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RESEARCH ARTICLE

A Study On Biochemical Characterization Of Listeria Monocytogenes In Milk And Milk By-Products.

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Abstract: ***Background***: The presence of pathogenic bacteria in raw milk have been a major factor for public health concern. Listeriosis was identified as an infection brought on by a Gram-positive bacterium Listeria monocytogenes. Its ability to not only survive but to grow as a psychrotrophic at 4°C makes this pathogen unique from other commonly found food borne pathogens. Globally, over the past few years, there has been an upsurge in Listeria food borne outbreaks due to the consumption of contaminated food products. Hence, this study was conducted focusing on isolation and biochemical characterization of L. monocytogenes in milk and milk by-product samples such as butter milk and curd. Milk, butter milk and curd samples were procured from locally available retailer shops in Bagalkot district head quarter. A Total of 62 microorganisms were isolated from samples of milk, butter milk, and curd. The isolated strains were positive for catalase, methyl red, citrate utilization, carbohydrate utilization (mannitol and dextrose), hemolysis, and CAMP's tests. Whereas, isolated strains were negative for oxidase, indole, and Voges-Proskauer. Furthermore, biochemical characterization of isolated strains revealed that out of total 62 isolated microorganisms 43 isolates were identified as L. monocytogenes based on CAMP's test. In conclusion, this study demonstrated that L. monocytogenes is prevalent in milk and milk by-products and sheds insights on the growth of food pathogens and spoilage of microbes in milk and milk by-products. This study results are helpful in estimating the risk associated with the processing and consumption of milk and milk by-products.

Keywords: Milk, Butter milk, Curd, Listeria monocytogenes, Listeriosis.

INTRODUCTION

Our everyday lives are greatly impacted by our surroundings. The environment is closely related to the production of milk and the processing of milk products.1 The fluid that mammals generate to feed their young is called milk. Water, fat, protein, and lactose are the main ingredients of milk. Milk is a great growth medium for microorganisms, including pathogens, due to its moderate pH (6.4–6.6) and greater water activity.2 One of the main causes of public health concerns has been the existence of harmful bacteria in raw milk. Dairy cattle, dairy equipment, and food handlers are the primary causes of contamination. Additionally, a number of Listeria monocytogenes, Salmonella sp., and Escherichia coli outbreaks are thought to be primarily caused by the intake of raw milk.3

A Gram-positive bacterium, later identified as L. monocytogenes, was thought to be the cause of listeriosis in the 1920s. An inquiry into the broad traits and pathogenic potential of this microbe was initiated when it was discovered in food and connected to an outbreak in Canada in 1981.4 According to Seeliger and Jones, L. monocytogenes is a foodborne pathogen that is commonly found in foods such fruits, vegetables, dairy products, and processed foods.5 Its ability to not only survive but to grow as a psychrotrophic at 4°C makes this pathogen unique from other commonly found food borne pathogens.3

Globally, over the past few years, there has been an upsurge in Listeria food borne outbreaks due to the consumption of contaminated food products.6 Presently, there are 27 different species of Listeria, out of which L. monocytogenes and L. ivanovii are pathogenic.7 However, L. monocytogenes is the only species that is capable of causing disease in both humans and animals.8,9 L. monocytogenes has been categorized by World Health Organization as the major notable foodborne pathogen associated with high case-fatality and hospitalization.6,10 Clinical manifestations in human listeriosis may range from critical sepsis in immuno-compromised individuals; febrile gastroenteritis, meningoencephalitis in infants and adults; abortions, stillbirth in pregnant women, and septicemia in neonates and the elderly.11

With these viewpoints, present study was conducted focusing on isolation and biochemical characterization of L. monocytogenes in milk and milk by-product samples.

MATERIALS AND METHODS

Sample collection

Milk and milk by-products like butter milk and curd samples were procured from locally available retailer shops in Bagalkot district headquarter in sterilized sample collection tubes, and were immediately taken to the laboratory and stored in the refrigerator for further processing.

Isolation and identification of Listeria spp.
Listeria spp. were isolated and identified according to the US Food and Drug Administration (FDA) protocol. Samples of milk, butter milk, and curd were first pre-enriched in buffered peptone water and incubated at 37°C for 48 hours. Then 25 mL from each pre-enriched sample were added to 225 mL of Listeria enrichment broth (Fraser broth) and incubated at 30°C for 48 hours. This Fraser broth provides optimum conditions for the growth of listeria. Peptone, tryptone, yeast extract, and beef extracts makes the media highly nutritive by providing essential nutrients including carbonaceous and nitrogenous substances. Phosphates maintain the buffering capacity of the medium.

Then a loopful from the enrichment broth was streaked directly onto Oxford agar or Placam agar and incubated at 37°C for 24-48 hours. Listeria species hydrolyze esculin (instead of esculin we used bile salt) to glucose and esculin. The latter mixes with ferric ions to ferric ammonium citrate, resulting in the formation of 7-dihydroxycoumarin and lithium chloride inhibits the enterococci. Three to four presumptive Listeria colonies were incubated overnight at 35°C for 24-48 hours on tryptic soy agar yeast extract (TSAYE) supplemented with 0.6% of yeast extract in general purpose plating medium adopted for isolation, cultivation, and maintenance of Listeria spp. Morphologically typical colonies were verified.

Collected different milk, butter milk, and curd samples are serially diluted at a concentration of 10-6 and 10-7 which are streaked on to the Palcam and oxford agar these plates were incubated at 37°C for 24 to 48 hours. Then, samples were direct plated on nutrient agar media and Oxford agar media and plates were incubated at 37ºC for 24 to 48 hours.

Gram's staining test
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. Clean grease free glass slide was used and thin smear of bacteria was made, heat fixed it and then air dried. Then the smear was spread with crystal violet stain and left for one minute and smear was washed with water and again added Gram's iodine reagent and left for another one minute. Again, washed the smear with 95% alcohol to removed excess stain and rinsed with water. Finally, the smear was spread with safranin stain for 45 seconds and washed with water. Prepared slide was observed at 100x under light microscope.12

Biochemical characterization

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. A clean glass slide was utilized to hold a loop containing organisms, onto which 4-5 drops of a 3% hydrogen peroxide solution were added. Employing a sterile inoculation loop, the organisms were thoroughly mixed with the peroxide solution. The slide was then noticed for the production of effervescence. This test serves to assess the catalase production activity of the organism.12

Oxidase test
Oxidase enzyme plays a significant role in the operation of the electron transport system in aerobic respiration. The test was performed by placing loopful of the species on to the oxidase disc taken in the sterile petri plate and observed for the progress of the purple colour organism after 30 seconds. This test used to detect the ability of the species to produce oxidase enzyme.12

Indole test
For assessing indole production by the organism, 24-hour-old cultures were introduced into tryptone broth. Following the incubation period, the cultures were scrutinized for the emergence of a cherry-red ring subsequent to the addition of Kovac's reagent.12

Methyl red test
To ascertain the glucose fermentation capability of microorganisms, MRVP (Methyl Red Voges-Proskauer) broth was formulated, and the organisms were aseptically introduced into the broth. Maintained at 37°C for 24 hours, methyl red indicator was then added, and the observation focused on the development of a red color. The detection of the organism's fermentation ability is based on the resultant color change.12

Voges Proskuer test
In MRVP broth, 24-hour-old cultures were inoculated. Following incubation, Barrit's solution A (α-naphthol) and Barrit's solution B were vigorously added, and the appearance of a red color was observed to signify a positive result.12

Citrate utilization test
To evaluate the citrate utilization of microorganisms, Simmons citrate agar slants were prepared. Organisms were stab-inoculated into tubes and left for incubation. Post-incubation, the alteration in the medium's color from green to blue was observed as an indicator of citrate utilization.12

Haemolysis test
The isolated organism was tested for the haemolytic activity using the standard protocol. The blood agar medium was prepared and sterilized, and poured on to the petri plates. The isolated organism was streaked on the blood agar medium and incubated at 37°C for 24-48 hours. Observed for zone of inhibition around the colonies.12

Christie–Atkins–Munch-Peterson (CAMP) test

This test is used to identify L. monocytogenes 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 2mm was observed for positive tests.12

Carbohydrates fermentation test

This test serves the purpose of distinguishing Listeria species through carbohydrate fermentation. It is a medium devoid of carbohydrates, utilizing bromocresol purple as a pH indicator. Specific carbohydrates are introduced to the foundational medium, and upon inoculation with an organism capable of fermenting the added carbohydrate, acid production occurs, leading to a color shift in the medium from purple to yellow as specified by the pH indicator. In cases, the carbohydrate is not fermented, the medium's color remains unchanged.12.

RESULTS AND OBSERVATIONS:

A Total of 62 microorganisms were isolated from samples of milk, butter milk, and curd collected from local retailer shops located in and around Bagalkot district. 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).

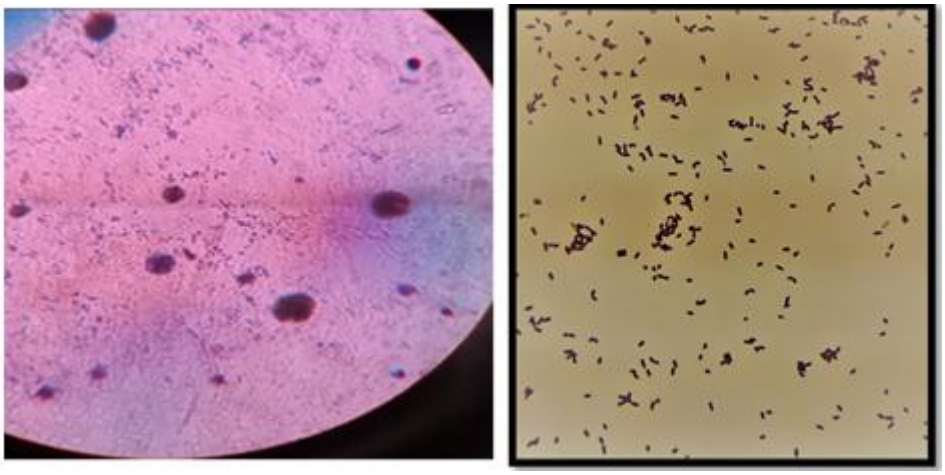

Fig 1: Showing gram staining of isolates

Biochemical characterization

The results of biochemical characterization of isolated strains were represented in Table 1. Results depicted that isolated strains were positive for catalase, methyl red, citrate utilization, carbohydrate utilization (mannitol and dextrose), haemolysis, and CAMP's tests. Whereas, isolated strains were negative for oxidase, indole, Voges-Proskauer, and carbohydrate utilization (fructose) tests. Furthermore, biochemical characterization of isolated strains revealed that out of total 62 isolated microorganisms 43 isolates were identified as *L. monocytogenes* based on CAMP's test.

Table 1. Showing biochemical characteristics of isolated microorganisms

Biochemical tests	Results	No. of Isolates
Catalase test	+	3
Oxidase test	-	2
Indole test	-	1
Methyl red test	+	4
Voges-Proskauer test	-	2
Citrate utilization teat	+	2
Carbohydrate utilization test		
A. Mannitol	+	1
B. Dextrose	+	1
C. Fructose	-	1
Haemolysis test	+	2
CAMP's test	+	43

DISCUSSION

Public health may be at danger from the food sector since L. monocytogenes may grow in a range of food products at low temperatures and can cause a number of human ailments. This pathogen is found throughout nature and is more resilient to several sanitizing agents, including quaternary ammonium compounds.13

The sources of L. monocytogenes contamination are milk processing facilities, which contain a variety of surfaces that food comes in touch with and where the pathogen may be able to build a biofilm. It must be identified and managed in milk and milk by-products at the earliest due to its importance and recent development as a food-borne pathogen.

Our study findings revealed that L. monocytogenes is common contaminants of a milk and milk by-products sold in local retailer shops located in and around Bagalkot district headquarter. The findings suggests that the public health qualities of the milk by-products are doubtful. These findings are in accordance with studies reported in the literature by Bille and Chukwu et al., who independently reported the isolation of L. monocytogenes in ready-to-eat dairy products.14,15 The findings in this 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 ice cream which are contaminated with L. monocytogenes.

CONCLUSION

In conclusion, our study clearly demonstrated that L. monocytogenes is prevalent in milk and milk by-products and sheds insights on the growth of food pathogens and spoilage microbes in milk and milk by-products. Our study results are helpful in estimating the risk associated with the processing and consumption of milk and milk by-products.

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