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Review Article

APPROACHES USED FOR DETECTION AND IDENTIFICATION OF *LISTERIA MONOCYTOGENES* IN FOOD

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ABSTRACT

Foodborne diseases have become a major public health issue. Listeriosis is also one such disease which is caused by Gram positive bacterium, *Listeria monocytogenes*. This bacterium can result in high fatality than any other food borne associated bacteria. Therefore, detection of these strains accurately is essential in outbreak detection and for the institution of appropriate treatment options. For the detection, a variety of methods are available range from phenotypic to genotypic methods. So this mini review focuses on different approaches for the isolation and identification of *Listeria monocytogenes* in food.

INTRODUCTION

Listeria monocytogenes is a Gram-positive anaerobic bacteria that is catalase positive and oxidase negative that forms a rod shape structure [1], with low G+C content [2]. It is a common bacterium that can be isolated from soil, water, animals, plants, mammalian intestines & food sources [3],[4],[5]. Listeria can live and multiply in harsh environments, such as extremely cold or hot temperatures, highly acidic or basic condition, high salt concentration as well as in low oxygen levels [1]. The bacterium is responsible for the highly infectious disease called listeriosis. It is well known serious food-borne illness that can be fatal and have a significant impact on public health[6]. There is a low incidence of listeriosis, but there is a high morbidity & mortality (up to 40%) [7], [8]. The disease may result into septicaemia, meningitis, infection of the central nervous system, and feto-maternal infections [1]. It can also cause encephalitis, abortion and stillbirth. The condition poses a significant risk to pregnant women, individuals with impaired immune systems as a result of T cell suppression and neonates, transplant and AIDS patients [9]. According to the WHO, people with HIV/AIDS are at least 300 times more likely to get sick than those with good immune systems, and pregnant women are around 20 times more likely to have listeriosis than healthy individuals [6] *Listeria* could be transmitted to human through ingestion of stale and contaminated food majorly RTE food, meat, poultry and seafood [9]. Before being consumed, the bacteria taint food items at several processing phases. Following ingestion, the pathogen crosses the intestinal barrier and enters the blood and lymphatic system, where it can grow in organs such as the liver and spleen [8],[10]. According to research, the primary sources of L. monocytogenes include raw milk, as well as other dairy products like cheese [11] Furthermore, food stored in changed atmospheres and refrigerators encourage the bacteria to reproduce in enormous numbers nearing the end of their storage life [12]. The isolate was recovered in a sample taken following pasteurisation (72.6°C for 15 seconds) at a high temperature for a short period of time. This might be due to milk contamination after pasteurisation or technological flaws during the pasteurisation process [13]. *Listeria* has ability to form biofilm in food industries especially in dairy industry and related environment. *L. monocytogenes* multiplies easily and quickly in appliances used in these environments and started forming biofilm within 20 minutes after contact with the surface. By this ability they are very much resistant to cleaning and disinfectant [14]. Hence, the main objective of this review is to study the *Listeria monocytogenes* with respect to its isolation and identification.

General Features

Listeria monocytogenes is a Gram-positive, rod-shaped bacterium that is small and motile having diameter between (0.5–4 μm). It is an intracellular pathogen that is facultatively anaerobic, catalase-positive, oxidase-negative and does not

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form spores. This bacterium can be found in the environment and can be found in soil, stable water (including drains, coolers, washing areas), plants, animals, the intestinal tract of many mammals, and various food sources [4],[15]. It may grow when subjected to extreme environmental conditions like low temperature, low pH, high salt content & anaerobic conditions. Listeria monocytogenes, Listeria ivanovii, Listeria seeligeri, Listeria innocua, Listeria welshimeri, and Listeria grayi are the six species that make up the genus Listeria. The *L. ivanovii* and *L. monocytogenes* are pathogenic for mice and some other animals. Nonetheless, L. monocytogenes is the most significant species that causes human listeriosis, but infection with other species such as L. seeligeri, L. ivanovii and L. innocua has been recorded occasionally [16].

Serotypes

L. monocytogenes has thirteen recognised serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7. Serotype1/2a, 1/2b & 4b are most often associated with human illness [17]. These serotypes are distributed among 4 strains categorised as (I, II, III and IV). Most of L. monocytogenes (about 98%) recovered from food samples and patients belong to strains I & II.

1. Strain I has serotypes 1/2b, 3b, 4b, 4d, and 4e, with serotypes 1/2b and 4b identified as the causal agent of human listeriosis.
2. The serotype of Strain II consists 1/2a, 1/2c and 3c; from which serotype 1/2a found to be associated as sporadic case in human. However,
3. Strain III has serotypes 1/2c and 4c, whereas strain IV contains serotypes 4b, 4d, and 4e.

Listeriosis

Foodborne illness Listeriosis is a potentially fatal disease caused by the intracellular bacteria Listeria monocytogenes, with a fatality rate of 20-30%, which is the highest rate observed among all foodborne microbial pathogens, such as *Salmonella* [18]. Individuals suffering from a serious illness like suppression of immune system, HIV/AIDS, long term illnesses like cirrhosis, women who are pregnant, neonates, and the elderly are the most vulnerable population to L. monocytogenes. The symptoms of this disease vary from flu-like sickness to serious consequences such as meningitis, septicaemia, abrupt miscarriage, or neonatal listeriosis [18]. L. monocytogenes strain I is responsible for the vast majority of human listeriosis outbreaks. This strain has has exotoxins such as phosphatidylinositol-specific phospholipase C (encoded by plcA), broad-range phospholipase C (encoded by plcB), and listeriolysin O. (LLO encoded by hlyA). P60, expressed by the iap (invasion-associated protein) gene, is a large 60-kDa autolysin involved in L. monocytogenes invasion [19].

Entry into host

L. monocytogenes enters the host via intestine. during uptake of food contaminated with *L. monocytogenes*. In this case, L. monocytogenes survives host proteolytic enzymes, bile salts, the extremely acidic pH 2.0 of stomach and certain non-specific inflammatory responses, owing primarily to the activities of several stress-response genes (opuCA, lmo1421, and bsh) and associated proteins [20]. Infections with L. monocytogenes begin in the intestinal epithelium, which is composed of polarised epithelial cells joined by adheren junctions. The bacteria begin invading a kind of epithelial tissue found at the tips of intestinal villi, where cells are actively extruded and lost. L. monocytogenes then adheres to and is taken in by cells with the aid of various proteins present on the surface. This proteins are known as internalins [2].

Virulence factor

Internalins are essential virulence factors that facilitate bacterial absorption by host cells by modulating eukaryotic cell attachment and invasion. Internalins also promote pathogen entrance into non-phagocytic cells like hepatocytes, fibroblasts & epithelial cells. After getting entry to host cells the bacteria evade host immune surveillance functions [2] here it located in a single-membrane vacuoles. The bacteria produce a unique soluble cytolysin called listeriolysin O (LLO, encoded by hlyA), it can activated in low pH.

- It is a pore-forming (which disrupts phagosomes to allow bacterial proliferation in the cytosol) ,
- Toxin that is triggered by thiol is required for bacterial pathogenecity.
- Phosphatidylinositol-specific phospholipase C (PI-PLC, protein encoded by plcA), in collaboration with phosphatidylcholine-specific phospholipase C (PC-PLC, a 29 kDa protein encoded by plcB), assists LLO in lysing primary vacuoles and allowing L. monocytogenes cells to be released from the phagosome to cytosol
- In addition, the pathogen grows intracellularly and multiplies intracytoplasmically. The bacteria move about within their cells and spread from cell to cell with the help of another surface protein designated as Act A. L. monocytogenes employs the protein ActA, which polymerizes actin into comet tails, resulting in a propulsive force by which bacteria is propelled into the cytoplasmic membrane.
- The bacterium are subsequently encased in structures similar to that of filopodium that are identified & swallowed by adjacent cells. This process takes place at the membrane and leads in the formation of secondary double-membraned vacuoles. The lysis of these vacuoles marks the start of a new cycle of infection. As a result of this, L. monocytogenes spread throughout the host tissues [21].
- L. monocytogenes exotoxins include phosphatidylinositol-specific phospholipase C (encoded by plcA), a broad-range phospholipase C (encoded by plcB), and listeriolysin O. (LLO encoded by hlyA). P60, expressed by the iap (invasion-associated protein) gene, is a large 60-kDa autolysin involved in the invasion of L. monocytogenes [22].

Characteristics Related to its Growth and Survival

L. monocytogenes is a facultative anaerobe that thrives in low oxygen environments with high carbon dioxide concentrations. The optimum pH of media for the growth of bacteria ranges from alkaline to neutral. L. monocytogenes grows best between 30 and 37°C; however it may also thrive between 1 and 45°C[17]. *L. monocytogenes* also have the potential to grow at 0°C. However the number of bacteria reduced in food during refrigerated storage.

Current Standard Methods

Standard culture processes are employed in the food sector. The most extensively used culture reference techniques for detecting L. monocytogenes in all food categories are the

1. International Organization of Standards (ISO) 11290 method,
2. To isolate Listeria spp. from seafood, vegetables, and dairy products, the FDA developed a bacteriological and analytical technique (BAM);
3. Listeria spp. isolation from meat, poultry products, and environmental samples using the USDA standard technique (USDA, 2002).

In all of these procedures, 25 grams of sample (food) is supplemented in a broth selective in nature. After this inoculation on selective agar & biochemical identification of targeted colonies can be performed [23].

Collection of Samples

Samples from the different sources such as milk (raw or pasteurized), milk product, fruits, vegetable, meat and poultry, are collected in a sterilized bag and preserved in a cooler with ice packs or in a refrigerator at 5°C. Samples should be processed and analyzed within 24hrs of collection. 25g/25ml of sample is the standard amount used for isolation of *Listeria* spp. The sample (if liquid) is homogenized and mixed with 225ml enrichment broth. In case of solid, the sample is first blended/minced and then homogenized before adding enrichment broth [19].

Isolation by enrichment broth and plating

Enrichment culture is an isolation technique which is designed to provide favourable conditions for the growth of desired organism while unfavourable condition for the background organism. This method is used to enhance the number of desired microbe to a detectable level. *L.* monocytogenes can be isolated through enrichment broth that employ selective agents like cycloheximide (suppress Gram-positive bacterial growth), nalidixic acid (suppress Gram-negative bacterial growth) and antifungal agent acriflavine. Prior to plating on selective medium, these chemicals suppress the development of competing microorganisms [24].

One typical enrichment broth used for the isolation of L. monocytogenes is known as Fraser broth. According to ISO 11290 standard

- The sample is first enriched in half strength Fraser broth for 24 hours at 30°C (primary enrichment) and thereafter
- Full strength aliquot is transferred in Fraser broth for 48 hours at 37°C (second ary enrichment)

Fraser broth includes selective agents plus esculin, which facilitates in the detection of Listeria *β-d*-glucosidase activity, which causes the medium to blacken [*25]*. Further the enriched media are plated on selective agar plates like (Oxford, PALCAM, MOX).

Oxford agar medium is primarily used to isolate L. monocytogenes from faeces and it has also been suggested for Listeria isolation from foods.
It uses

1. Lithium chloride, acriflavine, colistin, fosfomycin, cefotetan and cycloheximide as the selective inhibitory components.
2. The esculin and ferric iron as indicator systems

Listeria spp. hydrolyzes esculin, causing black zones to appear colonies formed as a result of the production of black iron phenolic compounds produced by glucon. By this, Gram negative bacteria growth is fully suppressed.

Many undesirable Gram positive species are inhibited as well; however certain coagulase negative staphylococci may emerge as aesculin negative colonies [26].

MOX is quite similar to Oxford agar medium used for L. monocytogenes isolation from foods. Lithium chloride level in MOX is lowered to 12 g/1, allowing certain more sensitive L. monocytogenes (e.g. ATCC 35152 and CDC F4561) strains to thrive. Incubation at 30ºC for 26 hrs is the preferred for many strains of Listeria [26].

PALCAM agar plate (Polymyxin Acriflavin Lithium chloride Ceftazidime Aesculin Mannitol agar) is suggested for L. monocytogenes isolation. It is highly selective for L. monocytogenes due to the presence of polymixin B, acriflavine hydrochloride, lithium chloride, ceftazidime. Due to the availability of a dual indication system having Mannitol & phenol red; Esculin and ferric ammonium citrate, PALCAM facilitates the differentiation of L. monocytogenes L. monocytogenes degrades esculin to produce esculetin & glucose. Esculetin combines with ferric ammonium citrate, forming a brown-black compound that appears as a black halo surrounding colonies. Listeria monocytogenes cannot ferment mannitol, however certain contaminants, including Enterococci, Micrococci & Staphylococci, may, as seen by a colour shift from red to yellow [27].

Identification and Confirmation

Following enrichment procedures, the enriched bacteria are isolated on selective plate media and identified up to species level based on their particular properties. (Figure 1). Isolates can be identified as a result of their colony morphology, carbohydrate fermentation & haemolytic properties [28].

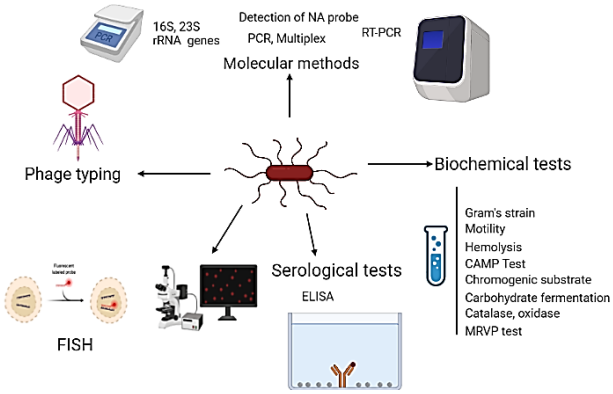

Figure 1 Various detection methods for Listeria monocytogenes

Biochemical methods

Gram staining

The L. monocytogenes gives purple color during gram staining, thus confirmed as Gram positive bacteria [5].

Motility

The motility of L. monocytogenes can be demonstrated by using stab inoculation in a semi-solid agar medium. Listeria spp. has little rods with a dynamic end-over-end tumbling/rotating action [30].

Hemolysis

L. monocytogenes' hemolysis characteristic is mediated by listeriolysin O, a secretory protein. Only three species of genus

Listeria i.e, L. monocytogenes, L. seeligeri and L. ivanovii exhibit hemolysis. A well-defined wide and clear zone or sometimes even multiple zone appear in case of L.ivanovii. L.monocytogene exhibit a narrow zone as compared to L.ivanovii while L. seeligeri produces even narrower zones of hemolysis [31].

CAMP (Christie-Atkins-Munch-Peterson) Test

This assay can be used to distinguish between the hemolytic strains of Listeria; L. monocytogenes, L. seeligeri and L. ivanovii. The plates should be observed for enhanced haemolysis by L. monocytogenes and L. seeligeri near the streak of S. aureus and another enhanced haemolysis by L. As compared to L. monocytogenes, the L. ivanovii produces β type of haemolysis [32].

Chromogenic substrate

Phosphatidylinositol-specific phospholipase C (PIPL-C) is an enzyme generated solely by Listeria monocytogenes & Listeria ivanovii [33]. On the basis of the results of trials, according to ISO/TC 34/SC 9, ALOA medium and Rapid'Lmono agar performed significantly better than the other media tested. The BCM chromogenic agar test and the CHRO-Magar Listeria test are two more commercially available chromogenic medium for detecting PIPL-C positive bacteria [34].

Carbohydrate fermentation

This test is used to identify whether the bacteria is able to ferment specific carbohydrate or not. It also helps to detect gas or acid production during carbohydrate fermentation by different strains of Listeria.

Maltose, dextrose and esculin is present in all Listeria species. Other than L. grayi, all Listeria spp. should be mannitol-negative [30].

Catalase test

All the species of Listeria are catalase positive showing bubbling effect when immersed in 3% hydrogen peroxide solution [30].

Oxidase test

All the species of Listeria are oxidase negative which do not change the color of the reagent [35].

Voges–Proskauer (VP) Test

Certain bacteria produce ***acetylmethyl carbinol*** from glucose fermentation. Listeria shows positive result for this test [35].

Methyl red test

The Methyl Red (MR) test employs methyl red, a pH indicator that aids in the detection of acidity when an organism ferments glucose. Listeria group contains the MR- positive strains [35].

Serological method

Species of Listeria are classified into serogroups according to the presence of particular antigens on their surfaces. The specific antigens are of two types,

1. Heat-stable somatic (O) and
2. Heat-labile flagellar (H) antigens

There are 15 somatic (O) antigen subtypes (I-XV) and four flagellar (H) antigen subtypes (A–D) in Listeria. Each Listeria strains' serotypes are determined by their distinct combinations of O and H antigens.

Based on this unique combination at least 13 serotypes (i.e. 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab,4b, 4c, 4d, 4e and 7) have been recognized in L. monocytogenes, several in L. seeligeri (e.g. 1/2a , 1/2b, 3b, 4a, 4b, 4c and 6b), one in L. ivanovii (i.e. 5) and a few in L. innocua, L. welshimeri and L. grayi (e.g. 1/2b, 6a and 6b) through examination of group-specific Listeria O and H antigens in slide agglutination [36].

Serotypes 1/2a, 1/2b, and 4b of L. monocytogenes have been found to be responsible for 98% of known human listeriosis cases, but serotypes 4a and 4c are seldom related with illness outbreaks[37]. Serotype 4b strains cause epidemic outbreaks of listeriosis, whereas serotypes 1/2a &1/2b cause sporadic L. monocytogenes infection.

Bacteriophage typing

Application of some defined bacteriophages to classify bacteria beyond the species & serotype is referred to as phage typing. This is feasible to distinguish strains of Listeria into separate phage categories and phagovars by examining bacteriophage-induced, host-specific lysis of Listeria [38].

Detection by Molecular Methods

Molecular approaches make it simpler to detect L. monocytogenes since they are accurate, sensitive and specific.

DNA based methods

Detection by nucleic acid probe

These probes are DNA or RNA segments that have been radioisotopes labelled or enzyme molecules that attach selectively to complementary nucleic acids (DNA/RNA). L. monocytogenes detection by nucleic acid probes is accurate and simple. By directing probes to virulence factor genes, Listeria species may be distinguished [2].This approach is more precise than methods based on phenotype as this method because distinguishes Listeria species at the genetic level.

Polymerase Chain Reaction

PCR has now been confirmed as a reliable approach for identifying Listeria spp. In addition, this method also helps in distinguishing L. monocytogenes from other Listeria species. This could be accomplished by using primers that target virulence factor genes or RNA subunit genes unique to L. monocytogenes [39]. Further, L. monocytogenes is promptly and accurately distinguished from furher species of Listeria and common bacteria by using molecular differences within 16S and 23S rRNA genes, intergenic spacer regions, hlyA, plcA, plcB, iap, and other genes (e.g. delayed-type hypersensitivity gene, aminopeptidase gene, and putative transcriptional regulator gene lmo0733) [27].

RNA based methods

Since DNA molecules are very stable, DNA detection technologies may identify DNA from both living and dead cells. This results into a false positive result [42]. To circumvent this, an RNA-based technique can be used in which RNA or mRNA is used as a target to identify pathogens specifically from food, as the presence of mRNA indicates that the cell is active or alive. Another pros of mRNA is that it can improve test sensitivity by including numerous copies of target genes. Instability of RNA creates difficulty during testing as appropriate conditions are required to prevent RNA from degradation. Another limitation of the RNA-based approach is the high cost of equipment and reagents, as well as the

requirement of thorough training to execute the test. RNA-based diagnostics have been shown to be as sensitive and specific as regular PCR [41].

RT-PCR

RT-PCR (Reverse transcription polymerase chain reaction) is an RNA-based approach for detecting Listeria spp. In the instance of Listeria spp, RT-PCR was done on particular genes such as prfA, iap, and hlyA. However this technique is not commonly used [42].

Real time PCR

Real-time PCR is a quantitative approach for identifying and quantifying the quantity of contaminated Listeria monocytogenes in samples of food and clinical origin, as well as for simultaneous Listeria detection. Because real-time PCR is a quantitative approach with substantial advantages over other molecular methods, it is widely used in food testing and epidemiological studies [42]**.**

CONCLUSION

Food-borne infections caused by microorganisms are one of the world's most important public health issues. The global prevalence of foodborne illness is difficult to measure but in many countries where there is information about foodborne illness, the overall number of cases has risen during the last 20-30 years. Unfortunately, in developing countries, not too much attention is paid to foodborne illnesses, and therefore, they support all issues related to the existence of a wide range of foodborne illnesses. One such important foodborne pathogen is *L. monocytogenes*. The occurrence of *Listeria* species has been reported from different types of food items. Due to their distribution, *Listeria* species have different manner to to enter the food manufacturing and processing industries environment and due to their psychotropic nature, they are capable of growing and multiplying even in food that has been refrigerated. In terms of its isolation, different phenotypic (Chromogenic media, selective plates, serotyping) and genotypic methods are available. Yet, one of the most essential properties of L. monocytogenes is that under the undesired condition, it transformed into a viable but non-culturable state. This bacterium, which is found in food, cannot be identified in culture medium. Hence, there is always room for an improvement in detection methodology. There is a lack of management capabilities at the early stage of HACCP and/or GMP application in developing nations such as India. GMP implementation on farms will aid in reducing pathogen burdens in animal production systems. In addition, training courses for food inspectors, food industry employee, staff in distribution centres, veterinarians, are needed on food safety and animal product regulations. Listeriosis is not considered a major public health problem in India therefore; any data on the incidence, detection, in different food items would be invaluable in assessing the status of food items contaminated with *Listeria* and thereby help in adopting appropriate measures.

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