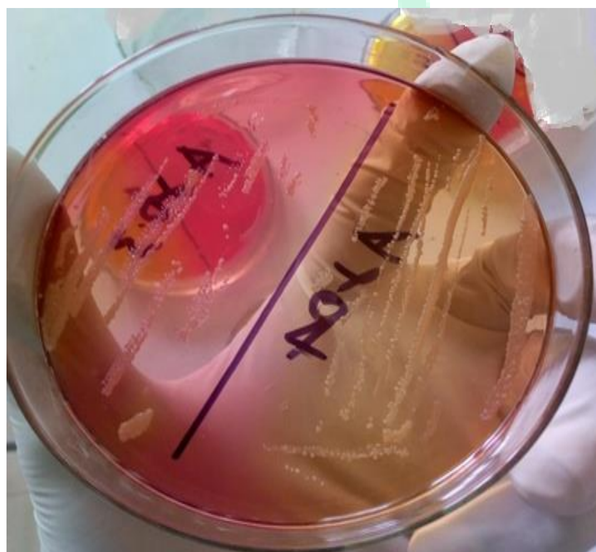

Figure 2: MacConckey Agar Tests of E.coli

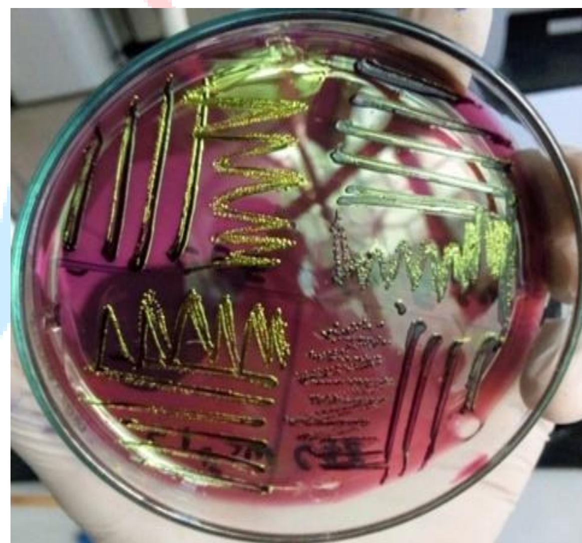

Figure 3: Eosin Methylene Blue tests of E.coli

Figure 4: Triple Sugar Iron Agar (TSI) test

Figure 5: The left side one is Simon's Citrate slant test

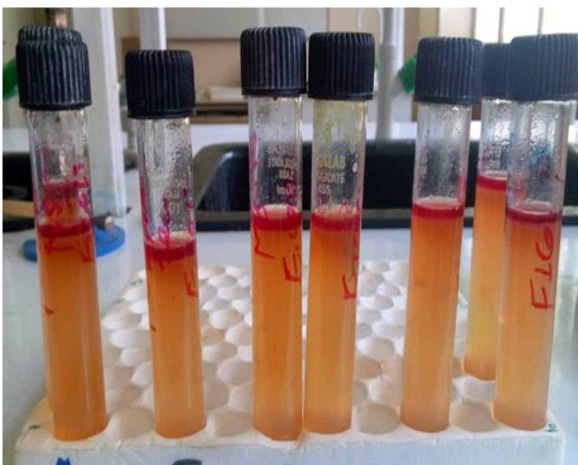

Figure 6: Indole test of E. coli

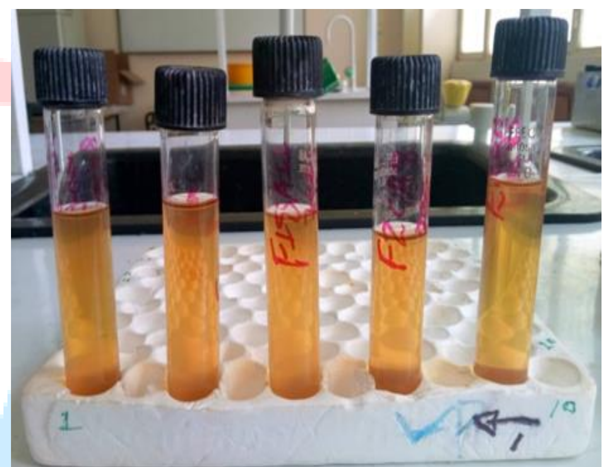

Figure 7: Vogas- Proskauer test of E.coli

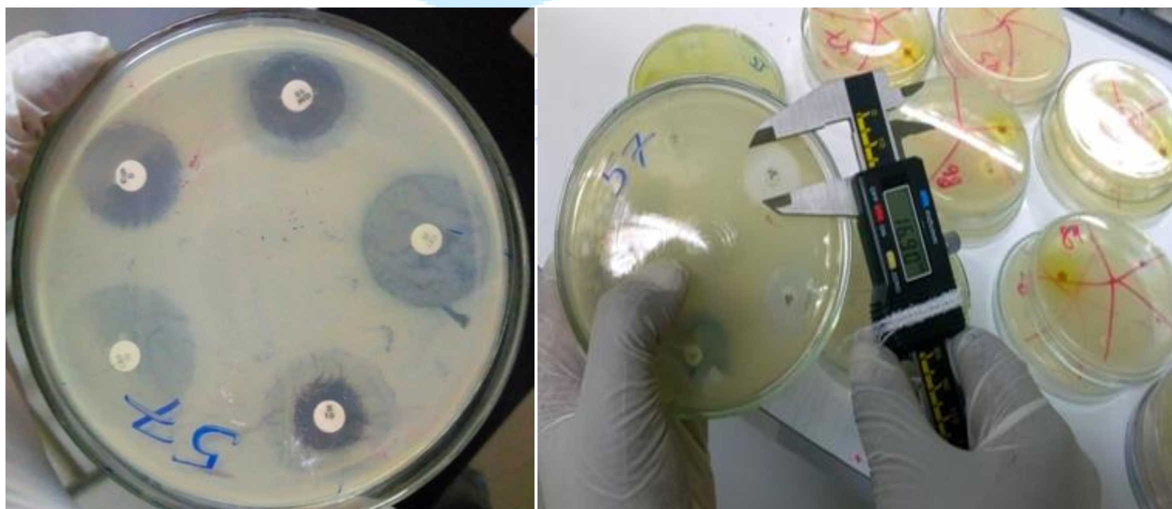

Figure 8: Antibiotic resistance profile and how to measure the inhibition zone of *E. coli* isolated from different samples.