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Isolation and Prevalence of *Listeria* Species in Milk and Milk Product Samples Collected from Bishoftu and Dukemtowns, Oromia, Ethiopia

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Abstract: Listeriosis is a serious and important emerging bacterial zoonotic disease worldwide. It is caused by ingestion of food contaminated with the bacteria, *Listeria* species. A cross-sectional study was conducted from November 2016 to April 2017, to estimate the prevalence of *Listeria* species in milk and milk product samples collected from Bishoftu and Dukem towns. A total of 200 samples were randomly collected and cultured for this study and 30% (n = 60) were positive for *Listeria* species. The species of *Listeria* isolated and identified using biochemical test in this study were *L. monocytogenes* 5% (n = 10), *L. innocua* 5% (n = 10), *L. welshimeri* 1.5% (n = 3), *L. seeligeri* 3% (n = 6), *L. ivanovii* 3% (n = 6) and *L. grayi* 7.5 % (n = 15). In addition, 5% (n = 10) unidentified *Listeria* species were also isolated. The overall prevalence of *Listeria* species isolated in this study were significantly (*p-value* = 0.01) higher in cheese (52.5%) than other samples. Samples collected from open market (53.3%) and hotels (41.2%) were also highly contaminated with different types of *Listeria* species compared to others. In general, the prevalence of the majority of *Listeria* species identified in this study was not statistically significant (*p-value*> 0.05) between different risk factors: site, sample type and sample source. However, the prevalence of *L. grayi* (29.4%) in samples collected from hotels was significantly higher (*p-value* = 0.03) than others. The occurrence of *Listeria* species in milk and milk product in this study is an indicator of the presence of public health hazards to the consumer, specifically to the high-risk groups. The possible causes for the occurrence of *Listeria* species are discussed and the results are compared with works done in Ethiopia and abroad. Finally based on the results obtained, good hygienic conditions during food processing and public awareness creation on food safety are recommended.

Key words: Ethiopia · *Listeria* Species · Milk · Milk Product · Prevalence

INTRODUCTION

Foodborne pathogens are one of the leading causes of illness and death in developing countries resulting in the loss of labor force which could have contributed in the economic growth [1]. Food borne diseases occur particularly in Africa because of the prevailing poor food handling and sanitation practices, inadequate food safety laws, weak regulatory systems, lack of financial resources to invest in safer equipment and lack of education for food handlers [2]. Of the food intended for humans, those of animal origin tend to be the most hazardous and are generally regarded as high risk commodity in respect of pathogen contents, natural toxins and other possible contaminants unless the principles of food hygiene are employed [3, 4]. Food of animal origins such as meat, milk and their products are very suitable for development of microorganisms, including zoonotic pathogenic bacteria [5].

Listeriosis is one of the important emerging bacterial zoonitic infections worldwide and arises mainly from the consumption of contaminated food products such as milk and milk products, meat and meat products, raw vegetables and sea foods. Of particular concern are ready-to-eat foods that are refrigerated before consumption and those that do not undergo any

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substantial heat treatment [6, 7]. Several studies have indicated that consumption of milk and dairy products contaminated with pathogenic speciesof *Listeria* can lead to individual cases or true outbreak of Listeriosis. Of all dairy products, soft cheeses and non-pasteurized milk are the most common sources of Listeriosis [8].

Bacteria under the genus *Listeria* are known to be responsible for cases of Listeriosis. The genus *Listeria* currently contains 17 species [9]. Among the different species of the genus *Listeria*, *Listeria monocytogenes* (*L. monocytogenes*) has been known to cause Listeriosis in humans and animals. The other pathogenic species is *L. ivanovii*, which causes abortion in animals [10]. Sporadic human infections due to *L. seeligeri* and *L. innocua* have also been reported [11]. In addition Listeriosis has also a major veterinary importance in animals used for food particularly cattle, sheep and goats. It causes encephalitis, abortion and septicemia in these animals [12].

Listeria species are ubiquitous in the environment and possess unique physiological characteristics that allow growth at refrigeration temperature that are usually adverse for most pathogenic food borne bacteria. The organism can also tolerate a pH between 4.5 to 9.6 [13, 14]. The available current literature shows that *Listeria* species have been reported from a wide variety of food types and clinical samples in various countries of the world [13].

Studies conducted by Molla *et al.* [15], Gebretsadik *et al*. [16], Firehiwot, [17], Muhammed *et al*. [18], Seyoum *et al*. [19] and Garede w*et al*. [20] have reported *Listeria* species with an overall prevalence range of 6.5% to 32.6 in different types of food of animal origin in Ethiopia. Despite veterinary and public health importance of Listeriosis, there is still lack of information on the occurrence and distribution of *Listeria* species in Ethiopia including in Bishoftu and Dukem towns. Therefore, the present study was conducted with the following objectives:

- To estimate the prevalence and speciesdiversity of *Listeria* in milk and other dairy products.
- To determine the risk factors for the occurrence of *Listeria* species in milk and other dairy products.

MATERIALS AND METHODS

Study Area: The study was carried out in Bishoftu and Dukem towns from November 2016 up to April 2017. Bishoftuis located in Oromia regional state, 45 kms South East of Addis Ababa, thecapital of Ethiopia. The area is located at 9°N latitude and 40°E longitudes at an altitude of 1850 meters above sea level in the central high land of Ethiopia. It has an annual rainfall of 866 mm of which 84% is in the long rainy season (June to September). The dry season extends from October to February. The mean annual maximum and minimum temperatures are 26°C and 14°C, respectively with mean relative humidity of 61.3%. Dukem is a town in central Ethiopia, located in the Oromia Special Zone of FinfinneSurrounding Addis Ababa, Oromia Region, 37 km southeast of Addis Ababa and 10 km northeast of Bishoftu. This town has a latitude of 8°48'N and longitude of 38°45'E and elevation of 1950 m above sea level [21].

Study Design and Sample Type: A cross-sectional study was conducted, to isolate and determine the prevalence of *Listeria* species in milk and milk product samples collected from Bishoftu and Dukem towns. Cheese, Ice cream, raw milk, yoghourt and pasteurized milk samples were collected by a simple random sampling technique from cafeterias, restaurants, milk processing unit, supermarkets, shop, open markets, milk collection centre and hotels located in the study areas based on owners/managers willingness and availability of sample.

Sample Size Determination: The sample size for this study was calculated following the formula described by Thrustfeild [22]. $n = (Ž\alpha/2)^2 (P(1-p)/Sd^2$. By taking 13% prevalence, average *Listeria* species from 4% incheese to 22 % in milk [16]. Where P is the prevalence of *Listeria* species in susceptible food sample, Sd is standard deviation of the observation which is acceptable with $Z \alpha/2$ (1.96), 95% confidence interval. $n = (1.96)^2 (0.13) (1-0.13)/(0.05)^2$. Accordingly, the minimum sample size was calculated to be 173. However, to increases the precision of the estimation, the sample size was increased and a total of 200 samples were collected for this study.

Sample Collection and Transportation: Approximately 100 gm or 100 ml of each type of samples were collected in a sterilized container and immediately transported under cold condition (in icebox) to the laboratory of Department of Microbiology, Addis Ababa University, College of Veterinary Medicine and Agriculture. Upon arrival, the samples were immediately processed and cultured for bacteriological examination or stored overnight in a refrigerator at 4°C, when needed, until cultured the next day. All samples were properly labeled and identified by date of collection, sources and sample type. Sample collection and transportation was done according to USFDA/CFSAN, [23] and Quinn *et al*. [24].

Culture and Identification: Raw/pasteurized sample (25ml or 25g) was enriched in 225 ml of *Listeria* broth-LEB (Difco Laboratories, Detroit, USA) (1:9 V/V ratio) and incubated at 30°C for 4 hours. After 4 hours of incubation, selective reagents (*Listeria* selective supplement, Difco) were added and incubated again at 30°C for 48 hours. After 48 hours of incubation, a loop full of aliquots was streaked onto PALCAM agar plates (Difco) and kept at 37°C for 24 hours. Growth of black or black green colony with a black halo and black sunken center was taken as positive for *Listeria* species. Colonies were transferred to tryptic soy agar (TSA, Difco) with yeast extract and incubated at 37°C for 24 hours. Then confirmation was performed using biochemical tests (gram stain, catalase, oxidase, carbohydrate fermentation tests (xylose, mannitol and rhamnose,) and CAMP test) [23-25].

Data Analysis: The collected data was entered in to Excel, 2007 spread sheet for storage. The SPSS statistical computer software version 20 was used to analyze the data. Descriptive statistics was used to describe the frequency of *Listeria* species from different food samples. Chi-square test was used to compare categorical variables.

RESULTS

From the total of 200 samples randomly collected and cultured, 30% (n = 60) were positive for *Listeria* species. The species of *Listeria* isolated in this study include *L. monocytogenes* 5% (n=10), *L. innocua* 5% (n=10), *L. welshimeri* 1.5% (n=3), *L. seeligeri* 3% (n = 6), *L. ivanovii*3% (n=6) and *L. grayi* 7.5 % (n=15). In addition, 5% (n=10) unidentified *Listeria* species were also isolated. Five or more *Listeria* species identified in this study were isolated from three or more of sample types: raw milk, cheese and yoghourt samples. In the contrary, only two different types of *Listeria* species were isolated from ice cream and pasteurized milk samples. In addition, *L. welshimeri* was isolated only from raw milk samples collected from milk collection centres. The overall prevalence of *Listeria* species isolated in this study were significantly (*p-value* = 0.01) higher in cheese (52.5%) than other samples. Samples collected from open market (53.3%) and hotels (41.2%) were highly contaminated with different types of *Listeria* species compared to others. The highest prevalence of *L. monocytogenes* (13.3%) and *L. seeligeri* (13.3%), *L. innocua* (10%) and *L. ivanovii* (9.5%) and unidentified *Listeria* species (9.5%)

Table 1: The frequency and prevalence of *Listeria* species isolated in this study

			Frequency (prevalence in %)							
Risk Factor		No	Positive	*L. monocytogenes*	*L. innocua*	*L. grayi*	*L. ivanovii*	*L. seeligeri*	*L. welshimeri*	Unidetified *Listeria* spp.
Site	Bishoftu	120	37 (30.8%)	5(4.2%)	7(5.8%)	10(8.3%)	4(3.3%)	4(3.3%)	1(0.8%)	6(5%)
	Dukem	80	23 (28.7%)	5(2.5%)	3(3.8%)	5(6.2%)	2(2.5%)	2(2.5%)	2(2.5%)	4(5%)
	Total	200	60 (30%)	10(5%)	10(5%)	15(7.5%)	6(3%)	6(3%)	3(1.5%)	10(5%)
	X^2(*p-value*)		0.99(0.75)	0.44(0.085)	0.44(0.51)	0.3(0.58)	0.11(0.73)	0.11(0.73)	0.9(0.34)	0.000(1.000)
Sample type	Cheese	40	21 (52.5%)	2(5%)	2(5%)	7(17.5%)	2(5%)	3(7.5%)	0(0%)	5(12.5%)
	Ice cream	25	4 (16%)	0(0%)	2(8%)	2(8%)	0(0%)	0(0%)	0(0%)	0(0%)
	R. milk	80	27 (33.8%)	7(8.8%)	4(5%)	5(6.2%)	2(2.5%)	2(2.5%)	3(3.8%)	4(5%)
	Yoghourt	30	6 (20%)	1(3.3%)	2(6.7%)	1(3.3%)	1(3.3%)	0(0%)	0(0%)	1(3.3%)
	P. milk	25	2 (8%)	0(0%)	0(0%)	0(0%)	1(4%)	1(4%)	0(0%)	0(0%)
	Total	200	60 (30%)	10(5%)	10(5%)	15(7.5%)	6(3%)	6(3%)	3(1.5%)	10(5%)
	X^2(*p-value*)		19.7(0.01)	5.2(0.27)	1.96(0.74)	8.7(0.068)	1.49(0.83)	4.6(0.34)	4.56(0.33)	7.5(0.11)
Sample source	Open market	15	8(53.3%)	2(13.3%)	1(6.7%)	2(13.3%)	1(6.7%)	2(13.3%)	0(0%)	0(0%)
.	M. C. C.	80	27(33.8%)	7(8.8%)	4(5%)	5(6.2%)	2(2.5%)	2(2.5%)	3(3.8%)	4(5%)
	Cafeteria	27	3(11.1%)	0(0%)	1(3.7%)	1(3.7%)	0(0%)	1(3.7%)	0(0%)	0(0%)
	Super market	21	5(23.8%)	0(0%)	0(0%)	1(4.8%)	2(9.5%)	0(0%)	0(0%)	2(9.5%)
	Hotel	17	7(41.2%)	0(0%)	1(5.9%)	5(29.4%)	1(5.9%)	0(0%)	0(0%)	0(0%)
	Restaurant	20	7(35%)	0(0%)	2(10%)	1(5%)	0(0%)	1(5%)	0(0%)	3(15%)
	M .P. U.	16	3(18.8%)	1(6.2%)	1(6.2%)	0(0%)	0(0%)	0(0%)	0(0%)	1(6.2%)
	I.C.S.	4	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
	Total	20	60 (30%)	10(5%)	10(5%)	15(7.5%)	6(3%)	6(3%)	3(1.5%)	10(5%)
	X^2(*p-value*)		13.3(0.65)	9.3(0.23)	2.6(0.92)	15.2(0.03)	6.4(0.49)	7.7(0.36)	4.5(0.71)	8.5(0.29)

N.B.: X^2 = Chi-Square, R. milk = Raw milk, P. milk = Pasteurized milk, M.C.C.= Milk collection center M.P.U.=Milk processing unit I.C.S.=Ice cream shop, No = Number of sample collected

were identified in samples collected from open market, restaurant and super market, respectively. The prevalence of *L. grayi* (29.4%) in samples collected from hotels was significantly higher (*p-value* = 0.03) than others. In general, the prevalence of different *Listeria* species identified in this study was not statistically significant (*p-value*> 0.05) among/ between different risk factors: site, sample type and sample source. The frequency and prevalence of *Listeria* species isolated in this study is shown on Table 1.

DISCUSSION

Out of the total of 200 samples randomly collected and cultured, 30% (n=60) were positive for *Listeria* species. This result coincides with the studies of Molla *et al*. [15], Seyoum *et al*. [19] and Firehiwot, [17], who reported *Listeria* species with an overall prevalence of 32.6%, 28.4% and 27.4% in different types of food samples in and around Addis Ababa, respectively. However, the finding of this study is higher than the results of Muhammed *et al*. [18], who reported *Listeria* species with overall prevalence of 6.5% in milk and milk product samples, in Jimma. The differences in the prevalence of *Listeria* species among these different studies might be attributed to differences in the type of sample investigated, food items composition or hygienic status of food production and processing plants. In this study six different species of *Listeria* namely *L. monocytogenes*, *L. innocua, L. welshimeri, L. seeligeri, L. ivanovii* and *L. grayi*were identified. In addition, 5% (n=10) unidentified *Listeria* species were also isolated. This was in accordance with the previous studies of Molla *et al*. [15], Seyoum *et al*. [19], Firehiwot, [17], Gebretsadik *et al*. [16] and Garedew *et al*. [20], who reported different species of *Listeria* from food samples including in milk and milk products in Ethiopia.

The overall prevalence of *L. monocytogenes* in this study was 5%, which is in agreement with the results of Seyoum *et al*. [19], Muhammed *et al*. [18] and Gebretsadik *et al*. [16] who reported prevalence of 5.6%, 4% and 3.6%, respectively. However, the finding of this study is higher than the results of Molla *et al*. [15] (2.8%) and Garedew *et al*. [20] (1.3%). Among the food samples tested, the contamination level of *L. monocytogenes* was high in raw milk (8.8%). It was comparable with the previous report of Muhammed *et al*. [18] (10%) but lower than the finding of Gebretsadik *et al*. [16] (13%). However, the finding of this study is higher than that of Seyoum *et al*. [19] (2.04%) and Garedew *et al*. [20] (4%) who reported *L. monocytogenes* from raw milk samples. Of the known *Listeria* species, *L. monocytogenes* is the most pathogenic to humans and animals and has been recognized as one of the most serious emerging bacterial diseases during the last two decades that is transmitted through the consumption of contaminated foods [26, 27]. And a number of foods have been implicated in the spread of *L. monocytogenes* including raw milk, milk products and meat which were repeatedly found to be the means for the occurrence and spread of the pathogen [28].

The prevalence of *L. grayi* in this study was 7.5%. It was higher than the previous studies of Seyoum *et al*. [19], Garedew *et al*. [20] and Gebretsadik *et al*. [16] who reported 4.3%, 0.88% and 0.5% prevalence of *L. grayi* in milk and milk product samples, respectively. The high prevalence of *L. grayi* was detected in cheese (17.5%) samples than others. It was in harmony with Seyoum *et al*. [19]. The prevalence of *L. innocua* isolated in this study was 5%. This result was in agreement with the findings of Garedew *et al*. [20] (6.7%), Firehiwot, [17] (6.7%) and Molla *et al*. [15] (6.5%), but higher than studies of Muhammed *et al*. [18] (1.5%) and Gebretsadik *et al*. [16] (2.5%). The prevalence of *L. seeligeri* and *L. ivanovii* in this study were 3% for each species. This result was in approach with previous result of Seyoum *et al*. [19], who isolated both *L. seeligeri* (2.7%) and *L. ivanovii* (4.3%) from milk and milk product samples. *L. welshimeri* was isolated only from raw milk samples with a prevalence of 1.5%. It was also in accordance with the finding of Seyoum *et al*. [19] (1.4%). Even though only *L. monocytogenes* is considered to be a significant human and animal pathogen, occasional human infections due to *L. ivanovii* and *L. seeligeri* have also been reported [11]. Report has also indicated that *L. ivanovii* can cause abortion in animals [10].

Variation in the prevalence of *Listeria* species was observed among the different sample sources. Samples collected from open market (53.3%) and hotel (41.2%) were highly contaminated with different *Listeria* species than those collected from restaurant(35%), milk collection centre (33.8%), super market (23.8), milk processing unit (18.8%) cafeteria (11.1%) and ice cream shop(0%). The difference in prevalence of *Listeria* species in samples collected from different sources might be due to variation in hygienic handling, transportation and storage of milk and milk product. For instance, considering the high rate of prevalence in the samples collected from open market might be due to poor hygiene handling and contamination

at market. And the low prevalence in sample collected from ice cream shop might be due to safe storage and handling of milk and milk product (personal observation).

CONCLUSION

Listeriosis has both veterinary and public health importance. In human it is associated with consumption of contaminated food. Pregnant women, neonates, adults with underlying disease, elderly and immunocompromised individuals are at highest risk of Listeriosis. In this study, the results of bacteriological examination showed that milk and milk products are contaminated with different types of *Listeria* species namely *L. monocytogenes*, *L. innocua, L. welshimeri, L. seeligeri, L. ivanovii*, *L. grayi*and 10 unidentified *Listeria* isolates. The isolation of these bacteria indicates the potential risk of infection with *Listeria* in people consuming these products since these foods are consumed directly after purchase with no further treatment applied. In addition, the presence of these bacteria also indicates the unhygienic handling, transportation and storage of milk and milk products and/or poor sanitary conditions of persons in contact with milk and milk product.

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

- Good and standard hygienic practices must be implemented along the supply chain to minimize the level of contamination of milk and milk products before reaching the consumer.
- Awareness of the community about Listeriosisshould be increased through public education.
- Further studies must be conducted to identify the major sources and factors that contribute to the contamination of the food and to determine the circulating serovars of *Listeria* species.

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