

Escherichia Coli O157:H7: Distribution, molecular characterization, antimicrobial resistance patterns and source of contamination of sheep and goat carcasses at an export Abattoir, Mojo, Ethiopia

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Research article

Keywords: Abattoir, CT-SMAC, E. coli O157:H7, IMS, Latex agglutination, PCR

Posted Date: July 17th, 2019

DOI: <https://doi.org/10.21203/rs.2.9605/v2>

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Version of Record: A version of this preprint was published on September 12th, 2019. See the published version at <https://doi.org/10.1186/s12866-019-1590-8>.

Abstract

Background: Cattle have been identified as a major reservoir of *E. coli* O157:H7 for human infection; the ecology of the organism in sheep and goats is less understood. This study was carried out to determine prevalence, source of infection, antibiotic resistance and molecular characterization of *Escherichia coli* O157: H7 isolated from sheep and goat. **Methods:** Systematic random sampling was carried out at Modjo export abattoir, Ethiopia, from November 2012 to April 2013 to collect 408 samples from 72 sheep and 32 goats. Samples collected were skin swabs, fecal samples, intestinal mucosal swabs and the inside and outside part of carcasses as well as carcass in contacts such as workers hands, knife, hook and carcass washing water. Then, samples were processed following standard bacteriological procedures. Non-Sorbitol fermenting colonies were tested on latex agglutination test and the positives are subjected to PCR for detection of attaching and effacing genes (*eae*) and shiga toxin producing genes (*stx* 1 and *stx* 2). All *E. coli* O157:H7 isolates were checked for their susceptibility pattern towards 15 selected antibiotics. **Results:** *E. coli* O157:H7 were detected in only 20/408 samples (4.9%). Among these 20 positive samples, 70% (14/20), 25% (5/20) and 5% (1/20) were from sheep, goats and knife samples, respectively. No significant associations were found between carcasses and the assumed sources of contaminations. Of all the 20 isolates virulent genes were found in 10 (50%) of them; 3 (15%) with only the *eaeA* gene and 7(35%) expressing *eaeA* and *stx* 2 genes. All the isolates were susceptible to Norfloxacin (NOR) (100%). **Conclusions:** The presence of virulent genes shows *E. coli* O157:H7 is a potential source of human infection in Ethiopia.

Background

Currently, microbial food borne illness, caused by a wide spectrum of pathogens, is a global concern though extensive scientific progress and technological developments achieved in recent years. Most of microbial pathogens are zoonotic and have reservoirs in healthy food animals from which they spread to an increasing variety of foods. This makes foods of animal origin major vehicles of food borne infections [1]. Microbial contamination of meat may originate from the feces and skin of animals presented for slaughter and can be transferred to the carcass during skin removal and evisceration [2,3].

Escherichia coli is a normal commensal microflora of the intestinal tract of animals and humans. In contrast, *E. coli* O157: H7, which is considered as a subtype of Shiga toxin-producing *E. coli* (STEC) strain, is known to cause human diseases as food borne pathogen and is determined by production of these virulence factors [4, 3]. The bacterium is known to cause the human illness such as haemorrhagic colitis (HC), haemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP) in all age groups, while children and elderly are more victims [5]. Intimin is responsible for the bacteria's intimate adhesion to intestinal cells, causing the appearance of attachment lesions and erasure of the microvilli of the brush border of enterocytes. Intimin is encoded by the *eaeA* virulence gene [6].

Cattle have been identified as a major reservoir of the organism and shed the bacteria in feces. The role of small ruminants as source of human infection through fecal shedding is being reported in a number of studies. So far, beef meat contaminated with *E. coli* O157:H7 has been linked to a number of large scale food poisoning outbreaks [7, 8]. Consumption of raw or undercooked foods, especially undercooked minced beef has been found to be the most common means of transmission [7]. Nevertheless, varieties of other foods have also been implicated in causing outbreaks [9].

Outbreaks of *E. coli* O157 caused infections have been reported in different parts of the world based on culture based identification, such as in South Africa, Swaziland, Central African Republic, Kenya, Uganda, Gabon, Nigeria and Ivory

Coast [9,10,11]. Little is known about the prevalence of this serogroup and associated genes in humans, animals or in foods of animal origin in Ethiopia [12, 13].

Antibiotic use in STEC infections is controversial because of the potential to increase production and secretion of Shiga toxins [14]. However, increase in antibiotic resistance has been noted over the last 20 years [15, 16, 17].

The rising incidence and the potentially serious nature of *E. coli* O157 infection are a cause for concern to public health authorities. In line with this, use of sensitive methods to detect *E. coli* O157 during investigations of outbreaks, surveillance and quality control are recommended [18].

In the presence of the above situations, very few attempts have been made to identify *E. coli* O157: H7 under Ethiopian conditions [12, 13]. Therefore, there is paucity of information regarding the prevalence, distribution, virulence characteristics and antibiotic resistance profile of *E. coli* O157: H7 in meat and abattoir house environments in Ethiopia. It has not yet been determined to what extent these environments serve as sources of *E. coli* O157: H7 particularly to red meat contamination. A study of such types would provide valuable information as to the major sites of contamination in abattoir environments and help in the implementation of strategies to minimize contamination levels.

Materials And Methods

Study Area

Lottery system was used to select from six (6) export abattoirs and the one in Modjo city was selected for this study. The study was conducted from November 2012 to April 2013 at the export abattoir in Modjo town, Ethiopia. Modjo is the center of Lume District, eastern Showa administrative zone of Oromia Regional State. It is located 73 km from Addis Ababa, with altitude of 1777 meters above sea level. The average minimum and maximum temperature are 18°C and 28°C respectively [19].

At this abattoir, 500-1500 goats are slaughtered every day along with 200-600 sheep twice per week. The export abattoir where the study was conducted is well equipped with modern facilities and it is certified of International Organization for Standardization (ISO) 22000. As an export standard abattoir, there is implementation of HACCP practices for maintaining hygienic standards of the abattoir. Sheep and goat are slaughtered separately and the same personnel are involved in slaughtering both groups of animals. All slaughtering operations are performed on overhead rails. Animals bled using *Halal* methods. The skins are washed by pressurized water, cleaned and trimmed and finally kept in chilling room. In the abattoir, there are clean areas for bleeding, dressing, evisceration and meat inspection. Animals slaughtered in the abattoir are exported to Arab countries, in Middle East.

Study Animals

The study was conducted on apparently healthy male sheep and goats slaughtered in the export abattoir during the study period. Animals are originated from different parts of the country mainly from Geanear (Bale), Somali, Awash-Metehara, Jima, Ambo, Borena, Arbaminch and Bati (Wollo) (Figure 1).

Figure 1 Catchment areas for sheep and goats slaughtered at the abattoir

Study Design

A survey was conducted to determine the prevalence of *E. coli* O157:H7 on skin, feces, intestinal mucosal swab and carcasses of slaughtered animals and abattoir environment particularly knives, water, hook and worker's hand. Swab samples were collected from November 2012 to April 2013.

Systematic random sampling was used to select the sampled animals. Fecal samples and skin, carcass and intestinal mucosal swab samples were collected from each selected animal following their rail (tag) along the line of operation. Swab from abattoir environment, which are in contact with the carcass, were sampled once on each sampling day. Knives, hook, workers hands and the tap water were considered to be carcass in contacts.

All samples were transported in icebox to Microbiology laboratory, College of Veterinary Medicine and Agriculture, Addis Ababa University (CVMA, AAU) and stored at 4°C until processed. All samples were processed in 12 hours interval. Culturing, isolation, identification and PCR were performed to determine the presence of *E. coli* O157:H7 in each sample and most effective drugs against the bacteria were selected after antibiotic sensitivity testing.

Sample Size Determination

The number of study animals was determined based on the expected prevalence of *E. coli* O157:H7 and the desired absolute precision according to the formula stated on Thrusfield [20];

$$n = 1.96^2 P_{exp}(1 - P_{exp}) / d^2$$

Where:- n=required sample size; P_{exp} =Expected prevalence; d = desired absolute precision

Based on a previous study done in Modjo and Debre-zeit export and municipal abattoirs, the prevalence of *E. coli* O157:H7 in goat and sheep were 2% and 2.5%, respectively [12]. Using these two expected prevalence, 95% confidence interval and 5% absolute precision; the number of sampled goats and sheep were estimated to be 31 and 38, respectively.

Sample Collection

Skin swab samples were taken according to McEvoy *et al.* [20], by using 2x3 cm sterile cotton tipped swabs soaked in approximately 10ml of buffered peptone water (Oxoid Ltd., Hampshire, England). Skins were swabbed from the neck of animals over the line of bleeding before slaughtering near the bleeding area at an area of approximately 10x10 cm. Skin of the ventral midline part of the animal was also swabbed similarly at the mid line to determine its contact to the carcass during flaying (Figure 2). The shaft of the swab was then broken by pressing it against the inner wall of the test tube and disposed. Finally this was repeated but with a dry sterile cotton.

Approximately 25g of fecal samples were taken after complete evisceration directly by opening the rectum according to the method described by Elder *et al.* [22]. The whole abdominal digestive organs were separated from the slaughtering line in a plastic bucket and the rectum was opened using a sterile surgical blade (Figure 2); the fecal sample was then put in to sterile universal bottle. Whereas for intestinal mucosal swab sampling, the distal colon was ligated and opened using a sterile surgical blade proximal to the rectum and the lumen was swabbed by using sterile swab. The swab was then introduced in to approximately 10 ml buffered peptone water in a sterile test tube.

The external parts of carcasses were swabbed from rump, midline and brisket area, just before chilling according to the methods described by McEvoy *et al.* [21]. Sterile cotton swabs soaked in approx. 10 ml of buffered peptone water was used to rub against the carcass first horizontally then vertically. A second dry sterile cotton swab was also rubbed at exactly the same area. For the internal part of the carcass, where contacts with other carcass is not possible, the thoracic and the pelvic parts of both sides, through the evisceration opening, were swabbed using the same procedure as above (Figure 2). Disposable sterile gloves were used for each carcass and changed.

Similarly, abattoir utensils and other carcass in contacts such as knife, hook, water and workers hands were also swabbed as a sample using sterile cotton swabs soaked in approx. 10 ml of buffered peptone water. A pooled sample was taken from knives used for evisceration and carcass trimming on each sampling day. Only hooks used to hang the sampled carcass were also swabbed each sampling day as a pooled sample. Whereas abattoir workers whose hands have direct access to the washed carcass were swabbed for sampling, at their palm surface and fingers. Moreover, 25ml of water sample was collected directly from the tap which is used for washing of the carcass.

For each and every sampling a sterile latex glove was used to avoid cross contamination and every procedure was done as aseptic as possible. All samples were transported in ice box to the laboratory.

Laboratory Work

Bacteriological sample processing

Fecal samples were measured for accuracy and placed in to sterile stomacher bag and a 1:9 ratio of a modified tryptone soya broth (Oxoid Ltd., Hampshire, England) containing 20 mg/l novobiocin (Sigma, Steinheim, Germany) (mTSB+n) was added in it. It was then put in to stomacher (Seward Stomacher 400, Seward, London, UK) for agitation at low speed for 30 seconds.

In to all other swab samples 90 ml of mTSB+n was added and homogenized using vortex mixer, and also for the 25 ml water sample 225ml mTSB+n was added just to keep the 1:9 ratio.

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

Microbiological samples for the isolation and identification of this bacterium were processed as described as follows.

Selective enrichment

Modified tryptone soya broth containing 20 mg/l novobiocin was used at 1:9 ratios as mentioned above, for selective enrichment of all the samples. Then, all the samples types were incubated at 41.5 °C for 24 h.

Isolation by immuno magnetic separation (IMS) and culturing of the isolates

After 24 h of incubation all enriched broth culture were subjected to IMS using Dynabeads anti-*E. coli* O157 (DynaL Biotech AS, ThermoFisher Scientific, Oslo, Norway) as follows. Both enriched broth culture and the paramagnetic beads were vortexed to be homogenized and 1ml of the enriched culture was put in to a sterile screw capped eppendorf tube. A 20µl of resuspended paramagnetic beads was then transferred in to the same eppendorf tube.

Then each tube was briefly vortexed on the dynal mixer (Dynal MX4sample mixer) at 20 rpm for 30 minutes at room temperature, for the bacteria to attach to antibody surface on the beads. The tubes were then put in to the manual magnetic particle concentrator (MPC-S) with the magnetic strip in place, inverted 3 to 4 times and left to settle for about 5 minutes. It was then gently rotated for the magnetic beads to concentrate at the back of the tube. The cap of the tube carefully opened and the supernatant was discarded by carefully aspirating it with sterile fine tipped pipette, without touching the back wall of the tube. Then magnetic strip was removed and 1ml of phosphate buffered saline containing 0.05% tween 20 (PBST, Sigma chemicals Co, Saint Louis, USA) was added to each tube using another disposable fine tipped pipette. It was then inverted 3 times after the tubes were clothed, the magnetic strip replaced and the above step repeated at least twice. To prevent cross contamination the PBST was put in different small containers and for each sample and each material transferring new pipette tips were used. Finally the supernatant was aspirated; the magnetic strip was removed and about 100µl of PBST was added in each tube and mixed gently.

Around 50 µl of IMS bead and bacteria complex were streaked onto Sorbitol MacConkey agar (Difco, Becton Dickinson, Claix, France) containing 0.05 mg/l cefixime and 2.5 mg /l potassium tellurite (Dynal Biotech ASA, Oslo, Norway) (CT-SMAC). Culturing was carefully to obtain pure colonies and plates were incubated at 37°C for 20–24 h. The CT-SMAC agar plates were examined for the presence of non sorbitol fermenting colonies [23, 24, 25, 26].

The non-sorbitol fermenting colonies on CT-SMAC appear as slightly transparent, almost colorless with a weak pale brownish appearance with a diameter of one mm [24, 23, 26]. Such colonies are sub cultured on CT-SMAC for further a confirmatory test.

Confirmatory test by latex agglutination

Latex agglutination was performed for confirmation of *E. coli* O157:H7 using latex kit. The latex kit consists of four components: latex test reagent, latex control reagent, the positive controls and negative controls. The test reagent is latex particles sensitized with specific rabbit antibody against O157 antigen and the control reagent consists of latex particles sensitized with rabbit globulin. The positive and negative controls are suspension of inactivated *E. coli* O157:H7 cells and inactivated non-specific *E. coli* cells respectively.

The test was performed according to the manufacturer instructions (Oxoid Ltd, Hampshire, England). But first the latex kit was checked for its performance by using the control suspensions in the kit, the test was continued after the positive control reacts with the test latex showing positive result. A drop of test latex and 0.085% sterile saline water were dispensed in to the reaction card separately. A few presumptive colonies of *E. coli* O157 were taken and emulsified in to the saline water on the latex card, then slowly mixed with the test latex and checked for agglutination within two minutes. Isolates showing visible agglutination by reacting with the test latex solution are again sub cultured for virulent gene identification.

Figure 2 Procedures followed during sample collection and processing

Determination of virulence genes by polymerase chain reaction

Multiplex PCR was conducted to assess the presence of virulence genes (*stx1*, *stx2* and *eaeA*) in *E. coli* O157:H7 colonies, which were confirmed by latex agglutination, by using the methods described in Mora *et al.* [16] and Inat and Siriken [27]. DNA extraction of isolates was conducted using boiling method. Briefly, each suspect colony was

inoculated on CT-SMAC and incubated for 24 h at 37°C to get fresh colony. Few colonies were then selected and suspended separately in 100 µl of sterile distilled water in eppendorf tubes, after which they were boiled at 92.5°C for 17 min in a water bath. The tubes were then centrifuged at 13000rpm for 10 min, after which the supernatant containing the template DNA was transferred into Dnase/Rnase-free eppendorf tubes. The extracted DNA samples were stored at -20°C until use.

Detection of the *stx1*, *stx2* and *eaeA* genes was performed according to the protocol indicated in Inat and Siriken [27] with slight modification. 2 µl of extracted DNA was used as a template in a reaction mixture with a final volume of 25 µl that contained 10 mM of each dNTP, 25 nM *stx1* primer, 25 nM *stx2* primers, 25 nM *eaeA* primer (Table 1), 1U of Taq DNA polymerase in 1× PCR buffer and 2 mM of MgCl₂. Amplification of DNA was conducted using initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 20 sec, annealing at 58°C for 40 sec, extension at 72°C for 1 min, and final extension at 72°C for 8 min.

For gel electrophoresis, the 10-µl amplicon mixture was loaded onto a 1.5% agarose gel. Electrophoresis was conducted at 125 V for 1 h. A 100 up to 1,000 bp molecular weight marker was used to identify the amplified products as a ladder, which was visualized by UV illumination.

Table 1 Primers' sequence used in multiplex PCR for amplification of *stx1*, *stx2* and *eaeA* genes

Antimicrobial susceptibility pattern

Antimicrobial susceptibility testing was performed following the standard agar disk diffusion method according to CSLI [30] using commercial antimicrobial disks. The selected antimicrobials their symbols and inhibition zone size interpretations are listed in table 2.

Pure colonies, incubated for 6 hours in Tryptone Soya Broth (Oxid, England) were processed to a turbidity of 0.5 McFarland standards (approximately 3×10^8 CFU per ml) in a sterile saline solution. Then, they were inoculated on Muller-Hinton agar plates (Becton Dickinson company, Cockeysville USA) using sterile cotton swab, making sure that all the surface of the media is immersed with the bacterial suspension. Antibiotic discs were then dispensed and plates were incubated for 24 hours at 37°C. Diameters of the zone of inhibition were measured and the results were classified as resistant, intermediate and susceptible according to CLIS [30]. *E. coli* ATTC 25922 type strains were used as a positive control.

Table 2 Antimicrobials used, their symbols and inhibition zone size interpretation for Gram-negative enteric bacteria.

Data collection, management and analysis

The establishment of computer database and the necessary manipulations such as variable coding was performed using MS Excel. The database was transferred to SPSS version 11.5 [29] for analysis. Descriptive statistics such as proportions, standard deviations and 95 % confidence intervals were performed. Over all and sample-specific prevalence were determined by dividing the number of positive samples to the total number of samples examined. Difference among and between proportions of the groups with certain determinant factors was determined by chi-square (χ^2) test. ORs were calculated using univariable logistic regression to determine the degree of associations of carcass contamination with fecal, skin and carcass in contact surfaces' status. A p-value <0.05 was considered indicative of a statistical significance difference.

Furthermore, Kappa statistics was performed to see whether there is agreement between fecal sample and intestinal mucosal as well as carcass inside and carcass outside swab *E. coli* O157: H7 status. Interpretation of the Kappa test was based on Rules-of-thumb for kappa: values less than 0.40 indicate low association; values between 0.40 and 0.75 indicate medium association; and values greater than 0.75 indicate high association between the two raters.

Results

This study was conducted on 40 and 32 apparently healthy slaughtered sheep and goats, respectively, at an export abattoir, Modjo, Ethiopia, from November 2012 to April 2013. Bacteriological examination was conducted on fecal, skin, intestinal mucosal, carcass outside and carcass inside swab samples, and swabs from knife, personnel's hand and hook as well as water samples.

Prevalence of *E. coli* O157:H7

From 408 samples examined for *E. coli* O157:H7 only 20 (4.9%) were found to be positive. It was present in feces, intestinal mucosal swabs, skins, inside and outside carcass swabs and knife used in the abattoir. From these 20 positive samples 70% (14/20) were from sheep and 25% (5/20) were from goats, the rest 5% (1/20) was from a knife.

Statistically significant difference ($p < 0.05$) was found in prevalence of *E. coli* O157:H7 between sheep and goats (Table 3). An animal was considered *E. coli* O157: H7 positive when it was positive for the bacteria on either fecal sample and/or intestinal mucosal swab; samples from fecal or intestinal mucosal swabs of goats were not positive. Skin, carcass inside, and carcass outside *E. coli* O157: H7 statuses were considered indicators of external contamination and were not used for the calculation of prevalence on animals. As a result, from the total 72 animals examined for the status of *E. coli* O157: H7, only 8 animals are considered positive. Statistically significant difference was observed between sheep and goat in harboring the bacteria; sheep being more prone to harbor *E. coli* O157:H7 than goats.

Of the 8 positive animals, only 2 (25%) were culture positive both for fecal sample and intestinal mucosal swab samples. The rest 6 (75%) were culture positive either for fecal sample or intestinal mucosal swab sample and were significantly different ($P = 0.033$). The agreement of the fecal sample and intestinal mucosal swab samples was measured using the Kappa statistics and the result indicated low agreement between the two (Kappa value = 0.357, 95% CI = 0.002 – 3.103).

Table 3 Prevalence of *E. coli* O157: H7 by sample types and species of animals examined

The statistically significant difference (p -value = 0.033) found between fecal sample and intestinal mucosal swab isolates indicates that sampling feces for isolation of *E. coli* O157:H7 is significantly different from sampling intestinal mucosal swab. Moreover, the bacterial ability to adhere on the intestinal wall may be a factor for the occurrence of this difference. This result was also supported by calculating the agreement between them by kappa statistics, which resulted in lower agreement, indicating both of them should be sampled for isolation of *E. coli* O157:H7 (Table 4).

Table 4 Comparative results by Pearson's X^2 test of species-specific *E. coli* O157: H7 prevalence in fecal sample and intestinal mucosal swabs

From all randomly selected 72 animals each type of samples were taken. Of the different sample types taken four fecal (5.55%), five skin swab (6.94%), six intestinal mucosal swabs (8.33%), one carcass inside swab (1.39%) and three carcass outside swab (4.17%) samples were found to be positive (Table 3). However, all the swab samples from personnel's hand and hook as well as water samples were found to be negative for *E. coli* O157: H7.

The internal part of the carcass was also sampled to determine the impact of carcass contamination by feces especially during evisceration. It was thought to be helpful in avoiding the risk of contamination of the carcass by other in contacts, like washing water, hand etc. and evaluate only the risk of carcass contamination by feces during evisceration. However, of all 72 samples of carcass inside samples taken during this study only 1(1.39%) was found to be positive for *E. coli* O157:H7 from sheep, with no significant importance.

The level of carcass outside contamination was considered as an outcome variable taking skin swab, fecal sample and knife swab as risk factors for carcass contamination. However, assessments using logistic regression analysis as well as Chi square test did not show significant associations between carcass contamination and the risk factors were (Table 5).

Table 5 Association of carcass outside contamination and *E. coli* O 157: H7 status of the risk factors

Even if there is no association with carcass contamination in our finding, a higher prevalence of *E. coli* O157:H7 from skin swabs (6.94 %) than from fecal samples of sheep (8.33%) was observed.

In the abattoir where these samples were collected, cold pressurized water wash was used to avoid visible contaminants such as blood clot, fecal debris, GIT contents so on. This was done after evisceration and inspection. The whole carcass surface was washed with this pressure wash. In addition to this, carcass trimming was performed using knives immersed in hot water after trimming of every carcass.. Carcasses are transferred manually by carrying from the overhead rails to the chilling room after weighing..

In addition, no positive carcasses were found from animals that were *E. coli* O157:H7 negative from their fecal, mucosal swab or skin samples.

Similarly, the level of carcass inside contamination was considered as an outcome variable taking skin swab, fecal sample and knife swab as risk factors for carcass contamination. However, such contamination sources were not significantly associated with carcass contamination and *E. coli* O 157: H7 status of the risk factors.

Table 6 Association of carcass inside contamination and *E. coli* O 157: H7 status of the risk factors

Detection of Virulence Genes on the Isolates

From the 20 *E. coli* O157:H7 isolates that were analyzed by PCR for the detection of virulence genes, 10 (50%) were found to be positive for having virulent genes. Among these 10 isolates, 7/20 (35%) of them are found to carry both *eaeA* and *stx2* and 3/20 (15%) carried only the *eaeA* genes; whereas no one of them carried the *stx 1* gene (Table 8).

Out of the six isolates of the intestinal mucosal swabs of sheep four (66.7%) of them expresses the virulence genes, but only one of the four fecal isolates (25%) have these genes as a virulent factor. These shows the significance of *eaeA* being more prevalent in mucosal isolates than faecal isolates in this study. Of the caprine isolates both the carcass outside isolates show *eaeA* genes, but only one of them are with the *stx 2*, whereas from the three isolates of

skin swabs, two of them show *eaeA* and only one was with *stx2*. The only one carcass in contact isolate, from Knife was also found to express the *eaeA* virulent gene.

Table 8 Summary of virulent gene expression of *E. coli* O157:H7 isolates

In our finding seven of the isolates from goat and sheep had both *eae* and *stx2* genes and three of them were with only the *eae* gene (Table 8), but none of them show the *stx1* gene.

Figure 3 *E. coli* O157:H7 *eaeA* (single arrow) and *stx2* (double arrow) virulence genes isolated from sheep goat carcass by PCR with 384 and 255 bp, respectively

Antimicrobial Susceptibility of the Isolates

Testing of all the 20 *E. coli* O157: H7 isolates for fifteen different antimicrobials showed susceptibility to one of the antimicrobial used, Norfloxacin (NOR) (100%). However, another two antibiotics show growth inhibition zones for all isolates, even if two of the isolates (10%) are moderately susceptible for Ceftazidime (CAZ) and another completely different two isolates (10%) showed intermediate resistance to polymixin-B (PB). Another antibiotic showed growth inhibition zones on four of the isolates (20%) was Kanamycin (K), even if it was not enough to consider it as an effective drug (Table 7).

Table 7 Antimicrobial susceptibility pattern of *E. coli* O157: H7 isolates by species and sample type. N.B: All isolates was resistant to the rest of antibiotics tested in this study

Discussion

Carcass contamination with *E. coli* O157:H7 may occur during slaughtering operations because of direct contact with contaminated materials such as skin, fecal material, knives, workers' hands and the likes [2,3].

Prevalence of *E. coli* O157:H7

While cattle are generally regarded as the main reservoir of *E. coli* O157:H7 for human infection on other studies [8], the results of the present study indicate that sheep and goats may also be contributing sources, with sheep being more significant. This finding is in line with several previous studies which have indicated sheep as a reservoir of this bacteria [8,31, 32, 33, 34]. Nevertheless, it is not in agreement with a previous study in another export abattoir in Modjo, Ethiopia, which showed that both species are equally potential important sources of human *E. coli* O157:H7 infections [13]. This is may be due to the lack of the previous study to consider intestinal mucosal swab as a sample, since it shows the highest number of isolates in this study.

A 10% fecal prevalence of *E. coli* O157:H7 from sheep in this study was a little higher than previous studies conducted in this country and in other countries. Mersha *et al.* [13] reported a relatively lower (5.4%) prevalence of *E. coli* O157:H7 in sheep feces. On the other hand, the bacterium was isolated from 4% of ewes and 4% of lambs in Netherlands [18], 3% of lambs in Spain [31], 1.4% of sheep with monthly variations of zero to 4.8% in UK [6, 34] and 0.2% in Italy [8]. However, a much more prevalence of 31% in USA [36] and 68% from sheep flocks in Australia [40] from fecal samples were reported. On the other hand, zero *E. coli* O157:H7 prevalence from sheep fecal samples were reported in Norway [37], Scotland [38], Ireland [34], Greece [39] and United States [41]. Similarly, this study have

smaller bacterial findings but on caprine. Closer results, however, are reported in Greece (1 out of 81) associated with human outbreaks from goats [39]. Similarly, Keen *et al.* [41] reported no *E. coli* O157:H7 from 526 goats' in US zoological parks. In contrast, higher prevalence observed in other countries. A prevalence ranging from 55 to 95% reported in France [42] and 40% in Australia [43] in goats by flocks.

Marked differences in the prevalence of *E. coli* O157:H7 from fecal samples were observed in both sheep and goats. The differences in the prevalence of *E. coli* O157:H7 observed between the current study and those previous reports are possibly due to differences in husbandry practices, agro-climatic variation, seasonal differences, sampling times, breeds and age of animals, sampling technique and so on. A number of studies have also shown that prevalence of *E. coli* O157:H7 shed from animal feces can vary significantly in relation to time, age of animals, nature of feeds etc [24, 7, 3]. Moreover, Battisti *et al.* [8] indicated sheep husbandry as a possible reasons for *E. coli* O157 fecal prevalence differences in US as compared to European countries.

The overall prevalence in skin swabs (6.9%) was comparable to the previous study done in export abattoir of Modjo, Ethiopia [13]. Isolation of *E. coli* O157:H7 from skin of these species is very rarely described. In Ireland as reported by Lenahan *et al.* [34], a 5.8% prevalence of *E. coli* O157:H7 from fleece, sampled by shaving was reported. However, in hides zero to 22% *E. coli* O157:H7 prevalence were reported from different sites of hides in US [22, 3].

E. coli O157:H7 prevalence of 4.17% from carcass outside swab in the current finding was comparable to a previous study done in east Showa of Ethiopia on meat samples of sheep and goat [12]. Lower prevalence than the present finding was reported in Ireland [34] and in England [7] that ranges between 0.7 and 4 % from goats and sheep carcasses. Similarly, Chapman *et al.* [7] in UK reported 0.21%, 1.22% and 1.17% prevalence of *E. coli* O157:H7 from minced meat, burger and sausages of lambs, respectively. On the other hand, much higher prevalence was reported from an export abattoir of Ethiopia by Mersha *et al.* [13], 8.7%, and in Australia by Sidjabat-Tambunan and Bensink [39], 29.2% (31/106) from sheep carcasses.

Differences in the reported prevalence could be due to differences in the method of sampling, number of samples, sampling sites, and so on. Thus, MacEvoy [21] recovered only one among the nine positive samples by swabbing but six by excision, showing sampling methods can be big factor. The surface swabbing method, which was used in this study, however, was also used by others [44, 13] and depending on the degree of abrasiveness the method is recommended as an alternative method since it is cost effective and nondestructive.

The internal part of the carcass was also sampled to determine the impact of carcass contamination by feces especially during evisceration. It was thought to be helpful in avoiding the risk of contamination of the carcass by other in contacts, like washing water, hand etc. and evaluate only the risk of carcass contamination by feces during evisceration. However, of all 72 samples of carcass inside samples taken during this study only 1(1.39%) was found to be positive for *E. coli* O157:H7 from sheep, with no significant importance.

The level of carcass outside contamination was considered as an outcome variable taking skin swab, fecal sample and knife swab as risk factors for carcass contamination. However, assessments using logistic regression analysis as well as Chi square test did not show significant associations between carcass contamination and the risk factors were (Table 5).

In contrast, the previous study on another export abattoir in Modjo showed a significant association with these risk factors except for carcass in contacts. This shows that the implementation of HACCP system in the abattoir has reduced the risk of contamination of carcasses during the slaughtering operation, as compared to the previous study with no HACCP system implemented. Even if no information is obtained about the slaughtering practice during

previous study, as it was observed during this study the abattoir management and MoARD takes the credit for proper implementation of strict prevention methods and careful handling on the slaughtering line.

Results of this study were supported by other studies for the absence of association between hide prevalence and carcass contamination [22]. However, in those reports hide prevalence of *E. coli* O157:H7 was much lower than fecal prevalence. Possible explanations for this apparent discrepancy might be a difference in the selection of skin sampling site. A difference in the sampling site could affect isolation rate of a given pathogen. For example, Reid *et al.* [3] isolated 3.3% from rump, 4.4% from flank and 22.2% from brisket of *E. coli* O157:H7 by swabs on the same animals. This might be due to difference in the survival rates of *E. coli* O157:H7 in different sites of the skin of animals. Moreover, it has been indicated that one site on the skin may have higher levels of contamination than the others and, therefore, posing greater risks for carcass contamination.

On the other hand, associations of these risk factors with carcass contaminations were also reported from different parts of the world: associations with feces by Elder *et al.* [22], [45] and Griffin *et al.* [46]; and with skin by Reid *et al.* [3]. On the contrary, absence of association between hide prevalence and carcass contamination was reported by Elder *et al.* [22], and in those reports hide prevalence of *E. coli* O157:H7 was much lower than fecal prevalence. Possible explanations for this apparent discrepancy might be a difference in the selection of skin sampling site as mentioned above.

Even if there is no association with carcass contamination in our finding, a higher prevalence of *E. coli* O157:H7 from skin swabs (6.94 %) than from fecal samples of sheep (8.33%) was observed. These observations were in contrast with Elder *et al.* [22], where they reported higher prevalence in feces (28%) than on hides (11%). The skin of animals could have a number of sources to carry *E. coli* O157:H7 such as the soil, feed, water, feces etc. but animals could shed *E. coli* O157:H7 seasonally in their feces and as a result might be negative for the organisms on sampling time. The skin of a given animal could be contaminated by fecal sources from themselves and other animals. Therefore, during transportation and in the lairage cross contamination of skins could occur due to a more close contact of animals and as result an increase in the apparent prevalence of *E. coli* O157:H7 on skin relative to feces could be observed.

Although pressure washing was applied on the carcass and also knives were immersed in hot water between trimming of successive carcasses, *E. coli* O157:H7 was isolated in one of the knives. This indicates that this could be a critical point for carcass contamination and the prevention methods should be applied strictly; in this aspect adequate temperature should be used for sterilization of knives. In addition, while transferring carcasses manually by carrying from the overhead rails to the chilling room, bacterial recontamination might occur and spread from one carcass to others.

In addition, no positive carcasses were found from animals that were *E. coli* O157:H7 negative from their fecal, mucosal swab or skin samples. This finding strongly suggests the presence of carcass cross-contamination in the abattoirs. Reducing the amount of *E. coli* O157:H7 in live sheep and goats will likely lower contamination not only of meat but also of other food and water supplies that are exposed to sheep and goat fecal matter.

Similarly, the level of carcass inside contamination was considered as an outcome variable taking skin swab, fecal sample and knife swab as risk factors for carcass contamination. However, such contamination sources were not significantly associated with carcass contamination and *E. coli* O157:H7 status of the risk factors.

Detection of Virulence Genes on the Isolates

It has been indicated that the virulence of *E. coli* O157:H7 has been mainly associated with the production of shiga toxins encoded by *stx1* or *stx2* genes, the presence of the pathogenicity island LEE, responsible for attaching and effacing lesions and the *ehxA* gene, encoding enterohaemolysin [47, 48]. In the current study, half of the positive isolates were having two of the virulence genes tested.

In our finding seven of the isolates from goat and sheep had both *eae* and *stx2* genes and three of them were with only the *eae* gene (Table 7), but none of them show the *stx1* gene. The higher numbers of isolates of this study showing the virulence genes with a lower prevalence, as compared to only one isolate, in the previous study in this area, with higher prevalence, are needed to be given an attention. In addition, it indicates the dynamicity of the bacteria over years and the capability of it to evolve and adapt to new environments. In other studies undertaken in Greece [39] only *stx2* from a single *E. coli* O157:H7 isolate was obtained from goat feces. Of the total 33 isolates in France [34] from sheep, only five of the isolates carried the *stx1* and *stx2*. The identification of these virulence genes in this study from half the isolates indicates the potential of small ruminant carcass as source of *E. coli* O157:H7 for human infections in the country and its effect to the growing meat export market. It also suggests the need for further detailed epidemiological studies on *E. coli* O157:H7 in Ethiopia involving different export abattoirs and species of animals.

Antimicrobial Susceptibility of the Isolates

This study showed complete susceptibility of all isolates to one of the antimicrobial used, Norfloxacin (NOR) and variable levels of resistance towards the other antibiotics tested. Hiko *et al.* [12], Faris [48] and Lula [50] have reported antimicrobial resistance patterns of *E. coli* O157:H7 isolates, from animal and human sources, in Ethiopia. Only three antibiotics namely Norfloxacin, Ceftazidime and Polymyxin B were seen to be effective against this bacteria but all the rest of the antibiotics used have no effect on it. A wider antibiotic resistance seen in this study is similar to other authors' explanations about the bacteria [51, 52, 53]. This higher percentage of resistance might be due to presence of only few isolates tested for susceptibility compared to overall study population and variability of resistant gene within isolates for particular antimicrobials. Although there is difference in the sources of isolates, the 20 (100%) of present isolates resistant to one or more antibiotics were higher than 41% of those previously tested isolate by Mora *et al.* [16] in Spain from human, cattle, ovine and food. Moreover, the overall drug resistant in the present study is much more higher than those isolates tested by Wilkerson *et al.* [53], forty-four (6.6%) of 663 of bovine and 29 (12.2%) 238 of human *E. coli* O157: H7 isolates from feedlots in the mid-western United States and the Public Health Departments of Washington, Oregon, Nevada, Wisconsin, Georgia, and Illinois states. This could be due to difference in the type of samples, number of isolate and genetic variation of the isolate among different geographical areas. Whereas the susceptibility of all the isolates to Norfloxacin, and 18 of them for polymyxin B and Ceftazidime encourages hope for finding the most effective antibiotic therapy against *E. coli* O157: H7.

Conclusions

In conclusion, *E. coli* O157: H7 was detected from the feces, skin, intestinal mucosal swab and carcasses of sheep and goats and knife at export abattoir. This pathogen was isolated from carcasses, from the inside and outside parts that indicated the presence of carcass contamination during slaughter operations. Thus, interventions to reduce the occurrence of *E. coli* O157:H7 and reduce carcass contaminations were not absolute even if the prevalence are lower in this study. Prevalence of *E. coli* O157:H7 is higher in sheep than goats with statistically significant difference. The presence of *eae* and *stx2* positive *E. coli* O157:H7 in sheep and goats carcass should be given attention. Thus, in addition to cattle, sheep and goats could serve as source of human infection.

Declarations

Authors' contributions

AT and SA designed the proposal, participated in the coordination and management of the study, collected, tested and analyzed the data and drafted the article; EC, TST study design, scientific advising of the overall work and edition of article. TST also participated in molecular part of the work and correspondence of the article's publication.

Acknowledgements

The financial support of VLIR-UOS project no. "ZEIN 2010 PR 372" "Promotion of the PhD program in veterinary public health at the Faculty of Veterinary Medicine", Belgium is highly acknowledged. We are extremely indebted to Professor Dr. Bruno Goddeeris, from the Catholic University of Leuven who led the VLI R-UOS project, for facilitation of the overall research grant, continuous support and follow up. We thank the Microbiology Laboratory, College of Veterinary Medicine and Agriculture, Addis Ababa University, for availing their laboratory facility and equipment. The authors also would like to extend their gratitude to the staff of the export of abattoir in Modjo for their collaboration during sampling.

Competing Interests

No conflict of interest declared

Availability of data and materials

Not applicable

Consent for publication

Not applicable

Ethics approval and consent to participate

This study was conducted on an abattoir. In addition, the research proposal was publicly defended and conduct of the experiment was permitted by the office of Graduate programs at College of Veterinary Medicine and Agriculture, Addis Ababa University, Debre Zeit/ Bishoftu, Ethiopia.

Funding

This research was financially supported by VLIR-UOS project no. "ZEIN 2010 PR 372" "Promotion of the PhD program in veterinary public health at the Faculty of Veterinary Medicine", Belgium.

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Tables

Table 1 Primers' sequence used in multiplex PCR for amplification of *stx1*, *stx2* and *eaeA* genes

Target gene	Primers sequence (5'-3') (Forward/reverse)	Amplicon size (bp)	Reference
<i>stx1</i>	ATAAATCGCCATTCGTTGACTAC/ AGAACGCCCACTGAGATCATC	180	[26]
<i>stx2</i>	GGCACTGTCTGAACTGCTCC/ TCGCCAGTTATCTGACATTCTG	255	[26]
<i>eaeA</i>	GACCCGGCACAAGCATAAGC/ CCACCTGCAGCAACAAGAGG	384	[47]

Table 2 Antimicrobials used, their symbols and inhibition zone size interpretation for Gram-negative enteric bacteria

Antimicrobial used a	Symbols	Diameter of zone of inhibition in millimeter			
		Resistant ≤	Intermediate	Moderately Susceptible	Susceptible ≥
Amoxicillin (25 µg)	AML	13	-	14-16	17
Bacitracin (10 µg)	B	14	15-16	-	17
Cefotaxime (5 µg)	CTX	14	-	15-22	23
Cefoxitin (30 µg)	FOX	14	-	15-17	18
Ceftazidime (10 µg)	CAZ	12	13-17	-	18
Cefuroxime Sodium(5µg)	CXM	14	-	15-22	23
Clindamycine (10 µg)	DA	14	15-20	-	21
Cloxacillin (5 µg)	OB	13	-	14-16	17
Doxycycline (30 µg)	DO	13	-	14-16	17
Kanamycin (30 µg)	K	13	14-17	-	1
Nalidixic acid (30 µg)	NAL	13	14-18	-	19
Nitrofurantoin (300 µg)	F	13	14-17	-	18
Norfloracin (10 µg)	NOR	12	13-16	-	17
Polymyxin B (300 unit)	Pb	8	9-11	-	12
Vancomycin (30 µg)	VA	14	15-16	-	17

a = all are Oxoid products (England).

Table 3 Prevalence of *E. coli* O157: H7 by sample types and species of animals examined

Sample Type	Number of samples		Goats			Sheep			Total		
	Examined	Positives (%)	95% CI	Examined	Positives (%)	95% CI	Examined	Positives (%)	95% CI		
Fecal	32	0 (0)	0-0	40	4 (10.0)	-0.39-20.39	72	4 (5.6)	2.9-10.91		
Skin Swab	32	3 (9.4)	-0.71-19.51	40	2 (5.0)	-2.55-12.55	72	5 (6.9)	1.05-12.75		
Mucosal Swab	32	0 (0)	0-0	40	6 (15.0)	2.63-27.37	72	6 (8.3)	1.93-14.67		
Carcass outside	32	2 (6.2)	-2.16-14.56	40	1 (2.5)	-2.91-7.91	72	3 (4.2)	-0.43-8.83		
Carcass inside	32	0 (0)	0-0	40	1 (2.5)	-2.91-7.91	72	1 (1.4)	-1.31-4.11		
Knife							12	1 (4.2)	-0.43-8.83		

CI= confidence interval

Table 4 Comparative results by Pearson's X2 test of species-specific *E. coli* O157: H7 prevalence in fecal sample and intestinal mucosal swabs

Sample type	<i>p-value</i>	Odds Ratio	CI for the Odds Ratio
Fecal	0.124	1.111	1.002 – 1.232
Mucosal swab	0.030	1.176	1.033 – 1.340

Table 5 Association of carcass outside contamination and *E. coli* O157: H7 status of the risk factors

Risk factors	<i>p-value</i>	Odds Ratio	95% Confidence interval for the Odds Ratio
Skin swab	0.999	0.928	0.868 - 0.991
Fecal sample	0.999	0.942	0.888 – 0.999
Knife swab	1.000	0.986	0.958 – 1.014

Table 6 Association of carcass inside contamination and *E. coli* O 157: H7 status of the risk factors

Risk factors	<i>p</i> -value	Odds Ratio	95% Confidence interval for the Odds Ratio
Fecal sample	0.999	0.944	0.892 – 0.999
Knife swab	1.000	0.986	0.959 – 1.014

Table 7 Antimicrobial susceptibility pattern of *E. coli* O157: H7 isolates by species and sample type. N.B: All isolates was resistant to the rest of antibiotics tested in this study

Species of animal	Type of sample	No of isolates tested	NORFLOXACIN			CAZ			PB		
			Sen. No. (%)	Int. No. (%)	Res. No. (%)	Sen. No. (%)	Int. No. (%)	Res. No. (%)	Sen. No. (%)	Int. No. (%)	Res. No. (%)
Goat	Skin	3	3	0	0	3	0	0	3	0	0
	CO	2	2	0	0	2	0	0	2	0	0
Sheep	Fecal	4	4	0	0	3	1	0	2	1	0
	Skin	2	2	0	0	2	0	0	2	0	0
	MS	6	6	0	0	5	1	0	6	0	0
	CO	1	1	0	0	1	0	0	1	0	0
	CI	1	1	0	0	1	0	0	1	0	0
	Knife	1	1	0	0	1	0	0	0	1	0
Total		20	20	0	0	18	2	0	18	2	0

CO= carcass outside, CI= carcass inside, Sen. = sensitive, Res. =resistant, Int. =intermediate resistant.

Table 8 Summary of virulent gene expression of *E. coli* O157:H7 isolates

Species of animals	Type of sample	Virulent genes expressed			
		<i>eaeA</i>	<i>stx1</i>	<i>stx2</i>	Both <i>stx2 & eaeA</i>
Sheep	Mucosal swab	4	-	4	4
	Fecal sample	1	-	1	1
Goat	Carcass outside	2	-	1	1
	Skin swab	2	-	1	1
	Knife	1	-	-	-
Total		10	0	7	7

Figures

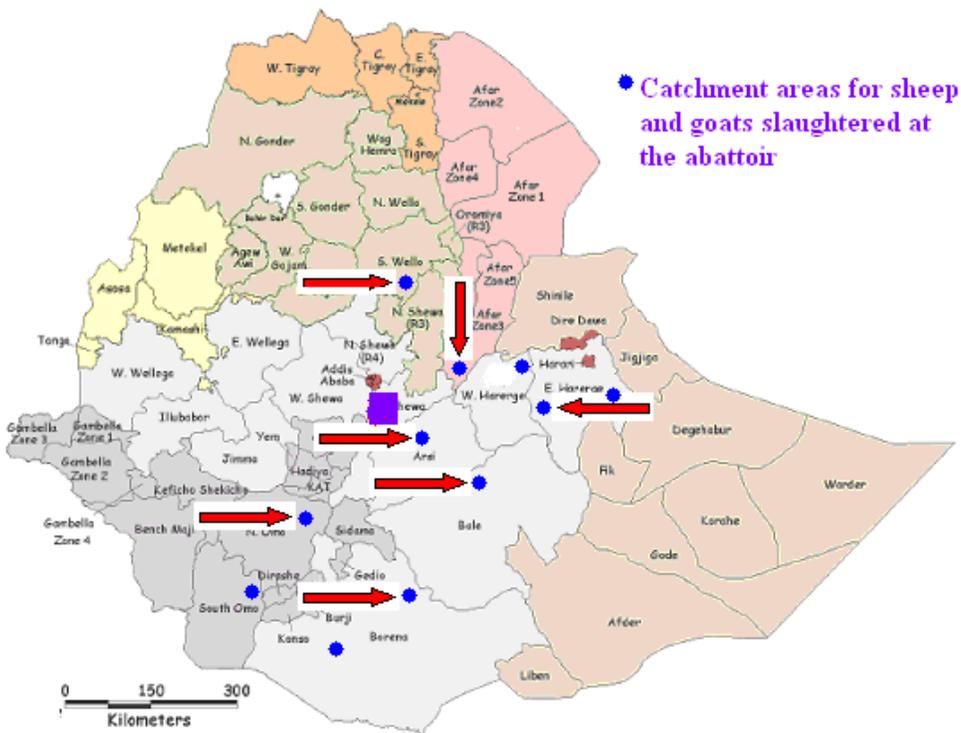


Figure 1

Catchment areas for sheep and goats slaughtered at the abattoir



A



B



C



D



E



F



G

Figure 2

Procedures followed during sample collection and processing: (a) skin swab sample collection; (b) intestinal mucosal swab sample collection; (c) fecal sample collection; (d) carcass outside swab sample collection; (e) carcass inside swab sample collection; (f) concentration of *E. coli* O157:H7 using IMS technique; (g) antimicrobial susceptibility test bacterial isolates

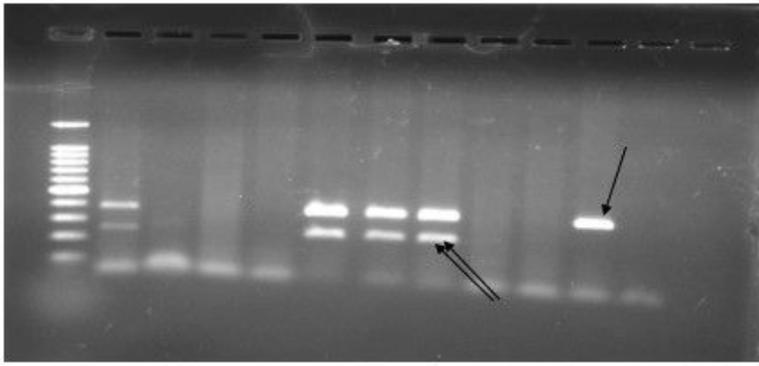


Figure 3

Amplification products of *eaeA* (single arrow, 384bp) and *stx2* (double arrow, 255bp) virulent genes of *E. coli* O157:H7 isolated from sheep & goat