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Identification and Biotyping of *Escherichia coli* from Diarrheic Lambs in and Around Debrebirhan Town, Ethiopia

Metasebia Aklilu[1]*, Tesfaye Sisay[2], GeneneTefera[3] and Belay Tekalign[4]

[1]*Department of Microbiology, Immunology and Veterinary Public Health, College of Veterinary Medicine and Agriculture, Addis Ababa University, P. O. Box 34, Debrezeite, Ethiopia*

[2]*Department of Health Biotechnology, Institute Biotechnology, Addis Ababa University, P.O.Box 30604, Addis Ababa, Ethiopia*

[3]*Institute of Biodiversity Center, Addis Ababa, Ethiopia*

[4]*Mereced University, California, USA*

Abstract

Background: Infectious diarrhea is the most significant cause of morbidity and mortality in neonatal dairy sheep throughout the world. This is the first study conducted on isolation and bio typing of *E. coli* from lamb diarrhea in and around DebreBirhan. The present study was undertaken with the objective of isolation, omnilog characterization and biotyping of *E. coli* isolates from faecal samples around Debrebrhan.

Methods: A cross-sectional study was conducted from October, 2012 to April, 2013. The study focused on lambs less than three months of age showing clinical symptoms of diarrhea. Standard cultural and Omnilog tests were done to identify *E. coli* species and biotypes; descriptive statistics and Chi-square were used to analyze the collected data.

Results: From a total of 100 diarrheic lambs examined, 84% were found to be positive for *E. coli*. On the basis of fermentation reactions of sugars, *viz.* dulcitol, raffinose, rhamnose, salicin, starch and sucrose, 69 isolates utilized one or more sugar, while 14 isolates failed to utilize any of the sugars. 69 *E. coli* isolates were bio typed into 15 different combinations. Association between different age groups and occurrence of lamb diarrhea caused by *E. coli* strains showed a significant association ($p<0.05$). From the questionnaire findings, 60% of all the health problems in lambs were due to diarrhea where young sheep were more affected.

Conclusion: Pathogenic *E. coli* has the potential to cause sheep diarrhea. The distribution of the *E. coli* isolates into different biotypes indicates the diverse nature of the organism. Therefore, further detailed study should be carried out to understand the role of *E. coli* in lamb diarrhea and identify the virulent strains involved.

Keywords: Biotypin; DebreBirhan; Lamb diarrhea; Omnilog; Sugars; Ethiopia

Introduction

E. coli is a multitalented, enteric Gram-negative bacillus, and best known as a noninvasive commensal that grows in mass culture in human and in animal gut lumen, perhaps keeping other more harmful bacteria away from proliferating [1]. Diarrhea is common in newborn calves, lambs and kids. The acute disease is characterized by progressive dehydration and death, sometimes in as few as 12 hrs. In the sub acute form, diarrhea may persist for several days and result in malnutrition and emaciation. Lambs are vulnerable to *E. coli* infection. Two age groups appear to be susceptible, lambs of 1-2 days of age and lambs of 3-8 weeks old. Symptoms include diarrhea, a rise in temperature, weakness and lack of appetite. This is soon followed by coma and death within a few hours. In older animals, there is a tendency or infection to localize itself in the joints of survivors. Lesions include enlarged, haemorrhagic spleens and the accumulation of synovial fluid, and sometimes pus in affected joints [2].

The *E. coli* infection is a disease of economic importance. The mortality rate due to *E. coli* infection in sheep ranged from 1-5% with an age distribution of 3-12 weeks old. Due to *E. coli* infection in sheep, wool and meat production declined dramatically. As a result, the farmers who are economically dependent on sheep rearing become loosers [3].

Cultural characterization of *E. coli* by using different media and biochemical characterization by observing variable reaction to different sugars and chemicals are the basic rules for their identification. Antibiotics are widely used in case of diseased animal in the treatment of Sheep diarrhea [4].

Knowledge of local antimicrobial therapy pattern is important in selecting the appropriate therapy. Various parameters, including the prevalence, isolation, identification and epidemiological investigation of *E. coli* was studied by various workers [5,6] conducted an experiment on *E. coli* isolated from human, cattle, sheep, goat, chickens, duck, pigeon, drain sewages and soil. For the prevention and control of any microbial disease, prior isolation, identification and characterization of that particular etiological agent in a country is a prerequisite.

E. coli are able to ferment a variety of carbohydrate substrates, generally by converting them to glucose or to a substrate on the fermentative chain of the breakdown of glucose. The ability to ferment a given sugar of the types described above by a strain of *E.coli* is dependent on the strain having the requisite enzymes to convert it to glucose, or to a substance on the degradative chain from glucose [7].

Intestinal lesions caused by AEEC are termed attaching and effacing (AE), because of their intimate attachment to the enterocyte and effacement of the microvillus border [8]. A chromosomal gene, EAEC, encodes the protein intimin, which is involved in activity [9]. AEEC which causes disease and does not produce enterotoxins or shiga toxins is referred to as enteropathogenic *E. coli* (EPEC).

***Corresponding author:** Metasebia Aklilu, Department of Microbiology, Immunology and Veterinary Public Health, College of Veterinary Medicine and Agriculture, Addis Ababa University, Addis Ababa, Ethiopia, E-mail: aklilumetasebia@gmail.com

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In the study area, the detection and identification of *E. coli* biotypes in diarrheic lamb has not been reported. Therefore, this research was proposed to study the distribution *E. coli* in lamb diarrhea in and around Debrebrhan with following objectives:

1. Isolation and characterization of *E. coli* from diarrheic lamb faeces.
2. Identification of biotypes of *E.coli* isolates using standard biochemical tests.
3. Determine the association between age of lamb and occurrence of lamb diarrhea.

Materials and Methods

Description of the study areas and population

Study area: The study was conducted in and around Debrebrhan. It is city and Wereda in central Ethiopia, located in Semen Shewa Zone of the Amhara region, about 120 kilometers north east of Addis Ababa on paved high way to Dessie. The town has latitude and longitude of 9°41'n 39°32' ecoordinates: 9°41'n 39°32'e and an elevation of 2,840 meters. It was an early capital of Ethiopia and afterwards, with Ankober and Angolalla, was one of the capitals of the kingdom of Shewa. There are large populations of sheep in these areas that supply meat and skin products for human consumption. It is good to mention here the economic importance of DebreBirhan wool factory and the contribution of the region for providing raw materials for wool industry. A sentence or two could be added to show the importance of the study in this area.

Study population: Animals that were included in this study are cross breed lamb, which are under three months old that were clinically affected with diarrhea, exhibiting signs of systemic disease (poor appetite, dehydration, decreased mentation and reduced suckle reflex) and had pasty watery faeces, which are found in DebreBirhan and its surrounding area.

Study design: The study was conducted in DebreBirhan veterinary clinic and the rural area around DebereBirhan from farms from November 2012 up to March 2013. Purposive type of sampling was employed, i.e. lambs showing diarrhea in the study area during the study period was used as source of sampling. Accordingly, a total of 100 lambs were sampled during the study. Faecal samples were collected from lambs that showed clinical cases of diarrhea. Approximately, 50 gm of fecal material was collected from the rectum of lambs by direct digital stimulation using a disposable latex glove. Samples were placed into sterile containers and refrigerated until shipping. Samples were then transported on ice box to the institute of Biodiversity Microbiology Laboratory for sample processing. The isolate was placed in nutrient broth at 4°C until Biolog is conducted.

Laboratory work

Isolation of *E. coli*: Faecal samples were inoculated on MacConkey agar and incubated at 37°C overnight. From each plate, isolated lactose fermenting colonies were inoculated on Eosin Methylene Blue (EMB) agar medium. For preliminary characterization, colonies showing characteristic metallic sheen on EMB agar were then be picked up and considered as presumptive *E. coli.* The purified cultures of *E. coli* were stored in nutrient broth for further identification for Biolog tests, biotyping and other studies. All the isolates were stained by Gram's stain to determine the cell morphology and purity of the isolates. After preparing the rainbow agar, the isolated colony from nutrient broth was inoculated to confirm the colony is *E. coli*. Here all the colonies on the rainbow agar had black or grey colors, which indicate the isolate is *E.coli*:O157:H7.

Isolation of *E.coli* by using Biolog: After growing in EMB, the purified cultures of *E. coli* were stored in nutrient broth for further identification by Biolog and sugar fermentation tests. After the *E.coli* was isolated on rainbow agar, it was inoculated on Bug agar for further confirmation.

Presumptive *E. coli* isolates were processed according to the procedures of the manufacturer. Briefly, a pure culture was isolated on Bug agar medium; inoculums are prepared at a specified cell density; the Biolog microplate was inoculated with the inoculums; the plate was inserted into the Biolog apparatus; the reaction pattern was entered and results were obtained from the apparatus.

Biotyping of *E. coli* isolates: Biotyping of *E. coli* isolates was conducted based on fermentation reactions of the isolates on six sugars, *viz.* dulcitol, raffinose, rhamnose, salicin and starch. One percent of each sugar in phenol red broth was used. For the test, isolates grown in phenol red broth were inoculated into each sugar medium. Tubes were incubated at 37°C for seven days and readings were then recorded every 24 hours. Production of yellow color was considered as positive reaction and proper controls were kept for each of the biochemical tests performed. Isolates showing similar fermentation reaction patterns on the six sugars were considered as belonging to one biotype.

Questionnaire survey

A questionnaire was administered to lamb owners to assess the general lamb husbandry practices. Generally, the questionnaire includes all practices which could have impact on the proper rearing of lambs. These include colostrums feeding, general health care, hygiene and sanitation of farms, occurrence of lamb diarrhea, preventive and control measures practiced in the farms.

Data collection, management and analysis

Data describing the diarrheal conditions suggestive of *E. coli* infection observed on sheep along with age was classified, filtered and coded using Microsoft Excel® 2007. The data were then be exported to SPSS windows version 17.0 (SPSS INC.Chicago, IL) for appropriate statistical analysis. The occurrence of *E. coli* from the total diarrheic lambs was determined by using descriptive statistics. Chi-square (χ^2) was used to measure the association between age and the occurrence of lamb diarrhea. Effects were reported as statistically significant if P-value was less than 5%.

Results

Isolation and identification of *E. coli* isolates

For isolation of *E. coli*, MacConkey agar (MCA) and Eosin Methylene Blue agar (EMB) were used as differential and selective plating media. One handerend (100) faecal samples were initially screened on MCA, EMB agar and omnilog. From100 diarrheic sheep, 84 samples (84%) were presumed to be positive for *E. coli*.

Omnilog'sidentification and characterization *of E. coli* isolates

The *E. coli* isolates were stained by Gram stain to check for the purity and were found to be Gram negative rods. And the isolate again cultured in to rainbow agar to confirm the isolate is *E.coli* (black or grey colors), and again cultured in to bug agar prepare for omnilog identification. The omnilog reading result revealed that four different species (*Escherichia coli, Salmonellagp1, Serratia odorifera* ,*Citrobacter freundii*) were identified and were found to be involved in lamb diarrhea (Table 1).

Type of species	Number	% of identified species
Escherichia coli	84	84
Salmonella gp1	9	9
Serratia odorifera	6	6
Citrobacter freundii	1	1

Table 1: Biolog's identification and characterization of *E. coli* isolates.

Biotype No.	Isolates	Total No. of isolates	Positive sugars
I	020,021,034,040,041,049,052,062,065,072,079,067,073,074,083	15	Dulicitol
II	006,007,027,076,048,053	6	Raffinose
III	015,019,060,055,054,082,085	7	Rhamnose
IV	035,050,047,058,061,076	6	Sucrose
V	010,039,046,	6	Dulicitol and rhamnose
VI	044,078,088	3	Raffinose and rhamnose
VII	029,087,0067	3	Rhamnose and salicine
VIII	022,066,070	3	Rhamnose and starch
IX	013,051,065	3	Rhamnose and sucrose
X	003,004,026,037	4	Dulicitol,raffinose and rhamnose
XI	009,069,079	3	Dulicitol,rhamnose and starch
XII	043,051,065	3	Raffinose, rhamnose and sucrose
XIII	001,025,080	3	Raffinose, salicine and sucrose
XIV	031,063	2	Raffinose,starch and sucrose
XV	032,096	2	Dulicitol, raffinose, salicine and sucrose

Table 2: Biotypes of E. coli isolates on the basis of fermentation reactions of dulcitol, raffinose, rhamnose, salicin, starch and sucrose.

Age	No. of lambs	No. of *E. coli* +ve samples	%of diarrheic lambs	% of *E. coli* +ve samples	OR (95% CI)	P-value
2-3 month	25	20	25%	23.6	-	-
1-2 month	35	28	35%	32.9	2.13(1.23,6.78)	0.043
<1 month	40	37	40%	43.5	1.92(2.45,4.16)	0.05
Total	100	85	100	100	2.26(3.2,6.75)	0.032

Table 3: Distribution of lamb diarrhea among different age groups.

Biolog characterization based on assimilation result

The equipped wotj 37-96 column panels contain carbon source for assimilation tests. Results from these tests were scored turbidmetrically. The last column panel 58-60 had wells that contain 2 elements, such as carbon and phosphate. These wells test for the co-utilization of various sources. Table 2 and 3 shows if the read is positive on the Micro Plate, <X>, and the database result for that well is negative, the printout shows <X- to indicate a mismatch where the database reaction is negative. If you have a negative read X with no brackets and database value for that well is positive, and hence, the well will read X+, indicating a positive reaction in the database. At the time of a read, the data is compared to the database to determine the ID.

Distribution of diarrhea among different agegroups of lambs

The study was conducted on lambs which were less than three months of age. During the study, a total of 100 diarrheic lambs were examined and from these, 84 lambs (84%) of lambs diarrhea caused by *E. oli* bacteria. From the whole sample which was under 1 month of age, 40 lambs (40%), for lambs between 1-2 month of age, 35 lambs (35%) for that 2-3 month of age 25 lambs (25%). The age distribution of diarrheic lambs positive for *E. coli* was 37 lambs (43.5%) for those which were under 1 month of age, 28 lambs (32.9%) for those between 1-2 months of age, 20 lambs (23.6%) for those 2-3 months of age (Table 4).

From the above age distribution, association between different age groups and occurrence of lamb diarrhea was calculated and showed a significant association was observed (P value<0.05). Similarly, association between different age groups and sheep diarrhea caused by *E. coli* were also calculated and showed no significant association (P value>0.05). Both tests were calculated using Chi-square at 95% confidence interval.

Findings of the questionnaire survey

In this study, all the farmers followed have almost similar management system. Due to the similarity in management, no statistical comparison was done for most of the factors, but some of the potential risk factors (age of lambs, colostrum feeding and hygiene of farm, treatment of cases, control measures taken) were included in the study.

From the above questionnaire results, the associations between lambs diarrhea and different age groups were calculated using chi-square at 95% confidence interval, and the result indicates there is significant association between age group and occurrence of lamb diarrhea (P value <0.03) (Table 4).

Discussion

Isolation and identification of *E. coli* isolates

In the present study, from a total of 100 diarrheic lambs, 84 (84%) of *E. coli* isolates were obtained from 84 rectal samples. The cultural characterization of all positive sheep *E. coli* revealed greenish black colony with metallic sheen in Eosine methylene blue agar, bright pink color smooth transparent colony in Mac Conkey agar, green color colony on rainbow agar. In Gram's staining, the morphology of the isolated bacteria exhibited Gram negative short rods arranged in single or paired.

Questionnaire data	Category	Total positive (%)	OR (95% CI)	P-value
Study area	Around DebrBirhan	53/65 (81.5)	Reference	-
	DebrBirhan	31/35 (88.6)	1.13 (0.59, 2.18)	0.626
Age	2-3 month	43/56 (76.7)	Reference	
	1-2month	26/28 (92.8)	4.46 (1.67, 11.89)	- 0.05
	Less than one month	15/16 (93.7)	2.01 (1.13, 3.59)	
Sex	Male	43/49 (87.7)	Reference	-
	Female	41/51 (80.3)	1.31 (0.52, 3.31)	0.315
Diarrhea	Bloody	12/15 (80)	Reference	
	Watery	42/51 (82.3)	1.10 (0.53, 2.29)	- 0.692
	Mucoid	30/34 (88.2)	1.47 (0.69, 3.14)	
Sheeping facility	Sheeping pen	2/7 (28.6)	Reference	-
	Same barn	82/93(88.2)	1.25 (0.64, 2.45)	0.231
Cholustum feeding	Yes	83/99 (83.8)	Reference	-
	No	1/1 (100)	1.31 (0.59, 2.90)	0.66
First feeding time	Six and above hrs	83/99 (83.8)	Reference	-
	Less than six hours	1/1 (100)	1.93 (0.61, 6.15)	0.661
Sheep lost due to diarrhea	Less than five	56/71 (78.9)	Reference	-
	Five and above	28/29(96.5)	2.42 (1.96, 6.06)	0.029
Treatment for sick sheep	Antibiotics	34/48 (70.8)	Reference	-
	Tradition	50/52 (96.1)	2.21 (1.03, 4.78)	0.001
Response to treatment	Recover	49/63 (77.8)	Reference	-
	Non recover	35/37 (94.6)	1.46 (0.62, 3.44)	0.071

Table 4: Description of farms based on questionnaires.

The prevalence of *E. coli* from lambs in this study was in disagreement with reports of survey conducted in other countries. *E. coli* was isolated from 4% of ewes and lambs in the Netherlands [10], 1.4% of sheep with monthly variation of zero to 4.8% in UK [11,12] and 0.2% in Italy [13]. However, a prevalence of 31% in USA [14] and 68% from sheep flock in Australia from faecal sample were reported. These reports are much lower than the present finding. On the other hand, zero *E. coli* prevalence from sheep fecal sample was reported in Norway, Scotland [15], Ireland [16], Greece [17] and United States [14]. This high prevalence in the current study may be attributed to delay in first colostrums feeding, unclean sheep houses and lack of implementation of proper preventive and control measures.

Biotypes of the *E. coli* isolates

Biochemical reactions have conventionally been used for identification of bacteria to the species level. Extensive studies of sugar fermentation reactions of bacteria have been done to introduce biochemical typing systems in epidemiological studies of bacteria [18,19].

E. coli are able to ferment a variety of carbohydrate substrates, generally by converting them to glucose or to a substrate on the fermentative chain of the breakdown of glucose. The ability to ferment a given sugar of the sugars described above by a strain of *E.coli* is dependent on the strain having the requisite enzymes to convert it to glucose, or to a substance on the degradative chain from glucose. It has been found that different strains of *E. coli* differ in their ability to perform these conversions. Thus, this is the basis of biotyping *E. coli*. These tests are easy to perform, by determining whether a strain of *E. coli* will produce acid following growth in the presence of carbohydrates [7].

In the present study, the fermentation reactions of carbohydrates by all the 84 *E. coli* isolates were found to be variable. Out of the 84 *E. coli* isolates obtained, 69 isolates were able to utilize one or more sugars, while 15 isolates failed to utilize any of the sugars tested. The isolates could be grouped into various biotypes considering the fermentation reactions of six different sugars, *viz.* dulcitol, raffinose, rhamnose, salicin, starch and sucrose. We followed the methods described by Pandey et al. [20] and Chachra and Katoch [21], using six sugars for biotyping of *E. coli* isolates.

In this study, the most commonly occurring biotypes were Biotype I (15 isolates), III (7 isolates), II, IV, V(6), VI,VII, VIII, XI,XII,XIII, IX,(3), XIV and XV(2).The distribution of the isolates in to different biotypes indicates a wide variety in the presence of enzymes that ferments a given sugar, which further implies the diverse nature of the bacteria.

Age association with lamb diarrhea

In the present study, the occurrence of lamb diarrhea among different age groups was assessed. Accordingly, young lambs (less than 1 month) of age were at a significantly high risk of being affected with diarrhea ($P<0.05$). This finding is also comparable with the result obtained from the questionnaire survey, which indicated young lambs were at a significantly high risk of being affected with diarrhea than the older age groups (2-3 month age).

The result can be associated with many factors at younger age; delay in first colostrums feeding and unclean lamb house was associated with higher risk of morbidity. The finding that delayed colostrums intake (latter than 6 hours of age) associated with high risk of morbidity agrees with other reports. Olsson et al. [22] found that each hour of delay in colostrums ingestion in the first 12 hours of age increased the chance of a lamb becoming ill by 10%. Matte et al. [23] found that 61% of colostral immunoglobulin containing 80 mg/ml of 43 IgG is absorbed in six hours and decreases sharply, thereafter. This indicates that the first six hours are the period in which maximum absorption of colostral immunoglobulin takes place.

The higher risk of morbidity related to delayed intake of first colostrums meal could be associated with Failure Of Passive Transfer (FPT) of cloistral immunity. The role of FPT on subsequent health and production of neonatal lambs was well documented by many researchers. There are many other similar studies which proved FPT

to be a risk for lamb mortality and morbidity. Perez et al. [24] found no difference in terms of morbidity between those ingested first colostrum before 3 hours and after three hours of age. This showed that colostrum can be still efficiently absorbed after three hours, and could not contradict with the finding of the present study or other similar findings. In this study, poor hygienic conditions of the observed farms may have contributed for the high occurrence of lamb diarrhea.

Conclusion and Recommendations

Lamb diarrhea is economically important health problem in sheep. During the present study isolation, identification and biotyping of *E. coli* were made from lamb diarrhea, for the first time to our knowledge. Accordingly, it was found that 84% of isolation rate of *E. coli* was found in lamb diarrhea in the study area. Based on their sugar fermentation abilities, isolated *E. coli* strains were grouped in to fifteen different biotypes. This biotyping indicated the diverse nature of the study organism in the study area.

Based on the above conclusion, the following points are recommended:

The current study indicated the potential role of pathogenic *E. coli* in lamb diarrhea. Thus, considering the complex nature of the disease and the diverse nature of the study organism, in depth study should be done to understand the distribution of *E. coli* biotypes and virulent strains in lamb diarrhea in Ethiopia.

➢ Further studies based on biotyping should be conducted to establish the association between fermentative enzymes of pathogenic E. coli and its pathogenicity in lamb diarrhea.

➢ Implementation of improved lamb management practices is greatly suggested to reduce the high level of lamb diarrhea in younger lambs. Special emphasis should be given to the time of colostrums feeding, hygiene of lamb house, preventive and control measures.

Competing Interest

The authors informed that they have no competing interest

Authors' Contribution

MA participated in designing of the proposal, coordinating and managing of the study, data and specimen collection, laboratory testing and drafted the article and with inputs from Genene Tefera, Tesfaye Sisay and Belay Tekalegn. Tesfaye Sisay, Genene Tefera and Belay Tekalegn actively participated in the study design and edition of article. Data analysis and interpretation is made by Metasebia Aklilu. All authors read and approved the final manuscript.

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