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RESEARCH ARTICLE
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ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF FOOD-BORNE *LISTERIA* SPP FROM HAVELIAN

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ABSTRACT
Listeria is food borne pathogen that causes listeriosis in humans and animals. Listeriosis is abundant, a clinically significant and sporadic illness because*of its severity and mortality rates can be as high as 20% to 30% even when appropriate medical care is provided. The pathogenic species of *Listeria* is *L. monocytogenes* and *L. ivanovii*. It is gram positive, purple, small rod shaped bacterium that can be found in environment and enter into food due to unhygienic conditions during food processing, handling and storage. The US Department of Agriculture and FDA implemented zero tolerance policy for RTE foods contaminated with *Listeria*. The present study was commenced to determine the prevalence and molecular characterization of *Listeria* spp. from different food samples sold at the market of Havelian. A total 150 food samples including vegetables, raw meat, poultry, raw fish, bakery and dairy products were examined and tested following the isolation procedure of direct and enrichment culturing method that were first confirmed on the basis of biochemical test including catalase, oxidase and motility test. Then isolates were subjected to further molecular identification using PCR technique and by sequencing strains of *Listeria* spp. was identified. In this study, *Listeria* spp. was confirmed in 32 different food samples with the prevalence rate of 21%. Among 32 isolates, 11 out of 62 vegetable samples, 6 out of 17 dairy products, 1 out of 7 chicken samples, 10 out of 47 bakery products, 2 out of 8 fish samples and 2 out of 9 meat samples were contaminated with *Listeria* spp. The isolates were gram positive, rod shaped, catalase positive, oxidase negative and motile at 25 ^{o}C. This study indicates a high level of contamination of *Listeria* in food samples signifies the risk of food borne illness associated to humans, especially pregnant women and immuno-compromised person. In this study, a heat-treated product (Kabab and chicken liver) was not contaminated with *Listeria* spp. Preventive measures such as properly washed and cooked food should be used by consumers to control the foodborne illnesses.

Keywords: Food contamination, listeriosis, prevalence, *Listeria* spp.

INTRODUCTION
Listeria is ubiquitous, small purple (gram positive), rod shaped (0.5- 2 μm in length and 0.5-4 μm in diameter), microaerophilic, no*spores forming, halo tolerant, facultative anaerobic, catalase positive, oxidase negative and has tumbling motility at 20-25 ^{o}C due to peritrichous*flagella[1]. By

2020, 21 species of *Listeria* had been identified: *L. monocytogenes*[2], *L. seeligeri, L. ivanovii* and *L. welshimeri*[3], *L. innocua*[4], *L. marthii* (first described in 2010), *L. grayi* (first described in 1966), *L. Fleischmannii*[5], *L. floridensis, L. aquatic, L. newyorkensis, L. cornellensis, L. Rocourtiae*[6], *L. weihenstephanensis*[7], *L. grandensis, L. riparia* and *L. booriae*[8].

Listeria are broadly distributed in different environments such as sewage, water, soil and frequently contaminate different foods include vegetables, milk, poultry, meat, seafood and dairy products etc. Moreover, it has exceptional characteristics such as the ability to grow at broad temperature range (ranging from 0.5-45 ºC), withstanding pH variation (between 4.3 and 9.8), low water activity (aw 0.91) and a special characteristics is osmotolerant that being able to survive in high salt concentrations (up to 20% w/v) introduce *Listeria* spp. as foodborne organisms [9]. *L. monocytogenes* is efficient in forming biofilms on surfaces made from stainless steel, polystyrene and glass so it can stay in food industries for several years where it contaminate food products, indicating a serious concern for food safety [10]. The Food and Drug Administration Center for Food Safety and the US Department of Agriculture impose a zero-tolerance policy for contamination of ready to eat foods with *Listeria*. This attempts led to 24% decline in the cases of invasive listeriosis and 37% decline in cases accompanying to pregnant women, since 1996 [11].

Listeriosis is abundant, a clinically significant and sporadic illness because of its severity and mortality rates can be as high as 20% to 30% even when appropriate medical care is provided [12]. Virtually all cases of human listeriosis are foodborne and connected with consumption of contaminated dairy products, grimy raw vegetables, and under-cooked meat, seafood and poultry products [13]. Foodborne listeriosis has three foremost clinical sorts i.e. meningitis, septicemia, and abortion. In healthy people, it can cause febrile gastroenteritis, but in susceptible ones (children, elderly, immune-compromised and pregnant women) it can lead to septicemia and meningitis[1]. *L. monocytogenes* saccounts for 10% of community acquired bacterial meningitis and fourth most common cause of meningeal infection sin adults after *S. pneumoniae*, *N. meningitides* sand Group B *Streptococci*. The most frequently reported cases of listeriosis causing encephalitis upsetting CNS in adults is 55-70%, common in animals but rare in adults. Additionally, bacteremia or septicemia (15-50% cases and up to 70% mortality rate) if it is associated with severe underlying debilitating conditions and atypical forms like myocarditis, pneumonia, peritonitis, pleuritis, hepatitis, arteritis, colecystitis, localized sabscesses etc are reported cases of accounts for CNS infection by *Listeria* are about 5-10%[14].

It is important to investigate the source and factors responsible for the food contamination. Recently, the Canadian Food Inspection Agency recalls 33 vegetable products over possible *Listeria* contamination [15]. To ensure food quality and safety, detection of pathogenic strains of *Listeria* spp. is necessary. The efforts to control the disease should be targeted at improving the food safety and quality. So this study will focus to characterized the strains at molecular level and determine the prevalence of *Listeria*.

METHODOLOGY

A total of One fifty food samples were collected in a sterile plastic bags from local market of Havelian, properly labeled and then brought to microbiology laboratory of the University of Haripur, Haripur.

SAMPLE PREPARATION

25 g or 25 ml from each food samples was taken in a sterile plastic bag and homogenized in peptone water. Distilled water is used to make dilutions for processing of samples after autoclaved at 121 ºC for about 1 hr. Hereafter, 10 test tubes were taken containing 9 ml of distilled water and 1 ml of homogenized sample was poured in the first tube. Then 1 ml from first test tube is taken and transferred to second test tube. In this way samples were serially diluted in all 10 test tubes.

ISOLATION OF *LISTERIA* SPP.

After homogenization, samples was inoculated into test tubes containing Listeria enrichment broth and incubated at 37 ^{o}C for 24-48 hr. After enrichment, enough growth was observed in broth then 25 ul pipette from broth were be taken and transfer into PALCAM agar plates while streaked it with the help of loop and incubated at 37 ^{o}C for 24-48 hr.

Serially diluted samples were spread onto nutrient agar plates after dilutions and then incubate at 37 ^{o}C for 24-48 hr. Then picked colonies from nutrient agar plates and streaked on Listeria selective agar plates, Columbia blood agar plates and PALCAM agar plates. Incubates these plates at 37 ^{o}C for 24-48 hr.

IDENTIFICATION OF *LISTERIA* SPP.

All the isolates were identified by colony morphology on the basis of size, color and shape seen on selective media and biochemical tests were done include gram staining, catalase, oxidase and motility test.

RESULTS

In this study, total 150 food samples including vegetables, raw meat, poultry, raw fish, bakery and dairy products were tested for the isolation and identification of *Listeria* spp. Out of total 150 food samples, 62 (41.3%) vegetables, 7 (4.7%) poultry, 9 (6.0%) meat, 8 (5.3%) fish, 17 (11.3%) dairy products and 47 (31.3%) bakery products were examined. *Listeria* spp. was confirmed in approximately 32 samples of food that determined prevalence rate of 21%.

Table 1 Total samples of food that was contaminated with *Listeria* spp.

Samples	No of samples	Positive
Vegetables	62	11
Dairy product	17	6
Poultry	7	1
Bakery products	47	10
Fish	8	2
Meat	9	2
Total	150	32

MORPHOLOGICAL CHARACTERISTICS

Listeria spp. was identified on the basis of their colony morphology on selective media. Isolated colonies were small size, dark brown with black halo on listeria selective agar plates and grey-green in color with black surrounding colonies on cherry-red medium as seen in figure 4 and 5.

BIOCHEMICAL TEST

All isolates were identified as gram positive, rod shaped, catalase positive and oxidase negative.

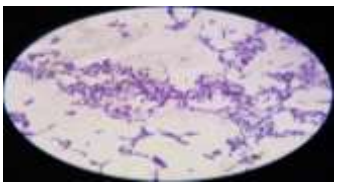

Figure 1 Morphology of Listeria spp. under microscope

Figure 2 Catalase test of isolated colonies

Figure 3 Oxidase test of isolated colonies

RESULT OF CULTURING

3 isolates of *Listeria* spp. that was confirmed by direct method of culturing on Listeria selective agar plates include cucumber, potato and tomato sample; 2 isolates from sample (1 chicken and 1 milk) was confirmed by direct method of culturing on Columbia blood agar plates. 10 isolates from sample (1 of each beetroot, curd, pea, coriander, milk and cauliflower; 2 of each butter and mayonnaise samples) was confirmed on PALCAM agar plates by direct method. By enrichment method of culturing, 17 isolates of *Listeria* spp. (1 of each tomato, carrot, pickle, milk, ketchup, cheese, bread, radish, cauliflower; 2 of each fish, juice, meat and ice cream) was confirmed on PALCAM agar plates mention in table 2.

Figure 4 Colonies of *Listeria* spp. on PALCAM agar plates

Figure 5 Colonies of *Listeria* spp. on Listeria selective agar

Of 62 vegetable samples 11 samples of these include 1 of 5 carrot, 1 of 4 beetroot, 2 of 3 cauliflower, 1 of 3 coriander, 1 of 4 cucumber, 1 of 3 pea, 1 of 4 potato, 2 of 7 tomato and 1 of 4 radish samples were tested positive for *Listeria* spp. by biochemical test. Conversely, brinjal, cabbage, capsicum, chili, ladyfinger, kaddu, lettuce, spinach and salad were tested negative. The study determined 3 of 8 milk samples were tested positive whereas other dairy products 1 of 6 curd samples and 2 of 3 butter samples were tested positive for *Listeria* spp.

However 10 out of 47 bakery products includes 2 of 6 juice samples, 2 of 8 ice cream samples, 2 of 5 mayonnaise samples, 1 of 4 bread, 1 of 6 cheese, 1 of 3 pickle and 1 of 5 ketchup samples were contaminated with Listeria spp. Conversely, *Listeria* spp. was not confirmed in these bakery

products i.e. custard, vermicelli, pizza, sauce and cream. Similarly no *Listeria* were contaminate heat treated minced meat despite 2 of 8 fish and 2 of 7 raw meat samples were positive contrasting 1 of 5 raw chicken except 2 samples of chicken products were contaminated with *Listeria* spp.

Table 2 Result of food samples that was contaminated with *Listeria* spp.

S No.	Sample of food	Date	Culturing method	Growth	Staining result	Biochemical test result
13	Cucumber	12/08/19	Direct LSA	Yes	Purple, rod shape	Positive
16	Potato	20/08/19	Direct LSA	Yes	Purple, Rod shape	Positive
19	Tomato	21/08/19	Direct LSA	Yes	Purple, Rod shape	Positive
48	Chicken	04/11/19	Direct CBA	Yes	Purple, Rod shape	Positive
63	Milk	02/01/19	Direct CBA	Yes	Purple, Rod shape	Positive
67	Tomato	09/01/20	Enrichment PALCAM	Yes	Purple, Rod shape	Positive
70	Carrot	13/01/20	Enrichment PALCAM	Yes	Purple, Rod shape	Positive
87	Beetroot	20/01/20	Direct PALCAM	Yes	Purple, Rod shape	Positive
88	Curd	20/01/20	Direct PALCAM	Yes	Purple, Rod shape	Positive
89	Butter	20/01/20	Direct PALCAM	Yes	Purple, Rod shape	Positive
92	Pickle	22/01/20	Enrichment PALCAM	Yes	Purple, Rod shape	Positive
96	Milk	22/01/20	Enrichment PALCAM	Yes	Purple, Rod shape	Positive
97	Mayonnaise	22/01/20	Direct PALCAM	Yes	Purple, Rod shape	Positive
98	Pea	22/01/20	Direct PALCAM	Yes	Purple, Rod shape	Positive
103	Coriander	27/01/20	Direct PALCAM	Yes	Purple, Rod shape	Positive
104	Butter	27/01/20	Direct PALCAM	Yes	Purple, Rod shape	Positive
105	Milk	27/01/20	Direct PALCAM	Yes	Purple, Rod shape	Positive
107	Mayonnaise	27/01/20	Direct PALCAM	Yes	Purple, Rod shape	Positive
111	Fish	06/02/20	Enrichment PALCAM	Yes	Purple, Rod shape	Positive
113	Juice	06/02/20	Enrichment PALCAM	Yes	Purple, Rod shape	Positive
115	Meat	06/02/20	Enrichment PALCAM	Yes	Purple, Rod shape	Positive
117	Juice	06/02/20	Enrichment PALCAM	Yes	Purple, Rod shape	Positive
123	Fish	11/02/20	Enrichment PALCAM	Yes	Purple, Rod shape	Positive
124	Meat	11/02/20	Enrichment PALCAM	Yes	Purple, Rod shape	Positive
125	Ketchup	11/02/20	Enrichment PALCAM	Yes	Purple, Rod shape	Positive
131	Cheese	18/02/20	Enrichment PALCAM	Yes	Purple, Rod shape	Positive
132	Bread	18/02/20	Enrichment PALCAM	Yes	Purple, Rod shape	Positive
133	Ice cream	18/02/20	Enrichment PALCAM	Yes	Purple, Rod shape	Positive
139	Ice cream	18/02/20	Enrichment PALCAM	Yes	Purple, Rod shape	Positive
146	Cauliflower	20/02/20	Direct PALCAM	Yes	Purple, Rod shape	Positive
149	Radish	20/02/20	Enrichment PALCAM	Yes	Purple, Rod shape	Positive
150	Cauliflower	20/02/20	Enrichment PALCAM	Yes	Purple, Rod shape	Positive

DISCUSSION

Food has been contaminated with *Listeria* spp. through food processing plants and environment such as soil, sewage and water. The occurrence of *Listeria* spp. in soil introduces products that play an important role and great risk for humans and animals. In Pakistan, 1,651 cases were reported from listeriosis and 292 deaths were reported from 2001-2011. Predominantly 58% cases were in adults aged group and 14% cases in pregnant women [16]. Pakistan has the third highest rate of infant mortality in the world and its control is one of sustainable development goal. Pregnant women, elderly persons, newborn, and immunocompromised person primarily affected by listeriosis [17]. The US Department of Agriculture and the Food and Drug Administration Center for Food Safety enforce a zero-tolerance policy for contamination of ready to eat foods with *Listeria*. These attempts led to 24% reduction in the incidence of invasive listeriosis and a 37% reduction associated to pregnant women infections, since 1996 [11].

The pathogenic species of *Listeria* are *L. monocytogenes* causes listeriosis and the outbreaks occurred during 2011 was caused by cantaloupes *L. monocytogenes* grown in Colorado. This outbreak sickened 147 people, 99% of the people affected were hospitalized and 33 were died [18]. Another outbreak of 224 was associated with listeriosis happens in different five multistate. The 14% cases were reported in residents of 38 states and total 58% cases with food borne listeriosis [16].

The consumption of vegetables acts the high risk of infection with *Listeria* spp. Szymczak *et al* inspected 1,000 soil samples originated from 15 different areas in which 210 samples of vegetable were collected. *L. monocytogenes* were detected in 15 % of potato samples [19]. 11 vegetable samples were contaminated with *Listeria* spp. in this study. In this study, a heat-treated product (Kabab and chicken liver) was not contaminated with *Listeria* spp. comparatively similar results by Kosek-Paszkowska *et al* (2005) that no strains of *Listeria* spp. was detected in any of 50 heat-treated products. It may consider that contamination with *Listeria* in cooked food items was lower [20].

CONCLUSION

In this study, 150 different food samples collected from the market of Havelian. Out of which 62 were vegetables, 7 poultry samples, 9 meat samples, 8 fish samples, 17 dairy products, and 47 bakery products. *Listeria* spp. is persisting in nature and has been isolated from different food source as it can survive in diverse conditions, withstanding high salt concentration and low temperature for long periods. From this study and results, it is concluded that *Listeria* spp. is present in high concentration in different samples of food that leads to higher the risk of developing listeriosis when consumed by humans. Newborn, pregnant women, elderly or immunocompromised individuals are at high risk. Preventive measures such as properly washed and cooked food should be used by consumers to control the foodborne illnesses.

High prevalence of *Listeria* in food needs to be controlled in order to lower the risk of listeriosis. So it is recommended to take necessary measures or to development of SOPs for food handlers, improved the GMPs and HACCP systems etc. It is also recommended Food and Health department may extend the knowledge of essential hygiene.

The officials may made teams to visit the local market and implement the rules, which are necessary for the control of food contamination and also spread awareness about listeriosis to conduct seminars. On priority basis the authorities should make routine schedule to collect food samples from the market to check the level of contamination and improve the food safety and quality.

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