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A STUDY ON ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF *LISTERIA MONOCYTOGENES* FROM READY-TO-EAT FOOD PRODUCTS

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Abstract

Ready-to-eat foods are those that do not require preparation by heat employment before being ingested by the consumer. The present study focused to assess the occurrence of *L. monocytogenes* in ready-to-eat food products available in the local markets in Chikkaballapura, Karnataka, and to improve the basic knowledge of the incidence and characteristics of *L. monocytogenes*. Samples comprising of locally prepared and sold ready-to-eat packed food products being consumed by Childrens were collected and subjected to serial dilution and inoculated to nutrient agar medium, Palcam selective agar base medium and Tryptic soy broth. The growth with the typical colony characteristics were further identified up to species level based on their morphological and biochemical characteristics. The bacteria isolated from different ready-to-eat food products was found to be gram-positive bacilli, motile. The results of biochemical assay of bacteria isolated from different ready-to-eat food products showed positive for catalase, oxidase, methyl red, Voges Proskauer, and CAMP tests. Whereas, isolated strains were negative for indole and citrate utilization tests. Based on the morphological and biochemical characterization, isolated strain was identified as *L. monocytogenes.* In conclusion, results of the presented study suggested that *L. monocytogenes* is prevalent in ready-to-eat food products and sheds new light on the growth of food pathogens and spoilage microbes in a variety of ready-to-eat food products. These findings could be considered in estimating the risks associated with the production and consumption of ready-to-eat food products.

Keywords: Ready-to-eat, Food products, *L. monocytogenes*, Pathogenic, Listeriosis

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Introduction

Listeriosis has been recognized in the 1920s as an infection caused by a Gram-positive bacterium later called *Listeria monocytogenes*. In 1981, an outbreak in Canada was first associated with the presence of this pathogen in foods, encouraging the development of research on the ubiquitous features of the microorganism and its pathogenicity.[1] Since then, it is considered an important bacterium in public health, responsible for foodborne disease that despite the low incidence, has a high mortality rate among affected individuals.

L. monocytogenes is extensively distributed in a wide range of agricultural environment such as soil, manure, and water.[2] This pathogen is generally transmitted to human through contaminated food and water. It is found as a saprotroph in soil, but it can transform into a pathogenic form when enters into the animal or human cell.[3] One of the possible sources of contamination of *L. monocytogenes* in food products and food industry is due to the cross contamination. *L. monocytogenes* is one of very few pathogenic organisms which can grow at refrigerated temperatures. The bacterium is widespread in nature and can survive and grow under low temperatures and pH, high concentrations of salt or bile, oxidative stress, carbon starvation, and other adverse conditions making it a potential hazard in foods.[4]

Listeriosis in human is very often associated with very high mortality which may be as high as 30%.[5] In USA, approximately 1,591 cases of listeriosis in human have been reported in a year and it contributes to about 0.1% of total food-borne illnesses, but is responsible for 2.6% of hospitalizations and 18.87% of the deaths caused by food-borne illnesses.[6] Both the outbreaks and sporadic human listeriosis is caused by transmission of this pathogen through contaminated food and the involvement of a wide variety of foods has been reported throughout the world.[7,8] In USA, the recent multistate outbreak of *L. monocytogenes* apparently through consumption of contaminated cantaloupe has drawn worldwide attention. A total of 146 persons were infected and 33 deaths were reported in this outbreak in which multiple states of USA were affected.[9]

Considering its immense pathogenic potentiality and high mortality rate, different regulatory agencies including USFDA have imposed zero tolerance for this pathogen in ready-to-eat food products.[10] The most widely used techniques for identifying *L. monocytogenes* are the conventional methods, which depend on the use of microbiological media to cultivate and count this pathogen in a targeted manner. These techniques yield both qualitative and quantitative results, are affordable, and are sensitive. There have also been reports of clinical cases of listeriosis in humans brought on by *L. monocytogenes* from Delhi and Karnataka states of India.[11,12]

Ready to eat foods are those that do not require preparation by heat employment before being ingested by the consumer.[1,13,14] According to Food and Agricultural Organization (FAO) guidelines,[15] a modern food safety system is proactive and based on risk. Therefore, it is necessary to identify the diseases that affect the population and the presence of pathogens in food to then establish risk mitigation measures. With this context, the present study has been undertaken to assess the occurrence of *L. monocytogenes* in ready-to-eat food products available

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in the local markets in Chikkaballapura, Karnataka, and to improve the basic knowledge of the incidence and characteristics of *L. monocytogenes*.

Materials and Methods

Sample Collection

Different types of locally sold ready-to-eat packed food products being consumed by Childrens were procured from different market places of Chikkaballapura, and immediately samples were brought to the laboratory and processed further within 3-4 hours of collection.

Isolation and Identification

Serial dilution involves the process of taking one mL of sample and diluting it through a series of standard volumes of sterile diluent, which can either be 9 mL of distilled water or 0.9% saline. Depending on the estimated concentration of cells / organisms in a sample, the extent of dilution is determined. 0.1mL of sample was taken from the dilution like 10^4 to 10^6 inoculated on to the respective plates by spread and streak plate method. Different medias such as Nutrient agar media, Selective Palcam agar media, oxford and tryptic soy broth was used to isolate the bacteria. After incubation on respective dilution different bacterial colonies were observed.

Morphological Characterization

Macroscopic and Microscopic

Macroscopic analysis involved the observation of physical appearance of the incubated colonies *viz.* size, shape, color and texture of microorganisms by naked eyes. Microscopic observation involves the observation of shape (Bacilli, Cocci), size, color, and texture of bacteria after staining.

Gram's staining reactivity

Morphological characterization of the potential isolates involved use of Gram's staining. The cells were studied based on their size, shape, arrangement and Gram's staining reactivity. A smear of the selected strains were prepared on a clean glass slide and the smear was allowed to air-dry and then heat fixed. The heat-fixed smear was flooded with crystal violet and after one minute, it was washed with water and flooded with mordant Gram's iodine. The smear was decolorized with 95% ethyl alcohol, washed with water and then counter-stained with safranin for 45 seconds. After washing with water, the smear was dried with tissue paper and examined under oil immersion under microscope.

Motility test

The hanging Drop method, which involves collecting live microorganisms and removing them from a liquid medium, has been the most widely used method for studying cell movement and morphology. Using a ring of adhesive tape, circular concavities was made in a glass slide. Vaseline was applied with a toothpick to the corners of the coverslip after placing a clean coverslip on its edges. In the middle of the coverslip, a loop of freshly made broth to test was transferred making sure to use a thin inoculum. To ensure that the vaseline is sealed within the concavity, the prepared glass slide or concavity slide upside-down (concavity downwards) was placed over the drops on the coverslip. The slide was flipped so that it is on top. The organism was allowed for 1 minute to settle. The droplet was seen suspended across the concavity.

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Biochemical Tests

Catalase test

Catalase is an enzyme that spilt up hydrogen peroxide into oxygen and water. Catalase is present in high concentration in majority of aerobic organisms, but absent in most obligate anaerobes. Heavy streaking of potential isolates were done on the surface of nutrient agar slant and incubated at 37°C for 24 hours. 1 mL of hydrogen peroxide was then added over the growth on agar slant. The slide was observed for the rapid appearance and sustained production of gas bubbling.

Oxidase test

Heavy streaking of all potential isolates on surface of nutrient agar slant was done and incubated at 37°C for 24 hours. Following incubation, a colony was picked up and a smear was made on a filter paper moistened with 1% tetraethyl phenylenediamine dihydrochloride solution. The formations of violet color within 45-60 seconds was observed.

Indole test

A loopful culture of selected potential isolates were inoculated in tryptone broth (1% Tryptone Water, 0.5 gm NaCl, 100 ml distilled water and pH 7.4) and incubated at 37°C for overnight. After incubation, 3-4 drops of xylene was added in medium and shaken vigorously. The two layers were allowed to separate and 1mL of Kovac's reagent was added slowly. The formation of pink color ring was observed.

Methyl red test

A loopful culture of selected potential isolates were inoculated in to glucose phosphate broth and incubated at 37°C for 48-72 hours. Following incubation, 5 drops of methyl red indicator was added in the medium. Development of red color was observed.

Voges Proskuer test

A loopful culture of selected potential isolates were inoculated in glucose phosphate broth and incubated at 37°C for 48-72 hours. Following incubation, 0.6 mL of α-naphthol and 0.2 mL KOH solution was added and shaken well. Development of cherry red colour was observed.

Citrate utilization test

Heavy streaking of all potential isolates was done on the surface of Simmon's citrate agar slant and incubated at slant position at 37°C for 48-72 hours. Development of deep blue colour within 24-48 hours was observed.

Nitrate utilization test

All potential isolates were inoculated in peptone nitrate broth and incubated at 37°C. 0.5 mL of α-naphthylamine reagent and sulphanilic acid reagent was added. Development of red colour was observed.

Haemolysis test

All potential isolates were cultured on the blood agar plate as spot or line and incubated at 37°C for 48-72 hours, and then observed for appearance of clear zone of hemolysis.

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Christie–Atkins–Munch-Peterson (CAMP) test

This test is used to identify *Listeria monocytogens* which also produces a positive CAMP reaction. A beta lysine producing strain, *Streptococcus aureus* was streaked down the center of a sheep blood agar plate. Incubated at 37°C for 18 to 24 hours. The streptococcal streak test organisms across the plate perpendicular to the *Streptococcus aureus* streak within 2 mm was observed for positive tests.

Urease test

Urease is a constitutively expressed enzyme that hydrolyzes urea to carbon dioxide and ammonia. A heavy inoculum from a 24 hour pure culture was inoculated to the broth. The tube was gently shaken to suspend the bacteria. The tubes were incubated at 37°C for 24 to 48 hours. Observed the broth for a color change.

Results

Morphological characterization

The shape of the bacteria (Bacilli, cocci), size, color, and texture of bacteria through fluorescent microscope after staining was observed. The pure colonies were observed under microscope by Gram staining technique (Figure 1 and Table 1).

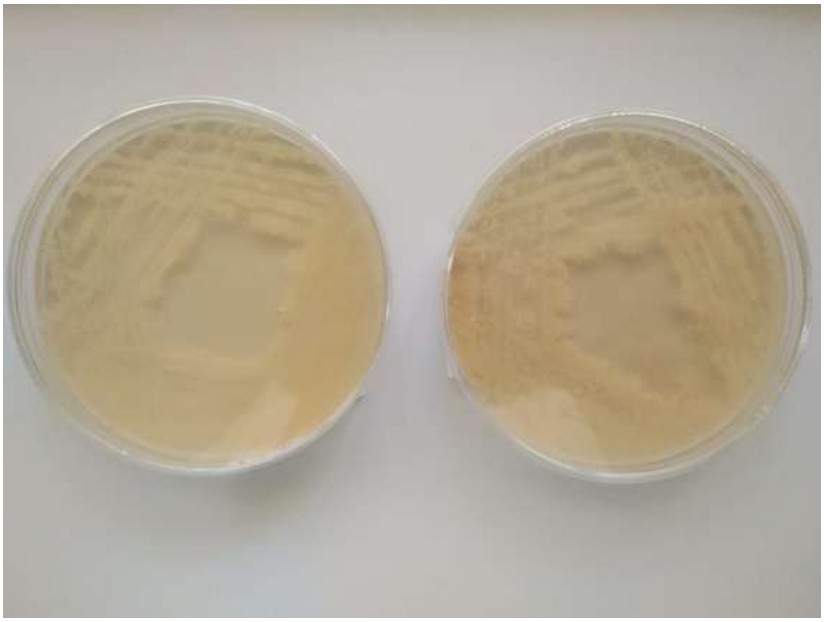

Figure 1: Showing colonies on nutrient agar plate

Table 1. Morphological characteristics

Bacterial Isolates	Colony Shape	Colony Colour	Margin	Elevation	Gram Staining	Motility test
1	Bacilli	Pale yellow	Regular	Convex	Positive	Motile
2	Bacilli	Pale yellow	Entire	Convex	Positive	Motile
3	Bacilli	Pale yellow	Regular	Convex	Positive	Motile

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4	Bacilli	Pale yellow	Entire	Convex	Positive	Motile

Biochemical characterization

The results of biochemical characterization of isolated strains were represented in Table 2. Results depicted that isolated strains were positive for catalase, oxidase, methyl red, Voges Proskauer, and CAMP tests. Whereas, isolated strains were negative for indole, and citrate utilization tests. Identification of *L. monocytogenes* through biochemical test was a useful technique to analyze the samples in less than a week.

Table 2. Biochemical characteristics of isolated bacterial strains

S. No.	**Biochemical Tests**	**Results**	
		Positive	Negative
1	Catalase test	+Ve	
2	Oxidase test	+Ve	
3	Indole test	-	-Ve
4	Methyl red test	+Ve	
5	Voges Proskauer test	+Ve	
6	Citrate utilization test	-	-Ve
7	CAMP test	+Ve	

Discussion

Because *L. monocytogenes* can grow in a variety of food products at low temperatures and can cause a variety of human illnesses, the food industry may pose a risk to public health. This pathogen is more resistant to various sanitizing agents, such as quaternary ammonium compounds, and is widely distributed in nature.[16] Food processing facilities, which include various surfaces with which food comes into contact and where the pathogen may be able to form a biofilm, are the sources of *L. monocytogenes* contamination. Given its significance and recent emergence as a food-borne pathogen, it needs to be detected and controlled in ready-to-eat food products as soon as possible.

Our study findings revealed that *L. monocytogenes* is common contaminants of a ready-to-eat food products sold in local markets of Chikkaballapura. The findings suggests that the public health qualities of the products are doubtful. The production is quite unwholesome for human consumption. These findings are in accordance with studies reported in the literature by Bille[17] and Chukwu et al.,[18] who independently reported the isolation of *L. monocytogenes* in ready-to-eat dairy products. To the best of our literature knowledge this is preliminary report of *L. monocytogenes* contamination in a locally sold ready-to-eat food products. The findings in this

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study are similar to the previous cases reported in processed meats and ready-to-eat dairy products like cooked salami, meat loaf, suya, cheese (gouda), unpasteurized milk sold as fura-de-nunu, ice cream which are contaminated with *L. monocytogenes*.

In the present study the bioload was apparently high, and may be justified by the fact that the raw materials including water used in the preparation might be heavily contaminated. It is worthy to note that ready-to-eat food products preparations have no quality control measures and critical control points, as such the contamination by highly pathogenic *L. monocytogenes* is also justified.

Conclusion

In conclusion, results of the presented study delineated that *L. monocytogenes* is prevalent in ready-to-eat food products and sheds new light on the growth of food pathogens and spoilage microbes in a variety of ready-to-eat food products. These findings could be considered in estimating the risk associated with the production and consumption of ready-to-eat food products.

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