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Enterohaemorrhagic Escherichia coli in Raw Milk and Dairy Products in Libya

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Abstract

An increased emphasis has been placed on the microbiological analysis of raw milk and dairy products designed to evaluate quality and to ensure safety for human consumption. Many microorganisms such as *Escherichia coli* can contaminate raw milk during milking process or via environmental contamination during different milk-processing methods. Enterohaem- orrhagic *Escherichia coli* O157 (EHEC) are a major cause of food-borne illnesses in humans. A hundred and seven samples of milk and milk products were collected from some regions (Janzour, Tripoli, Kryamia, Tajoura and Toubrok) in Libya. In the current study, samples were subjected to microbiological analysis for enumeration and isolation of *Escherichia coli*, which were detected by conventional cultural and molecular method using polymerase chain reaction (PCR) and partial sequencing of 16S rDNA. Out of the107 samples; 26 positive sample according to culture characteristics were found to be EHEC O157 included cow's milk 10 % (3/ 28), she-camel's milk 33%(3/ 9), goats milk 17 % (1/6) and 25 % (7/28) fermented raw milk samples. Samples of fresh locally made soft cheese (Maasora and Ricotta) were 43% (9/21) and 30% (3/10) respectively, while none of the ice cream samples revealed any growth, but 12 of these 26 sample were confirmed by partial sequencing of 16SrDNA as *Escherichia coli* (*E. coli*).

Key words: Enterohaemorrhagic *Escherichia coli*; raw milk; 16S rDNA.

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List of Abbreviations

ABBREVIATION	NAME
APHA	American Public Health Association
a_w	Water Activity
BAM	Bacteriological Analytical Manual
bp	Base Pair
CDC	Centers For Disease Control And Prevention
CFU	Colony Forming Unit
DAEC	Diffusely Adhering *E. coli*
rDNA	ribosomal Deoxyribonucleic Acid
E. Coli	Escherichia coli
EAEC	Enteroaggregative *E. coli*
EEB	Enterobacteriacae Enrichment Broth
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
g	Gram
HC	Hemorrhagic colitis
HUS	Hemolytic Uremic Syndrome
hly	Enterohaemolysin
HTST	High Temperature Short-Time
mL	Milliliter
NaCL	Sodium Chloride
STEC	Shiga Toxin Producing *Escherichia coli*
Stxs	Shiga-like Toxins
TC-SMAC	Tellurite-Cefixime-Sorbitol MacConkey

INTRODUCTION

1.INTRODUCTION

Milk is a high nutritional food for human beings consumption, it can be obtained from a multiplicity of animal sources such as cows, goats and camel, although it serves as an ideal medium for the growth of many organisms because of its near-neutral pH, complex biochemical composition and high water content (**Touch and Deeth, 2009**). Microorganisms can introduce to raw milk during and after milking which can be the main cause of microbial contamination **(Vissers and Driehuis, 2009**). Raw milk is still consumed by large number of farm families and the general population who believe the milk beneficial health effects **(Lejeune and Rajala-Schultz, 2009**).

Libya is not an exception where there is a high demand for consumption of both milk and dairy products. It is not unusual that Libyans purchase and consume not only raw cow's milk but also goat and sheep milk. Camel's milk is consumed raw, neither pasteurized nor boiled. Locally made dairy products, such as soft cheeses, fermented milk and ice cream are manufactured at small scale dairy parlors, where hygienic measures neither applied nor enforced. Fresh milk drawn from a healthy cow normally contains a low microbial count ($<10^5$ CFU/mL,g) according to **Libyan standards (2010)**, but the count may increase up to 100 fold or more once it is stored at impropriate temperature.

Therefore, keeping milk in clean vessels at refrigeration temperatures immediately after the milking process may delay the increasing of initial microbial count and prevent the multiplication of microorganisms in milk

during the interval between milking until milk transportation to the dairy shops **(Chye *et al.*, 2004**) During that period milk passes through various stages and the microbial contamination may occur specially when it has been handled improperly. The microbial count of raw milk can also negatively effect on milk quality and shelf life, which may cause an economic losses; in addition to health related influence in that the consumption of raw milk contaminated with pathogens can lead to, severe illness **(Oliver *et al*., 2009).**

Pasteurisation can destroy all the pathogenic and large number of non-pathogenic microorganisms, however if the initial flora present in large number in raw milk, it will be very hard to be killed **(Salmeron *et al*., 2002).**

Theodor Escherich, a German bacteriologist reported the isolation of a bacterium called Bacteria coli from a fecal sample in 1885. Later, in 1888, it was renamed *Escherichea coli* (*E. coli*) **(Bhunia, 2008).**

Pathogenic *E. coli* are classified into six virotypes: *Enterohaemorrhagic E.coli* (EHEC), *Enterotoxigenic E. coli*(ETEC), *Enteropathogenic E. coli* (EPEC), *Enteroaggregative E. coli*(EAEC), *Enteroinvasive E. coli* (EIEC) and *Diffuselyadhering E. coli*(DAEC).

Enterohaemorrhagic *Escherichea coli* (EHEC) are found in the faeces of healthy animals and their presence in raw milk is generally indicative of direct or indirect faecal contamination. However, organisms can be excreted through the udder when systemic infection results in mastitis **(Ahmed and Abouzeed, 2014).**

In spite of the fact that the majority of *E. coli* do not create a serious health hazard, there are some serotypes that could lead to food poisoning and alimentary intoxications. **(Picozzi *et al*., 2005).**

Escherichia coli O157:H7 has become an emerged pathogen in food, dairy industries and the public health **(McCarthy *et al*., 2001). Picozzi *et al*. (2005)** emphasized that Enterohaemorrhagic *E. coli* strains are the most dangerous among those serotypes, particularly, serotype O157:H7and has the ability to cause severe illness, especially, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP).

In Libya; a study by **Ghenghesh *et al*. (1997)** has detected EHEC O157:H7 (7%) linked to157diarrheal specimens samples from children. Another study from Tunisia which found EHEC in 10.4% from 73 of diarrheagenic symptomatic children **(Al-Gallas *et al*., 2007).** By using PCR based techniques **Al-Gallas *et al*. (2006)** have reported one isolate of *E. coli*O157:H7 from 11 isolates.

There is a clear evidence that raw milk samples and different dairy products are considered to be the primary reservoir of EHEC **(Hussein and Sakuma, 2005; Solomakos *et al*., 2009). Madic *et al*. (2011)** reported that most cases of *E. coli* outbreaks were due to consumption of raw traditional dairy products, or contaminated food by cross contamination or even undercooked foods plus personal hygiene.

Aim of this work

This study was planned out to evaluate the presence of Entero-haemorrhagic *Escherichea coli* O157:H7 in milk and dairy products in different localities in Libya by the following topics:

- To isolate EHEC from milk and dairy products marketed in different regions of Libya.
- To identify the isolates using conventional microbiological cultural methods.
- To identify EHEC isolates using molecular subtyping methods by PCR and partial sequencing of 16S rDNA gene.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. General characteristics of Enterohaemorrhagic *E. coli* (EHEC)

Escherichia coli is Gram-negative, non-spore-forming bacterium belonging to the family Enterobacteriaceae. It is found in the gastrointestinal tract of man and animals, which could contaminate water, soil and food due to faecal contamination. This association makes *E. coli* as an indicator organisms for bacteriological quality of milk and its products **(I.C.M.S.F., 1996)**, especially, it is able to resist many stressors such as acidic conditions, dehydration and high salt concentrations **(Alam and Zurek, 2004).** In addition, these characteristics facilitate its survival at different processing points (**BenjaminandDatta**, **1995**).

Generally it does not ferment sorbitol and does not have glucuronidase activity (GUD). Most of *E. coli* grow between 10 and 46 °C, while some strains are easy to grow at 8°C **(I.C.M.S.F., 1996).** However, some EHEC serotypes can grow in milk at temperatures as low as 6.5 °C **(Kauppi, 1996)**. A research has been conducted by **Vidovic *et al.*, 2011** showed that *E. coli* O157 strains possess inherent genetic mechanisms which enable growth at low temperatures (15 °C), compared to non-pathogenic *E. coli*. EHEC like other Gram-negative bacteria, do not exhibit unusual heat resistance under conditions of neutral pH and moderately high water activity (a_w: 0.95) **(Kaur *et al.*, 1998).** Pasteurisations of milk at 72 °C for 15 sec inactivates O157:H7 **(D'Aoust *et al.*, 1988).** In addition, strains resistant to pH 4.5 or below (pH 3.6–3.9) have been identified, and the organism is destroyed at 64.3 °C in 9.6 sec. during pasteurisation temperature 63°C for 30 min, but the cells survive well in food at −20 °C **(Bhunia, 2008).** In addition to the destruction of pathogens and

undesirable bacteria, pasteurisation also extends the useful life of the product with minimal alteration of flavor and physical characteristics.

Generally, a high temperature short time (HTST) process 72°C for 15 sec for fluid milk is preferable, instead of a low temperature long time treatment (63°C for 30 min), since HTST usually results in less nutrient destruction and fewer sensory changes(**CJ K Henry and Chapman, 2002**).

Environmental stresses can induce thermotolerance in some strains. Exposure to mild heat, or cross-protection induced during acid adaptation, has been reported to increase thermotolerance by up-regulating stress response genes **(Murano and Pierson, 1992; Ryu and Beuchat, 1998; Semanchek and Golden, 1998)**. Fat also has a protective effect against thermal inactivation **(Singh *et al*., 1980).** Three mechanisms in *E. coli* allow the cells to withstand acid challenge at pH 2.5 **(Castanie-Cornet *et al*., 1999).** Acid resistance of EHEC helps to survive the acidity of the stomach and to colonies the gastrointestinal tract. Furthermore, it also increases the survival of EHEC in acidic foods, enabling survival for extended periods, particularly at refrigeration temperature **(Semanchek and Golden, 1996)**.

EHEC is also referred as Shiga toxin producing *E. coli* (STEC), its ability to cause serious disease in humans is attributed to producing of different types of lethal toxins called Shiga-like toxins (Stxs) which is different among strains **(Law, 2000; Bhunia, 2008; Rizwan *et al*., 2015).**

2.2. Source and Transmission

Ruminants have been identified as a principal reservoir of *E. coli*O157:H7 **(Wang *et al.*, 1996;Gyles, 2007).** Bovines are STEC carriers and the main reservoir of serotypes O157:H7 and non-O157, many of them involved in HUS and HC worldwide.

E. coli O157:H7 has been isolated from many healthy cattle and has not been shown to be a pathogen in these animals. Cattle seem to lack vascular receptors for Stxs and these receptors provides insight into why cattle are tolerant reservoir hosts for *E. coli*O157:H7 **(Pruimboom-Brees *et al.*, 2000).**

Transmission of EHEC to humans occurs through consumption of undercooked meat, vegetables and water contaminated by feaces of carriers, person-to-person and contaminated environment contact. Dairy farms can contribute to the risk of EHEC infection in humans through the consumption of raw milk, dairy products, and contaminated meat from dairy cattle and through contamination of the dairy environment. Therefore, it is important to improve the control measures and management to prevent the transmission of EHEC strains among animals, environment and humans **(Fernandez and Padola, 2012).**

2.3. Public health significance of Enterohaemorhagic *E. coli* O157:H7

Escherichia coli are the predominant, facultative anaerobe of the human intestine. Beneficial strains of *E. coli* typically colonise the infant gastrointestinal tract within a few hours after birth. The presence of this bacterial population in the intestine suppresses the growth of harmful bacteria and is important for synthesising appreciable amounts of B vitamins. *E. coli* usually remains harmless when confined to the intestinal

lumen. However, in debilitated or immuno-suppressed persons, or when gastrointestinal barriers are violated, even non-pathogenic strains of *E. coli* can cause infection **(Paton and Paton, 1998).**

Jaeger and Acheson (2000) indicated that *E. coli* O157 is an emerging cause of food borne illness in the developed world.

EHEC O157:H7 is associated with outbreaks and sporadic cases of hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS), which considered as the most severe form of these human diseases. In Argentina, HUS is endemic, with 500 new cases per year and an incidence of 17/100,000 in children under 5 years of age, EHEC O157:H7 is the most frequently isolated serotype **(Fernandez and Padola, 2012).**

The mean annual incidence of haemolytic uremic syndrome in persons </=15 years of age in Italy from 1988 to 2000 was 0.28 per 100,000 population. Laboratory investigations showed that EHEC infection occurred in 73.1% of patients. EHEC O157 was the most common serotype, but a considerable number of cases were from infections by non-O157 EHEC **(Tozzi *et al*., 2003).** The infectious dose for O157:H7 is estimated to be 10 - 100 cell **(CDC, 1993).** On the other hand a study by **Tilden *et al*. (1996)** reported that 50–100 colony forming units (CFU) of EHEC is sufficient to cause disease in healthy individuals. In addition EHEC strains may cause human infections ranging from simple diarrhea to HUS **(Perrin *et al*., 2014).**

In 1996, an outbreak of *E. coli* O157:H7 in Japan affected over 6,300 school children and resulted in 2 deaths. At that time, these was the largest outbreak ever recorded for this pathogen **(Saudi, 2002).**

Denny *et al*. (2008) investigated an *E. coli* O157:H7 infection, that were related to consumption of unpasteurised milk or dairy products which

reported during the end of 2005 in Oregon and Washington states, 18 cases mostly among children aged 14 years.

Bielaszewska *et al.* (1997) reported an outbreak of people consumed unpasteurized goat's milk from the same farm within the week before the disease. Evidence of *E. coli*O157 infection was subsequently found in 5 of 15 regular drinkers of the farm's raw goat's milk.

Hence, contaminated cultured and fermented foods such as yoghurt and cheese have been implicated in sporadic cases and outbreaks **(Besser *et al.*, 1993; CDC, 1995; Baylis, 2009; King *et al.*, 2010).**

During cheese manufacturing, a salting step is usually applied to provide a supplementary drainage of whey, add to the flavor characteristics, help the rind formation and to regulate the water activity (a_w) in order to control microbial growth **(Mahaut *et al.*, 2000).**The minimum a_w for growth of *E. coli* is about 0.95 **(Salter *et al.*, 2000; Lindblad and Lindqvist, 2010)**. It has also been shown that tryptone soya broth **(Haversen *et al.*,2000)**, with 8.5 % sodium chloride (NaCl) inhibited the growth of *E. coli* O157:H7.However, the organism could grows in medium containing 6.5 % NaCl **(Glass *et al.*, 1992).**

2.4.Identification of *EHEC* by different molecular methods

Chiang *et al.* (2012) have stated that food products, such as milk and meat products including cheese, milk powder, fermented milk, sausage, etc. are susceptible to the contamination by pathogenic and deteriorative bacteria including *E. coli* O157:H7. Traditional methods for the detection of these microorganisms are laborious and time consuming. Therefore, rapid and accurate diagnostic methods are needed.

There are many DNA-based assay formats for the detection and identification of foodborne pathogens which have been an exploded in the past years, molecular-based *E. coli* assays have been advanced between 1980 and 1990; the hybridization using DNA probs to detect virulence determinants like EHEC virulence genes **(Campbell *et al.*, 2001; Bayardelle and Zafarullah, 2002).**

Conventional PCR be dependent on amplification of the target gene(s) in a thermocycler, separation of PCR products by gel electrophoresis, followed by visualization and analysis of the resulting electrophoretic patterns, a process that can take a number of hours. The specificity can be subsequently confirmed by sequencing the amplified fragment. PCR can be superior to detect the main pathogens in food samples **(Abubakar *et al.*, 2007).**

Shibasaki *et al.* (2000)have compared a multiplex real-time polymerase chain reaction assay with a culture-based approach in an on-farm quality assurance program for the detection of *E. coli*O157 in bulk tank milk. The results revealed that the real-time PCR was more sensitive in detecting *E. coli*O157 than the culture method (48% vs. 4% positive).They concluded that, the real-time PCR, by reducing analysis time to two working days, can be proposed as a useful tool in the raw milk primary production setting as a rapid and user-friendly screening method.

2.5.16S rRNA identification of *E. coli*

The 16S rRNA gene sequence has been determined for a large number of strains. GenBank has over 20 million deposited sequences which is the largest databank of nucleotide sequences; implicate over 90,000 of 16S rRNA gene. To facilitate thesearch and identify the species

that match the sequence of an unknown strain. With developing of researches; the 16S rRNA gene became an universal gene in bacteria, and relationships can be known among all bacteria **(Woese *et al.*, 1985).**

In general, the comparison of the 16S rRNA gene sequences facilitates the differentiation between organisms at the genus level across all major phyla of bacteria, in addition to classifying strains at multiple levels. The occasional exceptions to the usefulness of 16S rRNA gene sequencing usually relate to more than one well-known species having the same or very similar sequences **(Woese, 1987).**

The gene of 16S rRNA is present in all bacteria and the function of 16S rRNA gene has not changed overtime. It consists of about 1,542 nucleotide bases **(Janda and Abbott, 2007),**which can be quickly and cheaply copied and sequenced for accurate genetic identification of bacterial species.

2.6.Occurrence of Enterohaemorhagic *E. coli*O157:H7(EHEC)in milk and milk products

(Keene *et al.*, 1997) have reported that raw cow's milk can be potential vehicle for *E. coli* O157:H7 (EHEC) infection, however, pasteurized milk and yoghurt have also been implicated in outbreaks.

Conedera *et al.* (2004) have examined 3879 dairy products samples for the presence of *E. coli* O157 (EHEC O157). The dairy products were as follows: 657 pasteurized and 811 unpasteurized cow's milk samples, 477 pasteurised and 502 unpasteurized goat's milk samples, and 501 mozzarella cheese. EHEC O157 was not detected in any of the dairy products, while *E. coli* O157 *stx*, *eae* genes was isolated from one raw cow's milk cheese.

Garbaj (2004) examined 180 samples of milk and milk products and reported that; 30 isolates of EHEC strains were genetically confirmed by using PCR method.

Lira *et al.* (2004) examined 2144 milk samples and 432 cheese samples for the presence of *E. coli* and to determine the prevalence and molecular characteristics of EHEC in Brazil. 22 isolates of EHEC were detected (12.08%) from milk samples and 16 (3.7%) positive isolates which were obtained from cheese samples.

Picozzi *et al.* (2005) examined milk samples obtained from goats and cows were screened for the presence of *E. coli* O157 with cultural methods. Sorbitol-negative or slow-fermenting strains were subjected for detection of O157 gene; one strain was confirmed as O157.

Vernozy-Rozand *et al.* (2005)e valuate the large number of STEC strains recovered from the cheese samples which emphasizes the health risks associated with raw milk cheeses.

Roldan *et al.* (2007) have analyzed 150 milk samples from bulk tanks by selective enrichment and immunomagnetic separation. All milk samples were negative for EHEC O157.

Karns *et al.* (2007) have conducted a study in United State and EHEC subtype of *E. coli* was detected in 23% of strains. By using a real time PCR method, they proved that 5 samples were contaminated with O157:H7.

Seker and Yardimci (2008) have examined 213 raw milk samples obtained from the tanks and containers using standard cultural methods, and isolated *E. coli* O157:H7 from 3 samples (1.4%).

Solomakos *et al.* (2009) have collected several milk samples and tested for the presence of *E. coli* serogroup O157, 905 raw bovine milk, 460 caprine and 595 ovine bulk milk. In total 29 strains (1.4%) were isolated, of which; 21 were isolated from bovine (2.2%), 3 from caprine (0.7%) and 5 from ovine (0.8%) milk.

Brooks *et al.* (2012) analyzed 41 raw milk cheeses for detection the presence of *L. monocytogenes*, *Salmonella*, *E. coli* O157:H7, *Staphylococcus aureus*, and *Campylobacter* . All samples were obtained from retail specialty shops, farmer's markets, and on-line sources. The results revealed that five samples contained coliforms; two of those contained *E. coli* at less than 10^2 CFU/g.

Momtaz *et al.* (2012) isolated 102 (14.18%) from 719 samples which confirmed to be positive for *E. coli*. Out of 102 positive samples 13.72% were O157 in addition their results showed that raw bovine milk (20.9%) and soft cheese (23.58%) had a highest rate of *E. coli*, while raw camel (6.81%) and soft ice cream (10%) had a lowest.

Torres-Vitela *et al.* (2012) examined 200 samples to detect the incidence of Salmonella, Listeria, *E. coli* O157:H7, and staphylococcal enterotoxin in two types of fresh cheese (100 panela and 100 adobera). The incidence in the panela samples was 16% *E. coli* O157:H7, whereas in the adobera samples, incidence was 4% *E. coli* O157:H7. Handcrafted panela and adobera fresh cheeses in Mexico frequently contain pathogenic bacteria and therefore pose a public health risk.

Mohammadi *et al.* (2013) investigated raw milk samples which were collected from various cow farms in Kermanshah, Iran

during June - September 2009 for EHEC using PCR targeting. Their result indicated that out of 206 samples, 36 (17.47%) were EHEC positive.

Ruusunen *et al.* (2013) determined the occurrence of (EHEC) in raw cow's milk samples collected from bulk tanks at 183 Finnish farms. EHEC with Shiga toxin-encoding stx2 was detected in 2.7% of the samples. They found no relationship between *E. coli* or the total bacterial count and the presence of pathogenic bacteria.

Schoder *et al.* (2013) examined milk quality and safety of 109 of raw milk samples. Samples were analyzed for detection of foodborne pathogens such as *E. coli*O157:H7. Their finding demonstrated that 11of 109 (10.1%) of raw milk samples were positive.

Ivbade *et al.* (2014) investigated 202 milk and milk products samples for the presence of EHEC O157 in Ogun State, Nigeria, 10 (5%) were positive for EHEC O157 including 3 (6%) of 50 fresh local cheese samples, 1 (2%) of 50 fried local cheese samples and 5 (9.6%) of 52 fermented milk samples.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Collection of samples

One hundred and seven samples each of (28 cow's milk, 9 she-camel's milk and 6 goat's milk, 10 Ricotta, 21 Maasora,5 ice cream and 28 fermented cow's milk) were randomly collected from different regions in Libya (Janzour, Tripoli, Kremiya, Tajoura and Tobruk) between October 2013 and May 2014. Collected samples were transferred to the laboratory of Food Hygiene and Control Department, Faculty of Veterinary Medicine, University of Tripoli in an insulated ice-box with a minimum of delay to be immediately examined.

3.2. Preparation of collected samples

The preparation of collected samples was performed according to American Public Health Association (**APHA**). Briefly, 25 g/mL from each sample was aseptically transferred into a sterile polyethylene stomacher bag (Seaward medicals, UK) and blended with 225 mL of buffer peptone water (Catalogue # 610098, LIOFILCHEM) in a stomacher homogenizer (Stomacher 400, Seaward medicals, UK) at 230 rpm for 1 min. One ml of the previously prepared first dilution 1/10 was transferred to sterile test tube containing 9 ml of buffer peptone water with addition of sterile sodium citrate (2%) in cheese samples and mixed to obtain 1/100 dilution. From which one ml was added to another 9 ml sterilized diluents to obtain further tenfold serial dilutions, then incubated at 37°C for 18 hrs ±2

3.3. Enumeration of *E. coli* O157:H7 (BAM, 2001)

From the previously prepared decimal dilutions, one mL was transferred to 3 plates of Tellurite-Cefixime-Sorbitol-MacConkey. The inoculum was distributed over the surface of agar plates using sterile bent glass streaking rod. Plates were inverted and incubated at 37°C for 48 h. Plates with 25-250 colonies, were counted and recorded.

3.4. Isolation and identification of *E. coli* O157:H7 (BAM, 2001)

From the prepared samples, 25 g/mL were added to 225 mL of Enterobacteriacae Enrichment broth (EEB) (1/10 dilution). After thorough mixing, the inoculated broth was incubated for 24 hrs at 37 ±0.5°C with shaking. After incubation, 0.1 mL was spread evenly on a dried surface of Tellurite-Cefixime-Sorbitol MacConkey (TC-SMAC) agar plates, another plate of the TC-SMAC agar medium was streaked using a loopful. All inoculated plates were incubated at 37 ±0.5°C for 18-24 hrs. Typical colonies of *E. coli* O157 were colorless or neutral/gray with a smoky center and 1-2 mm in diameter, the inoculated plates were incubated at 37°C for 24h. Five typical colonies were picked up for further investigation.

To perform the completed test for *Escherichia coli*, a loopful from positive plates were streaked on a L-EMB agar plates and incubated for 24 h at 37°C. Plates were examined for suspected colonies of *E. coli* which represented as dark centered, flat, with metallic sheen. The positive colonies were picked up and stored at - 80°C in Ependorff tubes with glycerol (20 %) for further testing.

3.5.Purification of isolated *E. coli O157*

For purification, single colony of suspected EHEC from TC-SMAC agar was picked up and re-cultured on L-EMB agar for further investigation.

3.6.Identification of EHEC by PCR and partial sequencing of 16S rDNA

3.6.1. Chromosomal DNA extraction

GF-1 bacterial DNA extraction kit (Cat.# GF-BA-100, Vivantis, Malaysia) was used and the methods were performed according to the manufacturer's instruction (**Fig.**1). Briefly, a single colony from L-EMB plates was picked up and inoculated into 5 mL nutrient broth and incubated at 37°C for 48 hrs. A total volume of 2mL of bacterial culture was centrifuged at 10000 x g for 2 min, then supernatant was discarded. The pellet was then re-suspended by adding 100 µL of Buffer R1 (Cat. # GF-BA-100, Vivantis, Malaysia).The re-suspended cells were then spun down at 10,000 xg for 5 min before the supernatant was decanted completely. The protein of the pellet was denaturized by re-suspension in 180 µL of Buffer R2 (Cat. # GF-BA-100, Vivantis, Malaysia) and add 20µL of proteinase K. Then incubated at 65°C for 20 min with shaking every 5 min. Homogenization was achieved by adding 400 µL of Buffer BG (Cat. # GF-BA-100,Vivantis, Malaysia) and mixed thoroughly by inverting the tube gently, then incubated at 65°C for 10 min. 200 µL of absolute ethanol was added with immediate mixing to prevent precipitation of DNA due to high ethanol concentration. The sample was transferred (maximum volume 650 µL) into the column and centrifuged at 10000 x g for 1 min(BOECO, M 240 R, Germany). The flow was discarded. The column was washed by 750 µL of wash buffer (Cat. # GF-BA-100, Vivantis, Malaysia) by

centrifugation at 10,000 x g for 1 min. Then the flow was discarded. Finally, the DNA was eluted in 50 µL of elution buffer, which left for 5 min at room temperature, then centrifuged at 1,500x g 1 min, and DNA was stored at 4 °C or -20 °C.

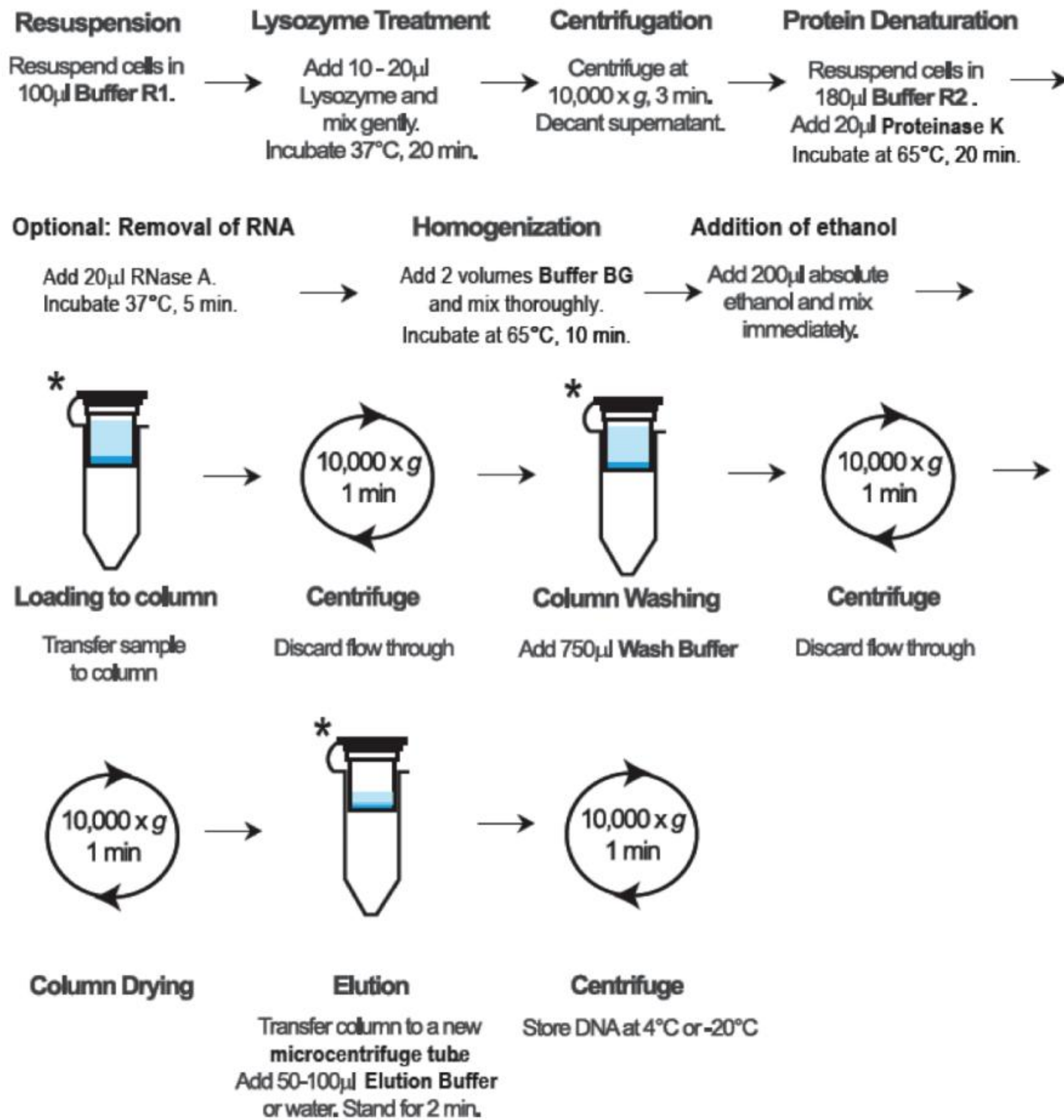

Figure 1.Steps of bacterial DNA extraction using GF-1 kit

(Cat.# GF-BA-100, Vivantis, Malaysia).

3.2. Amplification of 16S rDNA

Partial 16S rDNA was amplified using the universal oligonucleotides primers Forward S-D-Bact-0341-b-S-17 and Reverse S-D-Bact-0785-a-A-21 adopted from **(Herlemann*et al.*, 2011)**. Briefly, 0.2 µg of genomic DNA was add to 25 µL Maximo Dry PCR Master Mix (Cat. # S295, Gene ON). The mixture was then amplified in a DNA Thermal Cycler (TECHNE TC-512 Barloworld scientific Ltd, UK) using the following program: one denaturation step at 94 °C for 5 min; 35 cycles of denaturation, 92 °C for 30 s, annealing temperature for 30 s at 55°C, extension at 68 °C for 60 s; and a final extension at 72 °C for 10 min.

The PCR products were electrophoresed in 2% agarosegel (Cat. # 604-005, Gene ON, UK) incorporated with nucleic acid gel stain – 10000X (Gel RED, Cat. # S420, Gene ON, UK) at voltage 100 volt for one hour(SCIE-PLAS, UK). The sizes of the amplified fragents were determined by comparison with the Gel Pilot 100 bp increment Ladder (Qiagen, Cat.# 239035, Melbourne, Australia) a ready-to-use 6 fragents (100–600 bp) DNA marker. The gel was photographed with gel-documentation system micro DOC with UV-trans-illuminator (CSLUVTS312, Cleaver Scientific, UK).

3.6.3 DNA sequencing and analysis

The amplified 16S rDNA PCR fragent (464 bp) was excised from the gel and the DNA was extracted from the gel using GF-1 AmbiClean kit (Cat. # GF-GC-100, Vivantis, Malaysia) as described in (**Fig. 2).** Briefly, the net weight of gel slice was determined and 1 volume of Buffer DB was added to 1 volume of gel (A gel slice of mass 0.1g will have a volume of 100µL).

Then the gel was incubated at 50°C until the gel was melted completely. The melted gel was transferred to a column assembled in a clean collection tube. Centrifuge at 10,000 x g for 1 min. Discard flow through. The column was washed by adding750µL wash buffer and centrifuging at 10,000 x g for 1 min. and discarding flow through. Column was dried by centrifugation at 10,000 x g for 1 min to remove residual ethanol.

Table 1. List of oligonucleotides primers used in this study

Target Gene	**Name**	**Sequence**	**Ampilicon Size**	**GenBank Access Number**	**References**
16S rRNA	FOR S-D-Bact-0341-b-S-17	5 -CCTACGGGNGGCWGCAG-3	464	Universal primer	**(Herlemann *et al*., 2011; Klindworth *et al*., 2013)**
	REV S-D-Bact-0785-a-A-21	5 -GACTACHVGGGTATCTAATCC-3			

Finally, DNA was then eluted by adding 30 µl of elution buffer and mixture was left for 2 min, and DNA was stored at 4 °C or -20 °C until using.

The purified 16S rDNA amplicons underwent cycle sequencing by Big Dye® Terminator v1.1 kit (AB Applied Biosystems) and sequence reactions were separated on a four capillary ABI PRISM® 3130 Genetic Analyzer at IZSLER (Istituto Zooprofilattico Sperimentale della Lombardia edell 'Emilia Romagna, Bianchi, 9 - 25124 Brescia, Italy).

Sequences were assembled and edited using SeqMan module within Laser gene package, (DNA Star Inc., Madison, WI, USA). The obtained consensus sequences were subjected to BLAST search both at NCBI (http://www.ncbi.nlm.nih.gov/pubmed) and at 16S bacterial cultures Blast Server for the identification of prokaryotes (http://bioinfo.unice.fr/blast/).

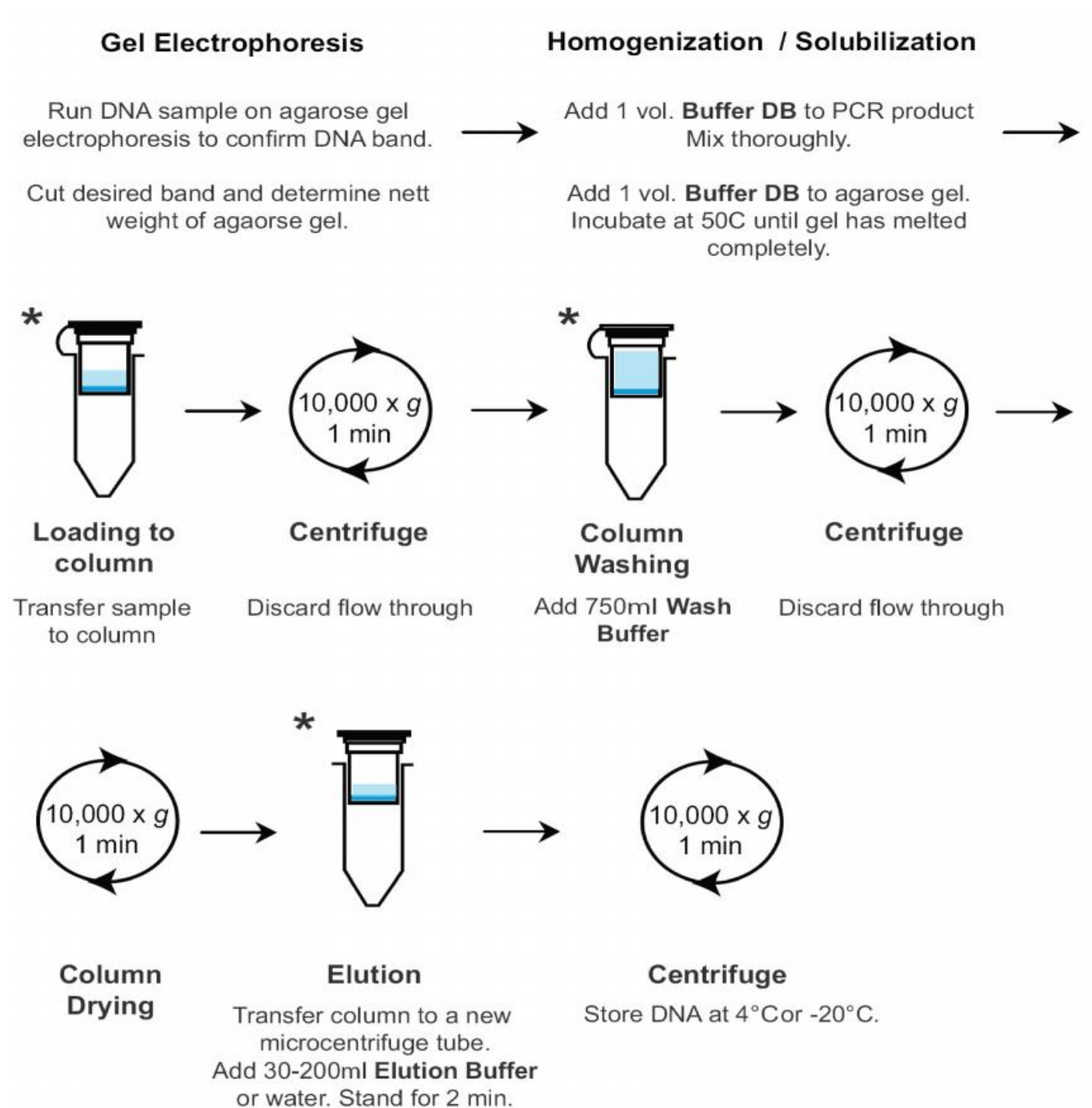

Figure 2. Steps of gel extraction of PCR products usingGF-1 AmbiClean kit (Cat. # GF-GC-100, Vivantis, Malaysia).

RESULTS

4.RESULTS

Among the 107 investigated samples, 26 were tested positive for *E. coli*. (**Table 2**). All suspected colonies which isolated from raw milk and milk products could successfully be cultivated on TC SMAC and EMB agar the morphology of colonies was colorless on TC-SMAC (**Fig. 5**)while the isolates showed green metallic sheen on EMB agar(**Fig.6**)and presumptively selected as EHEC. Under microscopic examination the bacteria was gram-negative, pink color, small rod shaped, single or paired.

With the exception of ice cream samples that revealed no growth on TC-SMAC, other samples showed different recovery rates of suspected colonies; the lowest rate was recorded in goat's milk 17 % (1/6) showed with counts ranging from 3×10^4 to 10×10^4 as shown in (**Table 2**).

Table 2. Count and Statistical analysis(CFU/mL) of EHEC in raw and fermented milk samples

Type of Sample	*Number of Sample*	*Number of Positive Sample*	*Isolation %*	*Max*	*Min*	*Average*
Cow's milk	28	3	10.70%	13×10^4	8×10^4	10×10^4
She Camel's milk	9	3	33.30%	15×10^4	1×10^4	7×10^4
Goat's milk	6	1	17%	3×10^4	3×10^4	3×10^4
Fermented milk	28	7	25%	17×10^4	1×10^4	9×10^4

Min=minimum, Max=maximum.

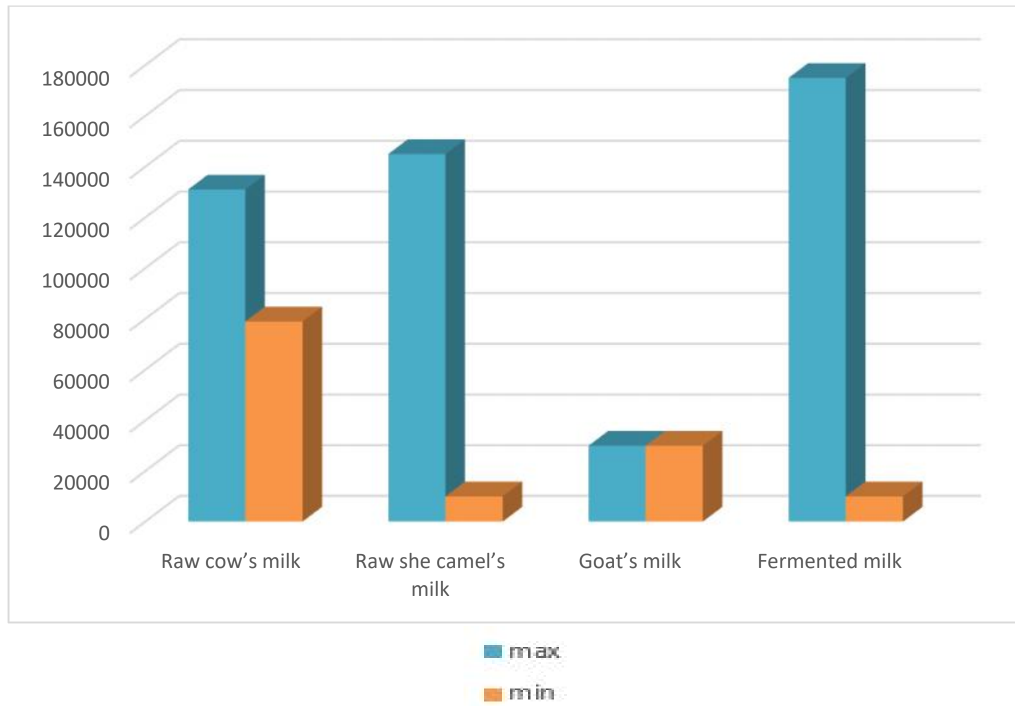

Figure 3:Mean count of EHEC in milk samples; CFU/mL.

Figure 3 showed that the fermented milk samples had the highest mean counts of EHEC, While; the she camel's milk samples had the lowest mean cunts of EHEC.

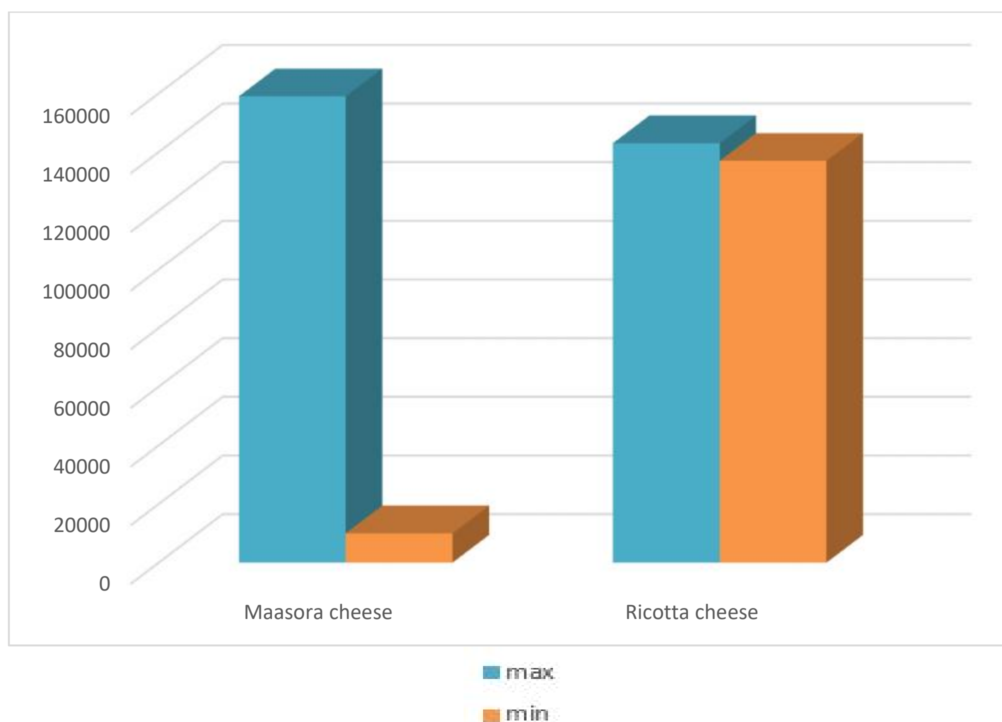

Figure 4:Mean count of EHEC in cheese samples; CFU/g.

It can be seen that the highest mean count of EHEC in Maasora cheese 43% (9/21) was 8 X 10^4 CFU/g of EHEC followed by Ricotta cheese30 % (3/10) with average count 12 x 10^4 CFU/g (**Table 3**).

Table3. Count and Statistical analysis(CFU/g) of EHEC in dairy products samples

Type of Sample	*Number of Samples*	*Number of Positive Sample*	*Isolaion %*	*Max*	*Min*	*Average*
Maasora cheese	21	9	43%	$15x10^4$	$1x10^4$	$8x10^4$
Ricotta cheese	10	3	30%	$14x10^4$	$10x10^4$	$12x10^4$
Ice cream	5	0	0	0	0	0

Min=minimum, Max=maximum

Presumptively selected colonies were recurrently streaked on the respective selected media (EMB agar) to check and confirm their purity. Recovered bacterial isolates (26) were subjected to molecular analysis by DNA extraction followed by partial sequencing of their 16S rDNA.

Results of partial sequence analysis (**Table 5**) showed that only 12 isolates (46%) were EHEC (**Table 5**&**Fig.7**). Among those 12 EHEC isolates: 6 (50%) were recovered from fermented cow's milk, 3 (25%) from raw cow's milk, while raw goat's milk gave 1 (8.3%) isolate and 2(16%) isolates from Maasora cheese (**Table 4&5**). There was no recovery from raw camel's milk, Ricotta and ice cream samples. That would result in an overall isolation rate of 11% (12/107) EHEC O157.

Table 4: Isolation and molecular identification of suspected EHEC in processed samples

Type of Sample	No. of Samples	No. of Suspected EHEC Growth on TC-SMAC	No. of Positive EHEC by 16S rDNA Sequencing
Raw cow's milk	28	3 (11%)	3
Raw she camel's milk	9	3 (33%)	0
Raw goat's milk	6	1 (17 %)	1
Fermented cow's milk	28	7 (25%)	6
Maasora cheeses	21	9 (43%)	2
Ricotta cheese	10	3 (30%)	0
Ice cream	5	0 (0%)	0
Total	107	26 (24%)	12 (11%)

Table 5: Identification of suspected EHEC by PCR and partial sequencing of 16SrDNA

Blast NCBI search results	Nucleotide identity %	Isolates code	Type of sample	Sampling area
Escherichia coli	100%	10426	Maasora	Tajoura
Escherichia coli	98%	9301	Raw cow's milk	Tobruk
Escherichia coli	100%	9404	Fermented cow's milk	Tobruk
Escherichia coli	100%	9405	Fermented cow's milk	Tobruk
Escherichia coli	100%	7401	Fermented cow's milk	Janzour
Escherichia coli	99%	6413	Fermented cow's milk	Janzour
Escherichia coli	100%	6412	Fermented cow's milk	Janzour
Escherichia coli	100%	6308	Raw cow's milk	Janzour
Escherichia coli	100%	6306	Raw cow's milk	Janzour
Escherichia coli	100%	3410.2	Fermented cow's milk	Kremiya
Escherichia coli	99%	3405.2	Maasora cheese	Kremiya
Escherichia coli	100%	3301.2	Raw goat's milk	Tripoli

Figure 5: TelluriteCefiximeSorbitol-MacConkey (TC- SMAC) agar plate showing *Enterohaemorrhagic E. coli* (colorless colonies).

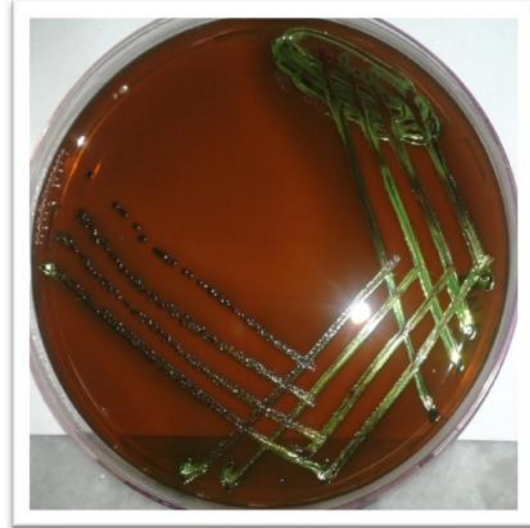

Figure 6: Levines – Eosin Methylene Blue (L-EMB) agar plate showing the characteristic EHEC colonies (green metallic sheen).

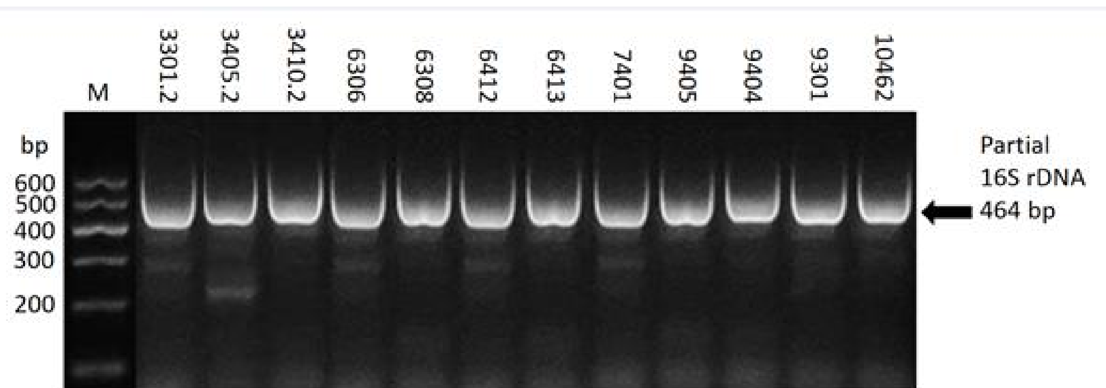

Figure 7:. Partial identification of 16S rDNA (464 bp) of isolated *E. coli* strains. First to Last lanes contain the 6 fragents (100-600 bp) DNA marker.

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.DISCUSSION

The number of people who consume raw milk has been increased dramatically, there is lack of studies to support these claims. Enhanced nutritional quality, taste, and health benefits have all been advocated as reasons for increased interest in raw milk consumption **(Oliver *et al.*, 2009).** Food-borne outbreaks due to consumption of dairy products constitute a chronic problem facing food hygienists. Milk and its products are subjected to different sources of contamination by many pathogens either from endogenous origin or exogenous source (directly and indirectly). The origin of contamination by food poisoning organisms varies with the type of product and the mode of production and processing. Treatment and processing of milk inhibit or encourage the multiplication of such organisms **(Garbaj, 2004).** All the nutritional components that make milk and dairy products important part of the human diet, and support the growth of these pathogenic organisms. With the purpose of microbiological risks associated with consumption of raw milk and its products this work was done.

Results obtained in this study showed that EHEC O157 has been isolated and identified from most of the examined milk and dairy products samples except from raw she camel’s milk, Ricotta and ice cream samples. In Libya; it is worth mentioning that it is quite difficult to compare our results from this study with others, simply because in the majority of other studies related to this one, EHEC has been identified according to conventional methods (biochemical tests and stereotyping), and not to sequencing of 16S rDNA. The isolation of EHEC from raw milk samples most likely is attributed to fecal contamination during the milking step

(Hussein *et al.*, 2005). On the other hand, the absence of EHEC in ice cream samples in this study could be due to the use of reconstituted milk powder or ready to use ice cream formulas rather than fresh milk.

On TC-SMAC agar, *E. coli* O157 did not ferment sorbitol and form colorless colonies. **(March and Ratnam, 1986)** reported that the detection of *E. coli* O157 on this medium had a sensitivity of 100% and specificity of 85% and it has been recommended as a simple, inexpensive and reliable means of screening *E. coli* O157.

All the 26 positive milk and its products samples showed growth of colonies with a metallic green sheen on Levine EMB agar, which was highly suspicious for *E. coli*.

Among all the various samples tested in this study, EHEC O157 was isolated from 50% (6/12) of fermented cow's milk, since the milk used in this type is raw without heat treatment; this is a high rate of isolation if compared to 9.6% (5/52) which reported by **Ivbade *et al.* (2014)** in Nigeria. The existence of EHEC in fermented milk samples could be explained by the acid tolerant property of the organism **(Benjamin and Datta, 1995).** The data of this study revealed that none of the raw she camel's milk samples were positive for EHEC, however, despite of scarcity of information about isolation of EHEC from she camel's milk, **Momtaz *et al.* (2012)** demonstrated that 6.8% (3/44) of she camel's milk samples were positive for EHEC. The results also showed that 8.3 % (1/12) of EHEC isolates were recovered from raw goat's milk samples, this is to the contrary of **Solomakos *et al.* (2009)** who found that the incidence of EHEC O157 in goat's milk was 0.7% (3/460) in samples collected in Greece, while no *E. coli* O157 was detected by **Almeida *et al.* (2007)** in

any of the cheeses made with raw ewe's and goat's milk samples from Portugal. Raw cow's milk yielded 25% (3/12) of EHEC O157. This result is slightly higher than that (17.4%) reported by **Mohammadi *et al.* (2013)**, however, much lower isolation rates were reported by other workers: 12% **(Lira *et al.*, 2004),** 10% **(Schoder *et al.*, 2013)**, 2% **(Ivbade *et al.*, 2014)** and 1.4% by **Seker and Yardimci (2008).**

Besides the risks associated with consuming raw milk there are concerns over the safety of cheeses made from raw milk. Cheese made from unpasteurized milk has been frequently implicated as the vehicle of transmission in outbreaks of infectious intestinal disease including *E. coli* O157:H7 (**Behravesh CB, *et al., 2012***).

Additionally; only Maasora yielded 16% (2/12) of EHEC O157 isolates recovered in this study, this result could be linked to the process of preparing Maasora (manufactured by simple method of casein precipitation using organic acids (Lemon juice or diluted acetic acid with heating then stay overnight under pressure)). **Ivbade *et al.* (2014)** reported the presence of EHEC O157 in 6% of locally made fresh soft cheese samples in Nigeria. Although cheese can be made safely with raw milk, there have been food poisoning outbreaks linked to raw milk cheeses caused by *Salmonella*, *Campylobacter*, *Staphylococcus aureus* and *E. coli* O157 **(De Buyser *et al.*, 2001)**. On the other hand, none of the Ricotta samples were positive.

6.CONCLUSION

It has been established that consumption of raw milk or its product directly is a high risk and that *E. coli* O157: H7 can cause severe disease and even death **(Mead and Griffin, 1998)**. In the U.S., the Centers for Disease Control and Prevention (CDC) estimates that EHEC O157:H7 causes approximately 73,000 illnesses, 2,000 hospitalizations, and 50-60 deaths each year **(Frenzen *et al*., 2005).** It is common to manufacture dairy products from raw milk in Libya and has increased in popularity. Despite the contrast of isolation rates of *E. coli* O157 from these products, however, this pathogen was isolated from these products and could be associated with human disease if consumed. However, it is not possible to link our findings with any of the food poisoning cases which are reported now and then in Libya because of the lack of documentation of such cases.

On-farm cross-contamination or direct contact with feces of animals are critical, and any failure in hygienic practices adversely impacting the microbial load in raw milk. Correct sanitizing procedures for packaging and effective cold chain management practices for the raw milk are important steps for minimizing cross-contamination and growth of any microorganism present in the raw milk. Findings of this study highlight the need to improve and implement the hygienic practices related to dairy production and to apply effective monitoring throughout from production to delivery. Moreover, further research is needed to fully study the incidence, prevalence and impact of toxins produced by *E. coli* O157 and other harmful microorganisms.

7.RECOMMENDATIONS

Libyan standards (2010) recommends that; milk must be held in a clean place designed and equipped immediately after milking, to avoid contamination and increasing the number of micro-organisms.

Moreover milk and dairy products must be cooled immediately at 4 °C and during transport, where the low temperature should be maintained from the milking at the farm to the table.

Apply educational program among farmers about hygienic practices at the farm level to reduce microbial count and overcoming the impact of the harmful pathogens like *E. coli*.

In addition, it is highly recommended to take the essential precautions into consideration during the production in the small plants, handling and distribution in the markets in order to carry out effective sanitary practices.

Implantation of Hazard Analysis of Critical Control Point (HACCP) plan built upon a solid foundation of prerequisite programs is required for each product for the safety production of such products.

A much needed data which will be used in further studies on EHEC as an emerging food borne pathogen and its virulence genes characteristics in human infection.

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8.REFERENCES

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9.APPENDIX

1. MATERIALS

1.1. Glassware

i. Flasks (250, 500, 1000 mL) were used for media, diluents and chemical solutions preparation.
ii. Media bottles (250, 500, 1000 mL) were used for bacteriological media preparation.
iii. Beakers used (250, 500 mL).
iv. Measuring Cylinders, (10, 100, 500, 1000 mL) were used for solutions preparation and measurements.
v. Pipettes (1, 5, and 10 mL) were used for sample, solutions and bacteriological media.
vi. 15 mL culture tubes with cap.

1.2. Plastic-wares

i. Disposable bacteriological Petri-dishes (Fisher-Scientific, USA)
ii. Disposable bacteriological loops (Looplast, LP Italiana, SPA, Milan, Italy).
iii. Disposable 1 mL pipettes.
iv. Disposable filter pipette tips (1 µl, 10 µl, 100 µl, 1000 µl) (Fisher-Scientific, USA).
v. Disposable L / T shaped plastic spreader.
vi. Plastic stomacher bags (Seward # 707, UK).
vii. Latex gloves.
viii. Culture tube racks.

ix. PCR tube racks.
x. Microfuge tubes (0.1mL, 0.5 mL, 1 mL) (Eppendorf, USA).
xi. Microfuge tube racks.
xii. Iceboxes.

1.3. Instruments

xiii. Micro-titer pipettes (1µl , 10 µl, 100 µl, 1000 µl) (Eppendorf, USA).
xiv. Water bath (Memmert, WB45 – Germany).
xv. Centrifuge (BOECO, M 240 R, Germany).
xvi. Autoclave (Hirayama, MFG, DY 1, Japan).
xvii. Hot air oven (Memmert, 100-800, Germany).
xviii. Deep Freezer (GFL, 6485 Germany).
xix. Fridge (White Westinghouse, USA).
xx. Stomacher (Seward # 400, UK).
xxi. Incubator (Memmert, 100-800, Germany).
xxii. Water Distillatory (GFL, 2302 Germany).
xxiii. Safety cabinet (HERA, KS 12, Germany).
xxiv. Thermal cycler (Strategene, USA).
xxv. Gel electrophoresis unit (box, tray, combs , power source)
xxvi. (Bio-Rad, USA).
xxvii. xiv. UV trans-illuminator (Cleaver, CSL UVTS 312, UK).
xxviii. Digital camera (Sony, Japan).

1.4. Chemicals, Media and Detection Kits

i. Distilled water.
ii. Molecular grade water (Siga-Aldrich, MO, USA).
iii. Peptone saline (Oxoid, UK).
iv. Tellurite-Cefixime-Sorbitol MacConkey (TC SMAC) agar (Park, UK).
v. Leveine's Eosin Methylene-Blue (L-EMB) agar (Park, UK).

2.Culture Media

Tellurite-Cefixime-Sorbitol MacConkey (TC -SMAC) agar

Pancreatic Digest of Gelatin	17.0 g.
Neutral Red	0.03 g.
Pancreatic Digest of Casein	1.5 g.
Crystal Violet	0.001g.
Peptic Digest of Animal Tissue	1.5 g.
Agar	13.5 g.
D-Sorbitol	10.0 g.
Cefixime	0.05 mg.
Bile Salts	1.5 g.
Potassium Tellurite	2.5 mg.
Sodium Chloride	5.0 g.

pH 7.1 +/- 0.3 at 25℃.

Sterilized by autoclaving at 121°C for 15 min

Peptone water

Peptone	10.0 g.
Sodium chloride	05.0 g.
Distilled water	1000mL.

pH 7.2 ± 0.2

Sterilized by autoclaving at 121°C for 15 min.

Levine's Eosin-Methylene Blue (L-EMB) Agar

Peptone	10.0 g.
Lactose	10.0 g.
Dipotassium hydrogen phosphate	02.0 g.
Eosin Y	0.4 g.
Methylene blue.	0.065 g.
Agar	15.0 g.
Distilled water	1000mL.

pH 6 .8 ± 0.2

Sterilized by autoclaving at 121°C for 15 min.

3.1.5: Molecular stuff:

- DNA extraction kit (Vivantis, GF1 # GF-BA-100, Malaysia).
- PCR master mix (Taq polymerase, dNTPs, Mag/Cal chloride) (Vivantis, Malaysia).
- 16 S rRNA universal primer (metbion, Germany)

  Forward: 5 -CCTACGGGNGGCWGCAG-3

  Reverse: 5 -GACTACHVGGGTATCTAATCC-3

ARABIC SAMARRY

الملخص العربي

10. ARABIC SAMMARY

نظرا لأهمية الألبان ومشتقاتها، تم التركيز على التحليل الميكروبيولوجية بهدف تقييم الحالة الصحية للألبان ومنتجاتهاوضمان سلامتها من الناحية الصحية لـ ستهلاك البشرى. هناك العديد من الكائنات الحية التى تلوث الألبان ثناء عملية الحلب أو التصنيع أو التداول الى ان تصل الى المستهلك، ومن ضمن هذه الكائنات الحية هى**الإشريكية القولونية المعوية النازفة** (O157) والتى تعتبر من المسببات الرئيسية لـ طريق الأغذية. لذلك تم تجميع عدد 107 عينة من الألبان وبعض مشتقاتها من ماكن مختلفة فى ليبيا لغرض فحصها ومعرفة مدى (O157). حيث تم الميكروبيولوجية لعزلها بـ ستعمال تقنية (PCR) وعملية للجين 16S. وأظهرت النتائج أن ميكروب **الإشريكية القولونية المعوية النازفة** متواجد فى حليب الأبقار بنسبة 10.7 (28/03) حليب النياق 33 (09/03) حليب 17 (6/1) 25 (28/07) (المعصورة والريكوتا) كانت 43 (21/09) 30 (10/03). ولم يسجلأى نمو للميكروب بالنسبة لعينات مثلجات اللبنية. من العزلات البكتيرية عن طريق الجزئي للجين 16 علي انها **الإشريكية القولونية المعوية النازفة** (107/12) 11 .

كلية الطب البيطري

قسم الرقابة الصحية على الأغذية

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/ . إيناس ماهر عوض

(بكالوريو بيطري 2010 - ليبيا)

الية (الماجستير)

قسم الرقابة الصحية علي الأغذية

كلية الطب البيطري

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كلية الطب البيطري

2016