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Identification and Antimicrobial Resistance Pattern of *Listeria Species* Isolated from Food Chilling Lines at Haramaya University, Ethiopia

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Abstract

Listeria species are worldwide zoonotic pathogens. Out breaks of listeriosis are linked refrigerated foods that are consumed without proper heating. Besides, indiscriminate use of antimicrobials leads to an increase in the rate of antimicrobial resistance (AMR) among *Listeria* species. However, the prevalence of *Listeria* species and their AMR pattern along food chilling lines at Haramaya University was not yet known. Therefore, a cross sectional study was conducted between the periods of October 2016 and April 2017 with the objectives to determine the prevalence of *Listeria* species and determine the antimicrobial profile of the isolates along food chilling lines at Haramaya University, Ethiopia. A total of 192 swab samples (96 food items and 96 refrigerator walls) were collected purposively. Isolation of *Listeria* from the samples was attempted as per the protocol of ISO 11290-1:1996. Disk diffusion method was used to assess antimicrobial profile of the *Listeria* isolates for five antimicrobials. The overall prevalence of *Listeria* species in food chilling lines was found to be 34.4%. The specific prevalence of *Listeria* species in food swab and refrigerator swab were 33.3% and 35.4% respectively but there was no statistically significant difference (P>0.05) on the prevalence of *Listeria* species in food and refrigerator swab. All the isolates were found to be resistance to penicillin, 80.4% to vancomycin, 64.8% to trimethoprim, 51% to tetracycline and 41.9% to cephalosporin. The high prevalence of *Listeria* species and their high antimicrobial resistance pattern along food chilling lines in this study represent a poor keeping quality and indiscriminate use of drugs at the study area. This suggests the need to implement sanitary measures and awareness creations to improve the hygienic conditions of refrigerators and food items kept in a refrigerator as well as proper use of antimicrobials in order to guarantee the quality of food products kept in refrigerators so as to safeguard the health of the consumers.

Keywords: Prevalence; Listeria species; Antimicrobial resistance; Food chilling lines; Haramaya university

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Introduction

The genus *Listeria* consists of six species: *L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seeligeri*, and *L. grayi*. All *Listeria* species are ubiquitously distributed in nature and can often be found in soil, decaying plants, sewage, silage, dust, and water. Two species, *L. monocytogenes* and *L. ivanovii*, are considered pathogenic for animals, with *L. monocytogenes* being predominantly associated with human illnesses such as meningitis, encephalitis, and sepsis [1].

Listerias are gram positive, non-spore forming, catalase-positive, oxidase-negative and facultative anaerobic bacteria. Among the risk groups (infants, pregnant women, elderly persons, and immune compromised individuals), the worldwide case fatality rate for listeriosis is estimated to be as high as 36%. Infection of humans predominantly occurs as a result of occasional contamination of ready-to-eat and raw food products [2].

Among food borne pathogens tracked by CDC in 2013 in USA, *Listeria* species had the second highest case fatality rate (21%) and the highest (90.5%) hospitalization rate. In recent years, it has emerged as a significant cause of human infection in industrialized countries causing approximately 1469 hospitalization each year in the United States which resulted in 260 deaths. The majority of the out breaks in the world are linked to refrigerated food and

ready to eat (RTE) food consumed without proper heating [3].

Growth temperatures for *Listeria* species ranges from 0°C to 45°C, and make it a food borne pathogen in chilled, refrigerated and ready-to-eat foods. Different authors from different countries reported high prevalence of *Listeria* species from domestic refrigerator and various frozen foods ranging from 1% up to 60% and from 1.3% up to 73.9% respectively [4].

Control of *Listeria* species contamination is particularly difficult, since the pathogen can survive and grow in adverse conditions like refrigeration temperatures, acidic pH and high saline concentrations. *Listeria* species form biofilms and embedded in a matrix of organic material that protects colonies from chemicals, increasing resistance to cleaning agents makes it difficult to eradicate from food storage and food contact surface [1]. Presence of *Listeria* species in refrigerator even at low concentration is of significant public health concern as the bacteria are capable of multiplying at this harsh condition. Unlike some other food borne pathogens, *Listeria* species can multiply in contaminated refrigerated food with one and half days doubling time in refrigerator [2].

Antimicrobial resistance is one of the crucial challenges that public health is facing today. The uncontrolled application of antimicrobials in the environment is leading to a constant increase in the rate of antimicrobial resistance among *Listeria*. Emergence of antimicrobial resistance is a result of the use, overuse and misuse of antimicrobials both in humans and animals. In Ethiopia, there are indications on the misuse of antimicrobials by health care providers, unskilled practitioners, and drug consumers. These coupled with rapid spread of resistant bacteria and inadequate surveillance contributed to the problem. Most pathogenic bacteria that are commonly involved in causing infections to human beings and animals showed considerable degree of resistance to commonly used first line antibacterial in Ethiopia, which is ranged from 0% to 100% [5].

Investigations on antibacterial resistance and on bacterial infections have shown that emerging antibacterial resistance threatens the management of bacterial infections. Some of the major consequences of resistance also include increased mortality, morbidity, costs of treatment, and loss of production in animals. Therefore, studying antimicrobial resistance in humans, animals and foods is important for detecting changing patterns of resistance, implementing control measures on the use of antimicrobial agents and preventing the spread of multidrug-resistant strains of bacteria [6].

However, the prevalence of *Listeria* species andtheir AMR pattern along food chilling lines at Haramaya University was not yet known. Therefore, this study was designed with the objectives to determine the prevalence of *Listeria* species along food chilling line and determine the AMR pattern of the isolates.

Materials and Methods

Study period and area

The study was carried out between the period of October 2016 and April 2017 at Haramaya University which is located in Haramaya district 5 km North East of Haramaya Town, East Hararghe, Oromiya region, Ethiopia. Haramaya is located at 513 km Eastern of Addis Ababa, capital city of the country and 12 km western of Harar town. The area has an altitude range between 1600-2100 meters above sea level. The rainfall of the District is bimodal, the short rain occurring between months of February to May and the long rain occurring between the months of June to September, followed by dry season from October to February. The mean annual rain fall is 492 mm ranging from 118 - 866 mm. The mean maximum and minimum temperatures are 23.4^{0}C and 9^{0}Crespectively [7].

Study design and source of samples

A cross-sectional study design was used for this study. There were a total of 96 public supply locations which have food chilling chain (student cafeteria and staff lounge resource center) all al of them were used as sample sources. These areas were selected because of their regular usage of refrigerators to preserve food both before cooking and after cooking. Also thousands of staff and students were using one of these locations daily. Meat were mostly preserved food in refrigerator before cooking in these cafeteria and lounges**.** In this cafeteria and lounges, most of the food items preserved in the refrigerators was beef and sometimes milk and butter. All of these selected public supply location use refrigerated and extended shelf life food. Also worker of chilling room have no enough information about food borne pathogen that can survive refrigerator temperature and result in cross contamination between ready to eat food and refrigerators to cause human health problems.

Sample type and sampling method

A total of 192 swab samples (96 food items and 96 refrigerator walls) were collected purposively. There were only a total of 96 refrigerators with in the University across all its college and all of them were sampled. Samples were taken by sterile swabs with cotton tips using the method described in ISO [8] by placing sterile swabs on specific sites of refrigerator and food items and placing in separate test tube containing 225 mL of Half Fraser Broth (primary enrichment medium).

All samples were correctly labeled using the date of collection, sources of sample and sample type. All samples were aseptically collected and put into a sterile screw capped bottles and was kept in an ice-box containing ice pack and taken immediately to Haramaya University College Veterinary Medicine, Microbiology Laboratory. In the laboratory the samples test tubes were transferred immediately from icebox to test tube rack and stored in refrigerator at +4^{0}C until processing.

Study Methodology

Cultural procedure

Isolation and identification of *Listeria* species was performed following the standard microbiological technique recommended by the International Standards Organization [8]. Samples which were kept overnight in a refrigerator at 4^{0}C were thawed for 3-5 hours at room temperature. The bacteriological media used for the study were prepared following the instructions of the

manufacturers. In order to get discrete separate colonies, the surface of the agar media was made dry by keeping the medium in the incubator for overnight.

Enrichment

Each sample was introduced aseptically into a sterile stomacher bag containing 225 mL of Half Fraser Broth (primary enrichment medium). The samples were then homogenized for 1 min at 260 rpm in a stomacher followed by incubation at 37^{0}C for 24 hours. After incubation period, 0.1 mL sub-sample from each Half Fraser Broth culture was added to 10 mL of Fraser Broth (secondary enrichment medium) and incubated at 37^{0}C for 24 - 48 hours.

Plating on selective media

A loopful of the Fraser Broth enrichment culture was streaked on PALCAM *Listeria* agar plate. The inoculated plates were incubated at 37^{0}C for 24 – 48 hours. Gray-green colonies with black halo were suspected to be of *Listeria.* The presumed colonies of *Listeria* (at least 3/plate) were sub cultured for further confirmation

Confirmation of isolates - biochemical characterization

From the isolation media, suspected colonies of *Listeria* were streaked onto Tryptone Soya Agar and incubated at 37^{0}C for 24-48 hours. The following tests were used for confirmation

- Gram's staining (Positive)
- Catalase reaction (Positive)
- Motility test (Tumbling motility)
- Oxidase test (Negative)

Antimicrobial resistance pattern test

Verified *Listeria* species that were grown on Nutrient Broth at 37^{0}C for 24-48 hours were used. Antimicrobial resistance pattern of the *Listeria* isolates was tested by the standard disk diffusion method following the procedure of Clinical and Laboratory Standards Institute [9] using Mueller-Hinton agar. The antimicrobials tested for resistance pattern were those which were proved to be often available and routinely used in the study areas for the treatment of animals. Four to five positively identified pure *Listeria* species' colonies of the same morphological type were selected from nutrient agar plate (NAP) and emulsified in 5 ml sterile tryptone soya broth in a sterile test tube.

The turbidity of the suspension was then adjusted by comparison with 0.5 McFarland turbidity standards which were in same amount in a similar test tube, in order to standardize the size of inoculums. A sterile cotton swab on an applicator stick was dipped into the standardized suspension of the bacterial culture, squeezed firmly against the insides of the test tube above the fluid level to remove the excess fluid and streaked and continuously brushed over the Mueller-Hinton agar plate and allowed to stand for 5 minutes to dry the flood. Thereafter, five different antimicrobial discs: penicillin (10 µg), tetracycline (30 µg), trimethoprim (5 µg), vancomycin (10 µg) and cephalosporin (10 µg) were placed on the agar with a distance of 24 mm from the center using sterile forceps and gently pressed down with the point of a sterile forceps to ensure complete contact with the agar surface. The plates were then allowed to stand for 30 minutes for diffusion of active substance of the agents. Plates were inverted and incubated at 37^{0}C for 24-48 hours. An inhibition zone diameter of each antimicrobial was then measured by using caliper and interpreted as 'Resistant', 'Intermediate' and 'Sensitive' by comparing with recorded diameters of a control organism, ATCC19111 **(Table 1).**

Statistical analysis

Microsoft excel spread sheet program was used to store all the data and Statistical Package for Social Sciences (SPSS) version 22.00 software was used to analyze the data. Prevalence of *Listeria* species was computed as the number of each sample items positive for *Listeria,* divided by total number of the samples examined. Analysis of antimicrobial resistance test result was by category agreement, where the zone diameters were divided into different categories (susceptible, intermediate and resistant). Chi-square (χ^2) was used to test the presence of association between variables When P value was less than 0.05, the presence of significance difference was considered.

Results

Prevalence of *listeria* species among food chilling lines

The overall prevalence of *Listeria* species in this study was found to be 34.4 % (66/192). Out of each 96 sampled collected from the refrigerator and food items, the prevalence of *Listeria* species were 35.4% and 33.3% respectively. This difference in prevalence of *Listeria* among sample types was not statistical significance (p>0.05) **(Table 2).**

The association of *Listeria* species between food items and refrigerator wall indicated that out of the 66 isolates, 20 swabs showed positive result for food items and refrigerator wall, 12 positive only from food items and 14 positive only from refrigerator wall **(Table 3).**

Antimicrobial resistance pattern of *listeria* species

A total of 31 isolates of *Listeria* species were tested for antimicrobial susceptibility. All the isolates showed complete resistance (100%) to penicillin (p10). Out of 31 isolates, 25 (80.6%), 20 (64.5%), 16 (51.6%) and 13 (42%) isolates were resistant to vancomycin, trimethoprim, tetracycline and cephalosporin respectively **(Table 4).**

Table 1 Inhibition Zone diameter interpretive standard for *Listeria* species.

Antimicrobial	Disc contents	Inhibition Zone Diameter (mm)		
		Resistant	Intermediate	Susceptible
Penicillin	10 µg	≤ 26	27-28	≥ 29
Tetracycline	30 µg	≤ 14	15-18	≥ 19
Trimethoprim	5 µg	≤ 23	24-27	≥28
Vancomycin	10 µg	≤ 12	13-14	≥ 15
Cephalosporin	30 µg	≤ 13	14-17	≥ 18

Table 2 Prevalence of *Listeria* species among food chilling lines.

Sample Type	Number Tested	Positive	Prevalence (%)	95% CI	χ^2	P value
Food Swab	96	32	33.33	25.62-46.84	0.09	0.76
Refrigerator Swab	96	34	35.42	26.72-48.62		
Total	192	66	34.4	25.45-46.76		

Table 3 Co-contamination of refrigerators and chilling food items by *Listeria.*

Food items	Refrigerator		
	Positive	Negative	Total
Positive	20	12	32
Negative	14	50	64
Total	34	62	96

Table 4 Antimicrobial resistance pattern rate of *Listeria* species to different antimicrobials.

Antimicrobials	Susceptible Number. (%)	Intermediate Number. (%)	Resistance Number. (%)
Penicillin	0	0	31 (100)
Vancomycine	4 (12.9)	2 (6.45)	25 (80.64)
Trimethoprim	4 (12.9)	6 (19.35)	20 (64.5)
Tetracycline	8 (25.8)	7 (22.58)	16 (51.61)
Cephalosporin	13 (41.9)	5 (16.13)	13 (41.9)
Resistance>1 drugs	0	0	31 (100)

Discussion

The presence of *Listeria* species in domestic refrigerators indicate both poor hygiene and possibility of cross contamination to foods stored in such refrigerators. The ability of *Listeria* species to contaminate interior surface of refrigerator may in turn contaminate foods stored in the [10].

The prevalence of *Listeria* species in food items in the present study (33.33%) was in agreement with a study carried out at Addis Ababa, Ethiopia that reported 32.6% prevalence of *Listeria* species in RTE foods [11] but it was lower than study that showed prevalence of 83.3% from raw minced meat in Turkey [12].

The prevalence of *Listeria* species in refrigerator wall in this study (35.42%) was in agreement with study conducted in Mexico by Beumer *et al.* who found prevalence of 36.5 but was lower than report of 61% [13] and Margaret [14] in Nigeria. This high occurrence of *Listeria* species from refrigerators walls in Haramaya University, Ethiopia might be due to inappropriate cleaning practices, keeping of unwashed fruit and vegetables as well as meat.

Our study indicated that all isolates (100%) of *Listeria* species were resistance to penicillin. This result was similar with Yuce et al. [12] who reported that all isolate of *Listeria* species were 100% resistance for penicillin but was higher than Lotfolahi et al. [15] and Navratilova et al. [16] who revealed 77.77% and 41% resistance for penicillin respectively. These variations in antimicrobial resistance could be attributed to difference in therapeutic practice among countries and types of sample.

The resistance of *Listeria* species to tetracycline in this study (51.6%) was in line with the 49% resistance reported by Mirriam et al. [14] but was lower than reported as 77.8% by Selamawit [17]. The result of the current study was higher than 8.16 % of tetracycline resistance reported by Gunja l [18]. The resistance of *Listeria* species to cephalosporin in this study (41.9%) was lower than 66.5 % resistance reported by Normanno *et al.* [19].

The resistance of *Listeria* species for trimethoprime in this study (64.5%) was found to be lower than Normannoet al [19] who reported 50% resistance of Listeria to trimethoprime. The resistance of Listeria species to vancomycine in this study (80.64%) was found to be lower Selamawit [17] who reported that *Listeria* species isolates showed 100% resistance to vancomycine.

All the isolates (100%) showed resistance to one or more antimicrobials (MDR). This was found to be in accordance with previous report that 100% *Listeria* isolates from RTE food by Mirriam et al. [14] were MDR. MDR in this organism could be attributed to antimicrobial selective pressure and gene transfer mechanisms between and among *Listeria* species [20].

The results of the present study on MDR isolates of *Listeria* species indicate that 100% of the isolates showed resistance to one or more antimicrobials. This was found to be in accordance with previous report that 100% *Listeria* isolates from RTE food by Mirriam et al. [14] were MDR. Thus, the resistance figures from different countries can considerably vary from very low to very high, probably reflecting the use of antimicrobials in those countries and regional differences in the use of antibiotics [21]. Multiple drug resistance in these organisms could also be attributed to antimicrobial selective pressure and gene transfer mechanisms between and among *Listeria* species and close relatives bacteria like *Enterococcus, Streptococcus* and *Staphylococcus* species [22].

Conclusion and Recommendations

This study revealed that food chilling lines (chilling food items and refrigerator wall) were highly contaminated by *Listeria* species and all the isolates were MDR. Occurrence of *Listeria* species in food chilling lines and their MDR pattern warrant public health concern and indicative to the problem of treatment and control of these pathogen infections. Based on the findings of this study the following recommendations are made.

- An extensive survey on the prevalence of *Listeria* species along food chilling lines should be undertaken
- Consumption of foods directly from refrigerator unless treating them with sufficient heat should be avoided
- Regular monitoring of AMR to *Listeria* should be practiced and the genetic mechanisms which mediate AMR in these bacteria should be further studied

- Judicious use of antimicrobials need to be adopted

Conflicts of interest

The authors declare that they have no competing interest

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