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Prevalence, Detection and Identification of *Listeria monocytogenes* in Retail Chicken Meat in Ludhiana, India by Employing conventional Isolation Techniques and Molecular Polymerase Chain Reaction (PCR) Assay

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A B S T R A C T

Listeria monocytogenes is one of the major food contaminants with potential to cause lethal food poisoning in both humans and animals. Among different food borne pathogens, *Listeria monocytogenes* has a high mortality rate and is therefore considered one of the most dangerous foodborne pathogen. The present study was aimed at finding the prevalence of *Listeria monocytogenes* in raw chicken meat purchased from different retail outlets and local butcher shops across the Ludhiana city. In the present study a total of 100 raw chicken meat samples were collected (80 fresh raw samples and 20 frozen chicken meat products). During this study, 100 chicken meat samples were inoculated in PALCAM selective agar for the selective isolation of *L. monocytogenes* and were later characterized by a combination of microscopic and biochemicaltests. Results of the study revealed that02 samples were containing *L. monocytogenes*, which is 02% of the total samples. These positive samples were subjected to molecular characterization using standard PCR technique to attest their presence in these meat samples. The PCR technique was found to be more specific/sensitive, reliable, precise and rapid technique to supplement the conventional methods of diagnosis of the *L. monocytogenes*.

Introduction

The genus Listeria includes Gram-positive, non-spore forming, catalase-positive rod-shaped bacteria, which were once classified into the family Corynebacteriaceae. Listeria species appear as small rods ranging in size from 0.4 to 0.5 by 1-2µm and sometimes are found to be arranged in short chains when viewed under the microscope. The growth of the organism on bacteriological media is enhanced by the presence of glucose or other fermentable sugars but is also dependent on the atmosphere and temperature in which they are grown. The organism can grow over a wide range of pH (4.3- 9.6), water activity (~

0.83) and salt concentrations (up to 10 %) as well. *Listeria* spp. is aerobic, microaerophilic and facultatively anaerobic and can be cultured over a wide temperature range. The organism has a growth temperature range of approximately 1°C - 45°C, making it a psychrotroph and a mesophile. There are, however, temperature-dependent growth factors.

The peritrichous flagella are formed at 20-25°C and cause the organism to be motile, whereas at 37°C the organism is weak or non-motile. Additionally, its ability to not only survive but to grow as a psychrotroph at 4°C makes this pathogen unique from other commonly found food-borne pathogens which are usually inhibited from growth at refrigeration temperatures. A coccoid appearance may be seen in direct smears. *Listeria* spp. produces flagella at room temperature and exhibits a tumbling motion when examined in broth and swarming motility can be observed in semi-soft agar at 30^{o}C. The wide distribution of *L. monocytogenes* in nature allows this bacterium to be easily spread and cause infection. *Listeria monocytogenes* can cause infection by several transmission routes such as ingestion of contaminated foods (e.g. unpasteurized milk or contaminated ready-to-eat foods). Many foods such as soft cheeses, hot dogs, and seafood have been implicated in listeriosis outbreaks, but *L. monocytogenes* also can be isolated from other foods such as beef, pork, fermented sausages, fresh produce, and fish products.

Materials and Methods

The predominant analysis of laboratory work was done at the Department of Veterinary Pathology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, from the meat samples collected.

Collection and processing of Samples

A total of 100 samples of poultry meat (80 raw chicken meat and 20 frozen meat) samples were collected from different retail shops in the vicinity of Ludhiana. About 100 grams of meat samples (muscle, liver, gizzard, kidney and heart) were collected in dry, clean and sterile polythene bags and transported to the laboratory for microbiological analysis within one hour of collection or refrigerated at 4°C till further analysis. These samples were then processed no later than 24 hours after collection. These samples were then swabbed with sterile cotton swabs and inoculated into the Brain Heart Infusion broth (BHI) and then incubated overnight at 37°C. After 18-24 hrs, the swabs from BHI broth were streaked onto the different media plates like Brain Heart Infusion Agar (BHI), PALCAM Selective Agar (PSA) for isolation of *Listeria* spp.

Identification of bacterial isolates

The bacterial colonies were isolated after incubation. These colonies were subjected to Gram's staining for identification and requisite biochemical tests were carried out to further confirm the presence of the pathogen. The final confirmation of the organism was done by using molecular techniques like PCR.

Biochemical characterization

L.monocytogenes suspected colonies were subjected to various biochemical tests like the Catalase test and *L.monocytogenes* identification kit (HIMEDIA) for Voges Proskauer, Catalase, Esculin hydrolysis, Nitrate reduction, Methyl red and various carbohydrate utilization tests including Glucose, Mannitol, Sucrose, Lactose, Rhamnose, Xylose, and a-Methyl-D mannoside tests.

Molecular characterization

The DNA was extracted from suspected colonies and tissues using Himedia DNA extraction kits. The extracted DNA was subjected to PCR for the detection of bacterial DNA in the samples using published primers and probes.

Polymerase Chain Reaction (PCR)

The DNA extracted was subjected to polymerase chain reaction using specific primers for *L.monocytogenes*. The 25 µl reaction mixture for PCR was prepared that consisted of 13 µl Mastermix (Promega), 1 µl each of 20 pmol/µl Forward primer and Reverse primer, 5 µl of DNA template and 5 µl of Nuclease free water. PCR was performed on C1000 touch thermocycler (Bio-Rad, USA) with the following conditions; an initial denaturation at 95^{o}C for 5 minutes and later 35 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 55^{o}C for 1 minute and extension at 72^{o}C for 1 minute. The final extension followed at 72^{o}C for 10 minutes. The PCR products were run on 1.5% agarose along with 50 bp DNA molecular weight marker (New England Biolabs, USA) at 5V/cm and visualized using a gel documentation system (AlphaImager, Alpha Innotech, USA).

Results and Discussion

Out of total 100 meat samples, 80 raw and 20 frozen meat product samples examined for the presence of bacterial pathogens. The *Listeria monocytogenes* was isolated from total 02 fresh samples i.e. 02% (Table 1) which were Catalase positive and later confirmed by PCR detection at 64 bp. The findings of the present study are in line with the observation of Kalorey *et al.,* (2005) who reported using a conventional culture and biochemical tests, a total of 08(8.5%) of all investigated samples being Listeria positive.The samples following the standard protocol were streaked on PALCAM Selective Agar (PSA) (Chapman, 1945) for selective culture of *L. monocytogenes* and black colonies surrounded with black zone in the media were obtained. The isolation results for *L. monocytogenes* are in concurrence with the findings of other workers.Franco *et al.,* (1995) used conventional culture methods and techniques to report the presence of *Listeria* spp. in chicken drumsticks, wings, breasts, and livers taken from a poultry processing plant to be 96% positive. Mahmood *et al.,* (2003) in a study performed on 320 samples of raw and frozen poultry meat and meat products found the prevalence of *Listeria* spp. to be ranging from 10 to 37.5%. Kalorey *et al.,* (2005) applied CAMP test and other culture characteristics to report 8.5% isolation of *Listeria* spp. out of the 94 samples examined. Reiter *et al.,* (2005) in a study used the automated mini-VIDAS system (Enzyme Linked Fluorescent Assay) to detect the presence of *L. monocytogenes* on the raw and frozen meat samples. *L. monocytogenes* were found in 35.6% of the 645 analyzed samples, respectively. Chemaly *et al.,* (2008) undertook a study involving two hundred laying-hen flocks and reported an estimated prevalence of 15.5% in laying-hen flocks. They also used the simple isolation techniques for the confirmation of the prevalence. Alsheikh *et al.,* (2013) employed biochemical tests as per conventional International Organization for Standardization methods for studying the prevalence of *Listeria* spp. on 250 broiler chickens and ready to eat meat products. *L. monocytogenes* was isolated to the tune of 13.6% besides other *Listeria* spp. viz. 20.8% for *L. ivanovi* and to minimal levels of 0.8% for *L. seeligeri*. Dahshan *et al.,* (2016) collected a total of 200 poultry farm samples and species wise isolated the organism using standard isolation methods wherein various species of listeria

were isolated and of which *L. monocytogenes* accounted for meager 1%. Maung *et al.*, (2019) collected a total of 85 and 50 chicken meat samples, including different body parts from different supermarkets in Fukuoka (Japan) in 2012 and 2017, respectively. Detection, isolation, identification, and characterization of *L. monocytogenes* were performed according to the conventional methods. Forty-five among 85 samples (53%) were positive for *L. monocytogenes* in 2012, while 12 among 50 samples in 2017 (24%) tested positive.

Table.1 Comparison of detection of *Listeria monocytogenes* in meat samples using various techniques

Techniques	Total fresh meat samples (80)	
	***Listeria* spp.**	**%**
Isolation	02	2.5
PCR	02	2.5
Total	**02**	**2.5**

Fig.1 Growth of *L. monocytogenes* on Palcam Selective Agar (PSA) medium with colonies appearing black with a black zone in surrounding medium

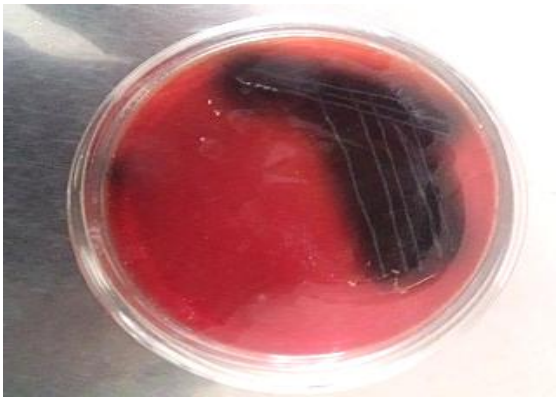


Fig.2 *Listeria* spp. from culture seen as numerous Gram positive rods. Gram's stain. 100X

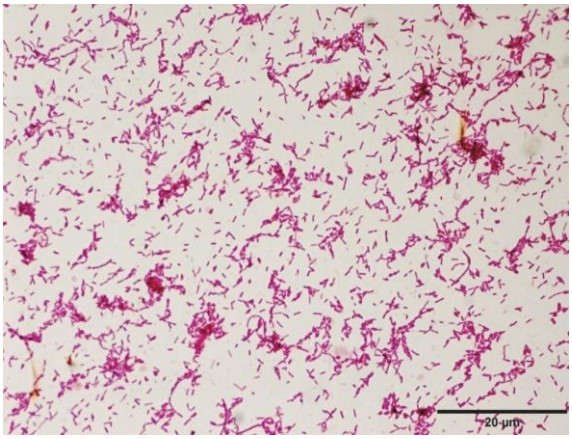


Fig.3 Catalase test for *L. monocytogenes*: Catalase test showing positive frothy effervescence

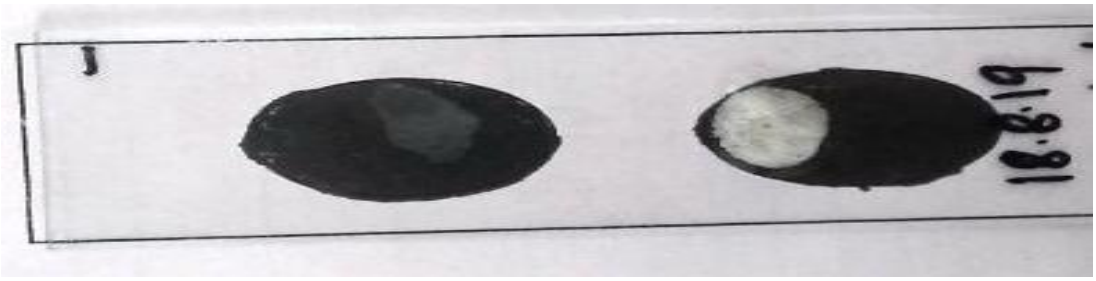


Fig.4 Biochemical test for *Listeria spp.* using Listeria identification kit by HiMedia

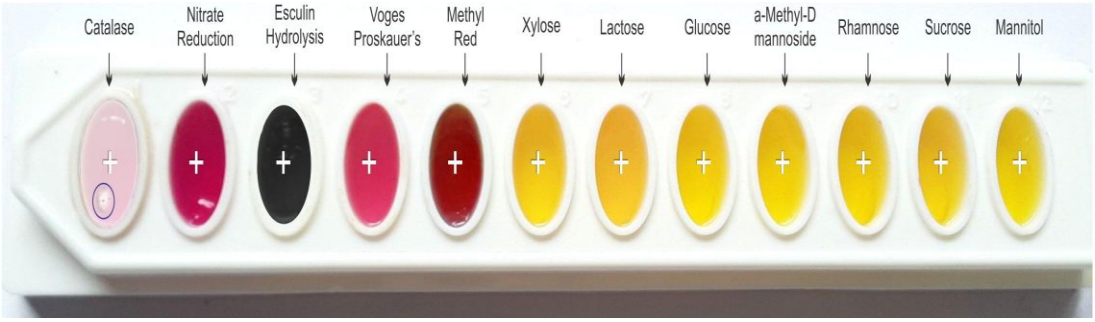
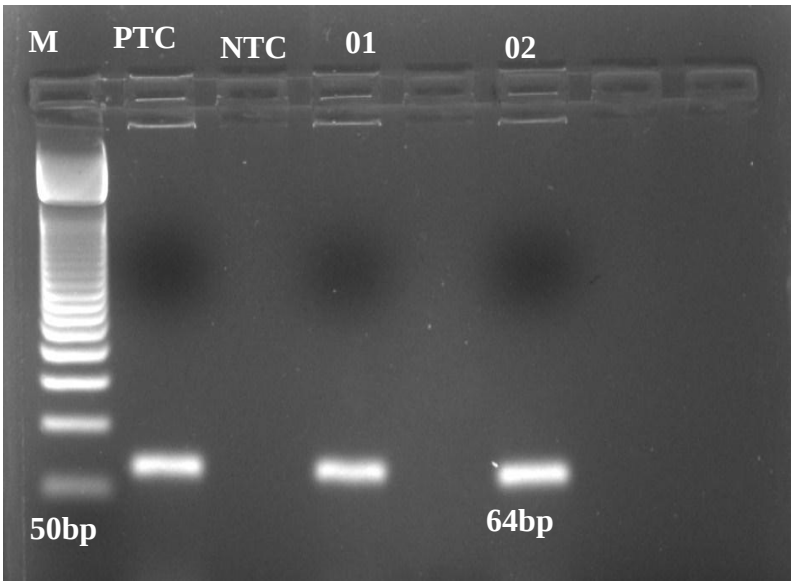


Fig.5 Molecular identification of *L. monocytogenes* at 64 bp targeting *hly-A* gene. M=50bp DNA ladder; L1 to L2= test samples showing distinct bands at 64bp; PTC= Positive template control; NTC= Negative template control



The colonies picked from PALCAM Selective Agar (PSA) were subjected to Catalase test which showed positive reactivity (Foster, 1996).The *L. monocytogenes* organisms exhibited greyish/black colonies with peripheral black zones when grown on Oxford and PALCAM agar, a selective media for their growth (Fig.1). Furthermore, the

Gram's staining performed on isolated colonies revealed numerous Gram positive rods(Fig.2).Catalase test for the bacteria showed frothy effervescence when positive culture of the bacteria was inoculated with H_2O_2 (Fig.3).Furthermore, biochemical test kit (Himedia) was used in the study for confirming the presence of *Listeria spp.*(Fig.4) with the help of 12 tests for identification of *S. aureus* namely Catalase, Nitrate Reduction, Esculin hydrolysis, Voges Proskauer's, Methyl red, Xylose, Lactose, Glucose, a-Methyl-D mannoside, Rhamnose, Sucrose, Mannitol. The results from the kit confirmed the presence of *Listeria* spp. The findings of the present study are in consonance with the observations of Ennaji *et al.,* (2008) in a study conducted at Casablanca, Morocco on the chicken meat samples (74) sold in supermarkets. In their study they found only 1 positive sample for *Listeria monocytogenes* with overall prevalence of 1.3%. Vasu *et al.,* (2014) who used biochemical tests on a total of 100 surface swabs (table tops and knives) from meat processing facilities and retail markets in Kerala and found 3% prevalence of *Listeria* spp.

Ahmed *et al.,* (2017) used common biochemical tests to detect the Listeria organism in chicken meat and reported that 04 samples out of 50 samples were positive for the presence of *L. monocytogenes*. In another similar finding Kureljušić *et al.,* (2017) conducted a six month study in republic of Serbia by using standard biochemical tests to report 3% prevalence of *Listeria* spp. in poultry meat samples.Soleimani *et al.,* (2019) by employing preferential selective culture media and various biochemical tests isolated *Listeria monocytogenes* which were later attested by PCR assay. Of the 247 samples 27% samples revealed *L. monocytogenes* besides other.

Standard PCR (Fig.5) was run to establish the presence of the pathogen and check the efficacy and priming conditions related to the primers and master mix used in the study. PCR was done to confirm the presence of *Listeria* spp. by targeting *hly-A* gene at 64bp using published primers (Lazaro *et al.,* 2004).

Osaili *et al.,* (2011) on a study done on 280 samples found 50% of samples contaminated with *Listeria* spp. of which *L. monocytogenes* accounted for 18.2% based on the routine conventional methods and supported by Polymerase chain reaction (PCR). Zeinali *et al.,* (2017) examined the chicken meat sold at different supermarkets by collecting 200 random fresh chicken carcasses and subjected them to isolation of *Listeria* spp. 40% of the samples did reveal *Listeria* spp. of which 18% was attributed to *Listeria monocytogenes.* This was further evidenced by use of multiplex PCR assay.

In conclusion the poultry meat sector aims at providing long shelf life ready to eat and other meat products, which are safe for human consumption but various biological hazards have been associated with poultry meat production and consumption. *Listeria*spp. has been ranked as one of the high risk pathogenin contaminated meat due to the severity of the illnessit causes and its impact on human health. The results of this study have confirmed that contamination of *L. monocytogenes* occurs due to insufficient hygiene and that there may be a serious risk in raw poultry meat for consumer health in India, because of the detection of *L. monocytogenes* in the samples. Therefore, the combination of high throughput detection methods with highly selective cultural methods and rapid, reliable and sensitive molecular techniques like PCR will be needed to identify the sources of meat contaminants and their dynamics during processing and storage.

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