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Application of MALDI-TOF Mass Spectrometry for the Assessment of Prevalence of *Listeria monocytogenes* in Raw Milk, Dairy Products and Freshwater Fishes

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ABSTRACT

Background: Foodborne infections like Listeriosis cover several disorders and are a worldwide public health emergency. *Listeria monocytogenes* has been isolated from various foodstuffs, including milk and fishes. MALDI-TOF mass spectrometry technique is known for its rapid and accurate identification of bacterial organisms.

Methods: In the current research, a total of 360 samples comprising raw milk (130), milk products (125) and freshwater fishes (105) were screened for the detection of pathogenic *Listeria* species by using the USDA method. The recovered *Listeria* isolates were characterized using conventional set of biochemical analysis along with sugar fermentation tests and further confirmed by MALDI-TOF MS. The virulent nature of pathogenic *Listeria* isolates was also assessed by *in vitro* tests like hemolysis on blood agar, CAMP and PI-PLC assay.

Result: In current study, on screening 360 animal origin food samples, three isolates were recovered from raw milk samples and identified as *Listeria monocytogenes* indicating an overall prevalence of *Listeria monocytogenes* to the tune of 0.83%. Excellent correlation was observed with identification of *Listeria* species using conventional phenotypic tests and advanced molecular tool Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) technique. The results depict dependability of advanced technique for rapid and reliable identification of *Listeria* species.

Key words: CAMP, *Listeria monocytogenes*, MALDI-TOF MS, PI-PLC assay.

INTRODUCTION

Listeriosis is an important foodborne disease in humans because it is associated with the ingestion of contaminated food and water with pathogenic *Listeria* spp. (Low and Donachie, 1997). It leads to severe invasive illness in humans; the main signs are septicemia, abortion, stillbirth, perinatal infections, meningitis, gastroenteritis and meningoencephalitis, particularly in aged and immuno compromised individuals (Posfay-Barbe and Wald, 2004). Pregnant women are more vulnerable to infection than non-pregnant women, which can result in abortion, stillbirth, or perinatal illnesses (Jackson *et al*., 2010). The incidence of listeriosis caused by this bacterium has skyrocketed in recent years. Most human listeriosis occurs when contamination levels of 102-106 CFUs/ml or/g of *Listeria* are present in food (Dawson *et al*., 2006). *Listeria monocytogenes* has been isolated from various foodstuffs, including milk (Barbuddhe *et al*., 2002). Several incidences of foodborne listeriosis have been reported caused by consuming contaminated meat (Lunden *et al*., 2003; Bhandare *et al*., 2007). Matrix-assisted laser desorption/ ionization-time of flight (MALDI-TOF) mass spectrometry (MS) is a single identification and source-tracking tool for *L. monocytogenes* (Jadhav *et al*., 2015). This technique examines the chemistry of major proteins, yielding profile spectra consisting of a series of peaks, a characteristic "fingerprint" mainly derived from ribosomal proteins and the fingerprinting has the potential for bacteria identification and subtyping (Barbuddhe *et al*., 2008). It has become the method of choice for bacterial species identification in clinical diagnostics due to its little hands-on and turnaround time, low costs and high accuracy (Angeletti and Ciccozzi, 2019; Rodrìguez-Saìnchez *et al*., 2019). The present study was

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planned to explore the prevalence of pathogenic *Listeria* species in animal origin foods like raw milk and freshwater fishes using conventional as well as advanced molecular tool like MALDI-TOF MS for the confirmation of reliable and rapid identification of organism.

MATERIALS AND METHODS

Sample collection

In current research work, a total of 360 samples comprising raw milk (130), milk products (125) and freshwater fishes (105) were collected from Udgir city of Maharashtra and screened for microbiological evaluation during the period of 2017-18 at College of Veterinary and Animal Sciences, Udgir. Milk products and fish samples were collected in a sterile zip lock bags while, raw milk samples were collected in sterile milk sampling bottles (35 ml, International Scientific Supplies Ltd., UK).

Bacterial strains

The standard strains of *L. monocytogenes* (ATCC 19115), *Staphylococcus aureus* (ATCC 12600) and *Rhodococcus equi* (ATCC 6939) were used in the present study which were obtained from Himedia, Mumbai.

Isolation and phenotypic characterization of *Listeria* species

The samples were collected aseptically and processed immediately after collection, for the isolation of *Listeria* species as per the protocol suggested by the USDA method as described by Curtis and Lee (1995) with suitable modifications, which includes two-step enrichment with the University of Vermont (UVM-I and II) and subsequent streaking onto polymyxin-Acriflavin-Lithium chloride Ceftazidime Aesculin-Mannitol (PALCAM) medium as a selective agar. Phenotypically, isolates were characterized by employing battery of biochemical and sugar fermentation tests. The results were validated along with standard strain of *L. monocytogenes* (ATCC 19115). Biochemical testing comprised catalase, oxidase Methyl Red-Voges Proskauer (MR-VP) and nitrate reduction tests, while sugar fermentation tests were carried out with Alpha-Methyl-D-Mannoside, Rhamnose and Glucose (Dextrose).

In vitro pathogenicity testing

In order to assess the pathogenic potential of recovered isolates of *L. monocytogenes* phenotypically, the isolates were subjected to *in vitro* pathogenicity tests like hemolysis on 7% sheep blood agar (SBA) (Courtieu, 1991), Christie, Atkins, Munch-Petersen (CAMP) Test (Christie *et al*., 1944) and Phosphatidylinositol-specific Phospholipase-C (PI-PLC) assay (Notermans *et al*., 1991).

Confirmation of isolates by MALDI-TOF-MS

The ionization method known as matrix-assisted laser desorption ionisation (MALDI) produces ions from big molecules with the least amount of fragmentation. (Hillenkamp *et al*., 1991). The *L. monocytogenes* isolates recovered in this study were also subjected to advanced molecular technique like MALDI-TOF-MS for species level confirmation. In this investigation, the samples were prepared by full protein extraction and processed as per the directions provided by Bruker Daltonics. The mass spectra were acquired and analyzed using MALDI Biotyper automation control and the Bruker Biotyper 2.0 software and library (version 2.0, 3,740 entries; Bruker Daltonics). Using the manufacturer's suggested bacterial test standard and following the manufacturer's instructions, calibration was completed. Identification score criteria were performed as recommended by Bruker Daltonics, which evaluated as per the protocol described by Shell *et al*., (2017). A score of 2.000 indicated species-level identification, a score of 1.700-1.999 indicated identification to the genus level and a score of 1.700 was interpreted as no identification.

RESULTS AND DISCUSSION

Prevalence of *Listeria monocytogenes* in animal origin foods

In this study, on microbiological analysis of 360 food samples comprising raw milk, milk products and freshwater fishes, three presumptive *Listeria* isolates were recovered and identified as *Listeria monocytogenes* (all from raw milk) giving an overall occurrence of *Listeria monocytogenes* to the tune of 0.83%. The other samples comprising milk products and freshwater fishes showed negativity for the presence of any of the *Listeria* species. The low prevalence of *Listeria* spp. in milk detected in the present research is in agreement with the results stated by Kalorey *et al*. (2008) with a 0.1% (2/2060) prevalence and with Aurora *et al*. (2006) who noted 1.69% prevalence of *Listeria* species in milk samples. The results also coincide with Sharma *et al*. (2017), Khan *et al*. (2013), Shakuntala *et al*. (2019) and Shantha and Gopal (2014), who reported 0.8%, 1.7%, 0.76% and 1.69% prevalence of listerial organisms in milk respectively. However, certain studies have reported quite a higher recovery of *Listeria* spp. in milk which includes Mary and Shrinithivihahshini (2017) with 52.7% (219/415) and Gebretsadik *et al*. (2011) with 22% (22/100) prevalence. *Listeria* spp. typically affects raw milk by contamination caused by unsanitary conditions in the environment, gastrointestinal tract and teat skin of animals. Besides, other factors like lack of hygiene, environmental contamination and poor milking practices also contribute to the *Listeria* contamination.

Biochemical characterization and *in vitro* pathogenicity testing of *Listeria* isolates

On analysis, three presumptive isolates showing the typical greyish green, glistening, iridescent and pointed colonies of about 0.5 mm diameter surrounded by a diffuse black zone of aesculin hydrolysis (Curtis and Lee, 1995) also revealed characteristics of Gram-positive coccobacilli morphology detected under the microscope on Gram staining, tumbling motility in hanging drop technique as described by Islam *et al*. (2016) and positivity towards

Table 1: MALDI-TOF MS analysis of *Listeria* isolates.

Source	MALDI-TOF MS Result	Strain	Score value
Raw milk	*L. monocytogenes*	*L. monocytogenes* CCUG 59664	2.246
Raw milk	*L. monocytogenes*	*L. Monocytogenes* CCUG 53269	2.308
Raw milk	*L. monocytogenes*	*L. monocytogenes* CCUG 33548	2.216

catalase, MR-VP and nitrate reduction test, while negativity to oxidase test. On sugar fermentation tests, isolates fermented only the Alpha-Methyl-D-Mannoside, Rhamnose, Glucose (Dextrose) sugars, suggestive of species confirmation of all three isolates as *Listeria monocytogenes* (OIE Terrestrial Manual 2021; Nayak *et al*., 2015). All three *L. monocytogenes* isolates were further tested for virulence character by *in-vitro* pathogenicity testing such as haemolysis on 7% sheep blood agar, CAMP and PI-PLC assay. All isolates showed characteristic beta (β) haemolysis on 7% sheep blood agar, CAMP positive reaction against *S. aureus* and typical blue-green colonies with a clearly defined opaque halo on ALOA (Agar *Listeria* Ottavani and Agosti) medium in PI-PLC assay, revealing their pathogenic nature.

Confirmation of isolates by MALDI-TOF-MS

In present investigation, three *Listeria monocytogenes* isolates recovered from milk samples were characterized using conventionally with battery of biochemical tests and sugar fermentation tests. These isolates were further subjected for confirmation by MALDI-TOF MS. On the basis of the score obtained and species identified by MALDI-TOF MS, all three isolates were designated as *Listeria monocytogenes* as shown in Table 1 and analyte ID 34, 35 and 36 in supplementary materials. The results obtained in molecular charecterization by MALDI-TOF MS were in complete agreement with the results of phenotypic biochemical characterization. This concurrence resembles with the study reported by Thouvenot *et al*. (2018), wherein researchers carried out a validation study of similar Bruker Daltonics system of MALDI-TOF MS Biotyper with phenotypic based identification of *Listeria* species. The authors further also confirmed its accuracy using average nucleotide identification identity BLAST (ANIb) of whole genome sequences of organisms. There are other several studies reported depicting use of MALDI-TOF MS for the identification of *Listeria* (Ojima-Kato *et al*., 2016; Jadhav *et al*., 2015; Hsueh *et al*., 2014; Rychert *et al*., 2013; Barbuddhe *et al*., 2008). However, The MALDI-TOF MS system from Bruker Daltonics (Bremen, Germany) has been successfully employed, albeit only on a small number of phenotypically characterized strains and to create mass spectral fingerprints of some reference strains (Barbuddhe *et al*., 2008; Hsueh *et al*., 2014). It depends on the MS database, which captures even single amino acid substitutions, to provide strain- or serotype-level microbial classification at a better resolution than that of conventional fingerprinting analysis, moreover we frequently observe small variations in MS peaks made up of the same proteins in closely related bacteria (Tamura *et al*., 2013; Ojima-Kato *et al*., 2015). The target bacteria's culture and/or growing conditions may still have a significant impact on fingerprinting analysis, which suggests that genetic sequence analysis be done to support the prototyping data for accurate identification (Wieme *et al*., 2014). The present results of MALDI-TOF MS for bacterial identification were accurate, with good repeatability and less time consumption (Seng *et al*., 2009; Valentine *et al*., 2005; van Baar, 2000). MALDI-TOF MS provided no identification in mixed cultures but reported identification in single major bacterial cultures (Croxatto *et al*., 2012).

CONCLUSION

To summarize, in current research, the prevalence of pathogenic *Listeria* species in animal origin foods like raw milk, milk products and freshwater fishes was noted to the tune of 0.83%. Besides, all the isolates examined for confirmation by MALDI-TOF MS, lead to the resemblance of results obtained by biochemical characterization designating all three isolates as *Listeria monocytogenes* suggesting the excellent utility of techniques like MALDI-TOF MS for rapid detection of significant bacterial pathogens. This study illustrates the entire dependability of MALDI-TOF mass spectrometry as a quick method for identifying *Listeria* species that are pathogenic, with 100% accuracy. However, the challenges like incomplete databases, close relatedness of species of interest and quality of spectra needed to be addressed.

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Conflict of interest

Authors declare that there is no conflict of interest.

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