

Original Research Article

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Occurrence of *Listeria* spp. in Ready to Eat Meat Products from Retail Shops of Punjab Region

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ABSTRACT

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In the present study, an attempt was made to detect potentially pathogenic *Listeria monocytogenes* along with other *Listeria* spp. From Ready to Eat meat products from retail markets of Punjab. A total of 200 samples of Ready to Eat meat products comprising 17 chicken seekh, 26 chicken nuggets, 52 chicken salami, 14 chicken sausage, 6 bacon, 7 pork sausage, 10 chicken garlic fingers, 1 chicken lollipop, 11 mutton shammi kebab, 10 chicken shammi kebab, 13 chicken Tikka, 1 mutton Tikka, 32 seekh kebab were processed. None of the sample found positive for *Listeria* spp. It was confirmed through biochemical tests as well as by molecular methods also. Absence of *L. monocytogenes* indicates good sanitation practices in food processing plants and ensures that the food is fit for consumer consumption in Punjab region.

Introduction

Listeria monocytogenes is a Gram positive, non sporulating, facultative intracellular exquisitely adaptable environment bacterium with powerful array of regulated virulence factors and it causes one of the serious worldwide food infections called Listeriosis. *Listeria monocytogenes* able to form biofilms on various surfaces and this ability is thought to contribute to persistence in the environment and on contact surfaces in the food industry (Zetzmann *et al.*, 2015). *L. monocytogenes* bacteria can sustain wide ranges of pH (4.0–9.5) and temperature (<1–

45 °C) and are able to survive in the presence of high salt concentrations (up to 10% NaCl) (Liu *et al.*, 2004). This makes the occurrence of *L. monocytogenes* in ready-to-eat (RTE) foods, such as cooked, raw-cured and dry-cured salted meat products, of particular concern.

Ready-to-eat (RTE) food products are those foods that do not require further heat treatment to significantly reduce the microbial load before consumption. *L. monocytogenes* has been isolated from a wide variety of ready-to-eat (RTE) foods and is responsible for several outbreaks of listeriosis linked to

the consumption of contaminated food stuff such as meat (chevon, mutton, poultry, pork, beef and carabeef) dairy product, processed meat products either raw or partially cooked and vegetables. Even when *L. monocytogenes* is initially present in small quantities in a foodstuff, it can multiply at varying rates during chilled storage depending on the type of food product, both under aerobic and anaerobic conditions, adapt to disinfectants and adhere to various surfaces. It is of major concern especially in the RTE meat and poultry processing industries as they are ubiquitous organisms and are able to multiply at refrigeration temperatures and under anaerobic conditions. It gets eliminated by the heating process but RTE products may become re-contaminated during production and post-processing steps such as slicing, peeling, and packaging as it is commonly found in the environment of processing plants.

Picture of RTE items bearing the risk of *L. monocytogenes* is also not cleared. The potential risk of *L. monocytogenes* in different foods has led to recalling of many products like RTE meat products, ice creams, vegetables etc worldwide which causes huge economic losses. Therefore, it is necessary to assess the prevalence of *L. monocytogenes* in RTE meat products because of the public health implications of potential spread of pathogenic *L. monocytogenes*. Moreover, in India, the occurrence of *Listeria* spp. among RTE meat products has not been studied to a large extent. It is in this context that this study was proposed with the following objectives:

To study the prevalence of *Listeria* species in ready to eat (RTE) meat samples from retail shops of Punjab, India.

Molecular characterization of isolated *Listeria* species from RTE meat samples.

Materials and Methods

Calculation of sample size

The selection of sample size was done according to Simple random sampling method, using the formula given below (Thrusfield, 2007).

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

where, n = sample size, P_{exp} = expected prevalence (as per the prevalence data available), d = desired absolute precision (d=0.05 at 95% level of confidence). *Listeria* spp. were isolated from 15.38% of sausages (Doijad *et al.*, 2010). P_{exp} will be 0.1538. Employing the above formula, n = 200.

Collection of samples

A total of 200 RTE meat samples were collected from retail shops of Punjab. Samples of different products were collected aseptically in sterile zip lock bags (Table 1). After labeling, samples were immediately transported to laboratory in ice box maintaining cold chain.

Enrichment of samples

RTE meat sample (25gms) was put in a sterile mortar to which 5-10 ml of autoclaved UVM 1 broth was added. Then with the help of sterile pestle, sample was triturated. After trituration, the entire mixture was transferred to a sterile flask and remaining volume of UVM 1 was added to the flask to make up the final volume to 225ml. The flask was incubated at 30°C for 24 hrs. After incubation, 1 ml of UVM-I was transferred to 9 ml of UVM 2 which was further incubated at 30°C for 24 hrs.

Isolation and identification

From UVM 2 a loopful of enriched sample was streaked directly onto selective medium polymixinacriflavin lithium chloride ceftazidineaes culinmannitol (PALCAM) agar plate (Hi Media Labs Pvt. Ltd, Mumbai, India) and incubated at 37°C for 24–48 hrs.

Confirmation of isolates by conventional techniques

The plates showing diffuse black zones of aesculin hydrolysis with typical green-yellow, glistening, iridescent, pointed colonies having 0.5 mm diameter were considered as presumptive *Listeria* spp. Then these colonies were subjected to Gram staining, catalase reaction, tumbling motility at 25°C, biochemical tests including methyl red-Voges Proskauer (MR-VP) reactions, nitrate reduction, sugar fermentation (rhamnose, xylose, mannitol, lactose, glucose and α -methyl- d-mannoside), and in-vitro pathogenicity tests such as phosphatidylinositol-specific phospholipase C (PI-PLC) activity on Agar *Listeria* according to Ottaviani and Agosti (ALOA), haemolysis on 7% sheep blood agar and CAMP test with *Staphylococcus aureus* and *Rhodococcus equi*.

Conventional PCR based detection of *Listeria* spp. and species *L. monocytogenes*

After performing conventional biochemical tests for detection of *Listeria* spp. and sugar fermentation, additional confirmation was done by molecular technique especially, PCR. The PCR reaction was carried out for amplification of *Listeria* targeting genus specific gene, putative phosphoribosyl pyrophosphate synthetase (*prs*) and *L. monocytogenes* specific, haemolysin (*hlyA*) gene. The PCR for amplification for *prs* and *hlyA* gene was carried out in a total reaction

volume of 25 μ l containing 12.5 μ l Master mix (EmeraldAmp®GT PCR Master Mix, Takara Bio Product), 0.5 μ l of 10 pmol/ μ l of primer set containing forward and reverse primer, 2 μ l of DNA template and rest sterilised nuclease free water to make up the reaction volume. The reaction mixture was vortexed and then flash spun in a micro centrifuge to settle the reaction mixture at the bottom. The DNA amplification reaction was performed in Mastercycler Gradient Thermocycler (Eppendorf, Germany) with pre-heated lid.

The cycling conditions for *prs* gene included an initial denaturation of DNA at 94°C for 1 minute followed by 35 cycles each of denaturation at 94°C for 30 seconds, annealing at 53°C for 45 seconds and extension at 72°C for 45 seconds followed by final extension at 72°C for 2 minutes and finally reaction hold at 4°C.

The cycling conditions for *hlyA* gene targeting PCR included an initial denaturation of DNA at 94°C for 2 minutes followed by 35 cycles each of denaturation at 94°C for 30 seconds, annealing at 53°C for 1 minute and extension at 72°C for 1 minute 30 seconds followed by final extension at 72°C for 10 minutes and finally reaction hold at 4°C. The standard strain of *L. monocytogenes* (ATCC 19115) was used as positive control, while nuclease free water as negative control. The amplified PCR products were analysed by using agarose gel electrophoresis and the bands in the gel were visualised by Gel Documentation System (Syngene, USA). The nucleotide sequences used in primer sets are listed in table 2.

Real Time PCR for detection of *Listeria* sp.

Real time PCR based detection of *Listeria* spp. and *L. monocytogenes* was done by targeting *prs* and *hlyA* gene respectively, the

primer sequence for the same is mentioned in table 2. The qPCR amplification was carried out in LightCycler 96 Roche (Germany). The reaction was carried out in 25 µl reaction mixture containing 12.5 µl of Power SYBR® Green PCR master mix (Thermo Fisher Scientific, UK), 1.0 µl of 10 pmol/µl of each primer set containing forward and reverse primers, 2 µl of DNA template and 8.5 µl sterilised nuclease free water to make up the reaction volume. After carefully adding the components, strips were briefly spun and loaded on to the Roche thermocycler.

The cycling conditions for qPCR included a single cycle of initial denaturation at 94°C for 5 minute followed by 40 cycles each of denaturation at 94°C for 30 seconds, annealing and extension at 60°C for 30 seconds. The final result of each reaction was expressed in threshold cycle (ct). The qPCR software (LightCycler® 96 SW 1.1) was used for data analysis. The qPCR monitored the fluorescence of reaction mixtures and recorded the cycle number at which

fluorescence crossed a specific threshold value and reached the exponential phase of amplification, which was designated as threshold cycle (Ct). The nucleotide sequences used in primer sets are listed in table 2.

Results and Discussion

A total of 200 samples of Ready to Eat meat products comprising 17 chicken seekh, 26 chicken nuggets, 52 chicken salami, 14 chicken sausage, 6 bacon, 7 pork sausage, 10 chicken garlic fingers, 1 chicken lollipop, 11 mutton shammi kebab, 10 chicken shammi kebab, 13 chicken Tikka, 1 mutton Tikka, 32 seekh kebab were processed.

The suspected colonies were subjected to biochemical tests, in-vitro pathogenicity tests and also they were subjected to qPCR and conventional PCR. None of the sample was found positive for *Listeria* spp. Therefore, zero percent prevalence of *L. monocytogenes* was found from ready to eat meat products.

Table.1 Collection of Ready to Eat meat products from Punjab

Sr. No.	Name of the sample	Number of samples
1	Chicken Seekh	17
2	Chicken nuggets	26
3	Chicken salami	52
4	Chicken sausage	14
5	Bacon	6
6	Pork sausage	7
7	Chicken Garlic Fingers	10
8	Chicken Lollipop	1
9	Mutton Shammi Kebab	11
10	Chicken Shammi Kebab	10
11	Chicken Tikka	13
12	Mutton Tikka	1
13	Seekh Kebab	32
Total		200

Table.2 Nucleotide sequences of primer sets used for detection of *Listeria* spp. *L. monocytogenes* in the study

Primer Name	Primer Sequence		Product Size	Reference
Prs-2-deg (<i>Listeria</i>. Spp) For qPCR	Forward	5'- ATTTTCTCGCTAAATTCTAATCGTG- 3'	60 bp	Barbau-Piednoir <i>et al</i> (2013)
	Reverse	5'- CAATACCWACTTCTTTTCGCAATCTC- 3'		
<i>hlyA</i>-146-deg-tronc (<i>L. monocytogenes</i>) For qPCR	Forward	5' - AAATCTGTCTCAGGYGATGT-3'	103 bp	Hough <i>et al</i> (2002)
	Reverse	5'- CGATGATTTGAACTTCATCTTTTGC-3'		
<i>Prs</i> (<i>Listeria</i> spp.)for conventional PCR	Forward	5' - GCTGAAGAGATTGCGAAAGAAG-3'	370 bp	Doumithet <i>al.</i> , 2004
	Reverse	5' - CAAAGAAACCTTGGATTTGCGG-3'		
<i>hlyA</i> (<i>L.</i> <i>monocytogenes</i>) for conventional PCR	Forward	5' - GCAGTTGCAAGCGCTTGGAGTGAA-3'	4 56 bp	Pazaik -Domanskaet <i>al.</i> (1999)
	Reverse	5' - GCAACGTATCCTCCAGAGTGATCG-3'		

In India, Doijadet *al* (2010) conducted study on 52 pork sausages and found that 15.38% samples were contaminated with *Listeria* spp. Results of our study were similar to Abdelgadir *et al.*, (2009) in which prevalence of meat was 1.2%, and prevalence of sausages and frankfurters was only 0.4% only. Osaili *et al.*, (2014) revealed that overall prevalence of *L. monocytogenes* harboured from RTE chicken and RTE beef products was found to be 2%, with 2.7% and 1.5% prevalence in RTE chicken and beef products, respectively.

In contrast to our study, some of the researchers found that prevalence of *L. monocytogenes* in RTE meats were quiet high. Osaili *et al.*, (2010) found 50% of the samples of RTE meat products were contaminated with *Listeria* spp. Whereas, Alsheikh *et al.*, (2012) revealed that 38% of samples of retail ready to eat chicken products were contaminated with *Listeria* spp. A study was conducted in which effects of lactate and acetate were studied and they concluded that presence of lactate and acetate in RTE meat products were associated with low occurrence

of *L. monocytogenes* in RTE meats (Ahmed *et al.*, 2015). Essential oils of various spices used in the preparation of ready to eat foods are having antimicrobial roles. A research on carvacrol and nisinstated that they act in synergism in killing *L. monocytogenes* through various pathways (Charklam *et al.*,). It was also found that essential oils of conifers were having anti-listerial properties (Mourey *et al.*, 2020). Moreover, hygiene and sanitation also plays a major role in RTE meat products contamination during post processing including packaging, handling or also due to collective handling of raw as well as processed foods on the same table.

Good Sanitation and hygiene practices followed in the processing and manufacturing unit also play a vital role in the reduction of the microbial load in the final product.

In conclusion, *listeria* spp. could not be detected in RTE meat products, indicating that such products undergo hygienic processing and are fit for consumption in Punjab region of India.

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