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Review Article

A Review on Various Approaches to the Identification of *Listeriae*

Atul Kumar Gupta[1], Asima Zehra[2]* and Simranpreet Kaur[3]

[1]Assistant Professor, Determent of Veterinary Public Health and Epidemiology, Dr. G.C. Negi College of Veterinary and Animal Sciences, CSK HP Agricultural University, Palampur, HP, India

[2]Phd Scholar, School of Public Health and Zoonoses, Guru Angad Dev Veterinary and Animal Sciences University, Punjab, India

[3]Assistant Professor, School of Public Health and Zoonoses, Guru Angad Dev Veterinary and Animal Sciences University, Punjab, India

***Corresponding Author:** Asima Zehra, Phd Scholar, School of Public Health and Zoonoses, Guru Angad Dev Veterinary and Animal Sciences University, Punjab, India.

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Abstract

Historically, for a considerable period, *Listeria* spp. remained unnoticed as a major food pathogen which can be credited to its challenging isolation from food or other samples. But now *L. monocytogenes* is an established food-borne pathogen of public health significance and is widely present in food, environmental and clinical samples. The very first step for the isolation of the organisms are culture methods that characterise the organisms based on the morphology of the colony, fermentation of sugar and their haemolytic effects. These are the gold standards; but they are time consuming and may not be suitable for testing of perishable foods. As a result, more rapid and sensitive serological (ELISA) and molecular (real time PCR or DNA hybridization) tests were developed. These tests not only provide a measure of cell viability but they can also be used for quantitative analysis. In addition, a variety of tests can be used for sub-species characterizations, which are particularly useful in epidemiological investigations. Early typing methods differentiated isolates based on phenotypic markers, such as MLEE, phage typing and serotyping. These phenotypic typing methods are being replaced by molecular tests, which reflect genetic relationships between isolates and are more accurate. These new methods are currently mainly used in research but their considerable potential for routine testing in the future cannot be overlooked.

Keywords: *L. monocytogenes; Serological Assays; Molecular Assays*

Introduction

The genus *Listeria* covers a group of opportunistic, aerobic/facultative anaerobic, gram-positive, non-sporulating, motile (tumbling motility at 25°C), bacilli of 0.4 - 0.5 µm × 1 - 1.5 µm in size with a low G+C content (36 - 39%). Till date, in addition to *L. monocytogenes*, 16 other closely related bacterial species (*L. ivanovii, L. innocua, L. seeligeri, L. welshimeri, L. grayi, L. marthii, L. rocourtiae, L. fleischmannii, L. weihenstephanensis L. floridensis, L. aquatica, L. newyorkensis, L. cornellensis, L. grandensis, L. riparia, and L. booriae*) with ubiquitous distribution have been identified within the genus *Listeria* [1]. Out of these species, *Listeria monocytogenes* is a facultative intracellular pathogen of veterinary and human importance, *L. ivanovii* primarily infects ungulates (e.g. sheep and cattle), and the other species are primarily free-living saprophytes. *L. monocytogenes* has been differentiated into 13 serotypes; serotypes 1/2a, 1/2b, and 4b have been involved in more than 95% of reported human listeriosis cases [2]. Most isolates from food have a place with serogroup 1/2a and prevail

in instances of sporadic listeriosis, yet a large portion of the episodes of human sickness are brought about by isolates of serotype 4b. However, non-monocytogenes *Listeria* species including *L. ivanovii, L. seeligeri, L. innocua, L. welshimeri* and *L. grayi* have been occasionally implicated in human clinical cases, mainly in individuals with suppressed immune functions and/or underlying illnesses [1].

There are assortments of diagnostic techniques at present accessible for the recognition and distinguishing proof of *L. monocytogenes* and other *Listeria* spp. For animals and people, customary bacteriological strategies are significant for different reasons: their utilization brings about an uncontaminated culture of the organism, which is valuable for administrative, epidemiological surveillance and flare-up the executives purposes. They remain the 'gold standards' against which different techniques are looked at and approved. These strategies are generally sensitive and they don't require advanced and costly hardware, permitting across the board use. Some of the disadvantages of this group of methods include the relatively long period of time that the protocols require for completion, several 'hands-on' manipulations, the requirement for many different chemicals, reagents and media, the possibility of contaminating microorganisms masking the presence of the target ones, including overgrowth, the potential overlook of atypical variants of the target organism and the relative subjectivity involved when interpreting bacterial growth on selective and differential agar plates [3].

For the rapid epidemiological tracking and control of listeriosis outbreaks, it is important to distinguish between pathogenic and non-pathogenic *Listeria* spp., as well as between pathogenic and non-pathogenic *L. monocytogenes* strains. While conventional methods have contributed to the identification and detection of *Listeria* organisms in the past, they are now largely overtaken by new generation molecular techniques that demonstrate superior sensitivity, specificity and speed. There is a wide scope of traditional, serological, molecular and epidemiological strategies accessible for the distinguishing proof of *Listeria* spp. in test samples from the food chain and clinical samples and some routinely utilized techniques will be talked about in this review.

Methods/Approaches for the Identification of *Listeria*

Laboratory diagnosis of listeriosis is primarily based upon isolation of the organism. Apart from food of animal origin (milk, meat and their products etc.), samples (spinal fluid, blood, brain tissue, spleen liver, abomasal fluid and/or meconium) from clinical cases are cultured, depending upon signs, lesions and tissue available. Identification of *Listeria* spp. customarily included culture methods, in light of specific enrichment followed by the characterization dependent on morphology of its colonies, fermentation of sugar and its haemolytic properties. However, presently more rapid tests have been created dependent on antibodies (ELISA) or molecular techniques like PCR, DNA hybridization, reverse transcriptase-PCR, real time PCR, NASBA and so forth. In addition, a variety of tests (e.g. MLEE, phage typing, serotyping etc.) can be used for sub-species characterizations which are particularly useful in epidemiological investigations.

Conventional methods

Listeria are psychrophilic and can multiply at low temperatures. This fact has been utilized to isolate these microbes from clinical samples by incubation for extended periods at 4°C on agar plates (cold enhancement). This strategy for isolation takes as long as half a month and typically doesn't take into account the isolation of damaged *Listeria* cells, which won't endure and multiply when stressed. Additionally, *Listeria* cells are moderate growing and can be quickly out-grown by contenders, and subsequently bacteriostatic agents that explicitly act to suppress contending microflora, have been brought into enrichment media or particular agar [4]. But, there have been numerous reports of the unsafe impacts of these specific bacteriostatic agents on stressed or damaged *Listeria* cells [5]. Thus, to overcome this situation slight modifications in the methodology have already been made.

Two of the most broadly utilized culture reference techniques are the FDA Bacteriological and Analytical Method (BAM) and the International Organization for Standardization (ISO) 11290 method. Despite the fact that USDA protocol is oftenly utilized as the recommended technique for food of animal origin and ecological samples. These techniques are sensitive however frequently tedious and may

take a week long before the outcome is accessible. At present, suspect microbes are generally classified as *Listeria* in the event that they show the accompanying attributes: Gram-positive bacilli, motility temperature dependent, aerobic/facultatively anaerobic, non-spore forming, catalase-positive (despite the fact that there are reports of catalase negative *Listeria*), oxidase-negative, sugar fermentation and no gas end product on producing acid (Table 1).

		L. monocytogenes	*L. ivanovii*	*L. seeligeri*	*L. innocua*	*L. welshimeri*	*L. grayi*
Haemolysin		+	+	+	-	-	-
Catalase		+	+	+	+	+	+
Oxidase		-	-	-	-	-	-
Fermentation of:	L-Rhamnose	+	-	-	+/-	+/-	+/-
	D-Mannitol	-	-	-	-	-	+
	D-Xylose	-	+	+	-	+	-
	α-Methyl-Mannoside	+	-	-	+	+	+

Table 1: *Biochemical characterization of Listeria spp.*

Also, the CAMP (Christie, Atkins, Munch-Petersen) test can be used to differentiate between hemolytic *Listeria* spp.: *L. monocytogenes*, *L. ivanovii* and *L. seeligeri* [6]. *L. monocytogenes* is CAMP positive when cross streaked with β-hemolytic *Staphylococcus aureus* on 5% sheep blood agar i.e. hemolysis by *L. monocytogenes* and to a lesser degree the hemolysis by *L. seeligeri* is increased in and around the *S. aureus*. A comparable phenomenon is seen when *L. ivanovii* is cross streaked with *Rhodococcus equi*.

Chromogenic media are progressively picking up acknowledgment by administrative specialists. Phosphatidylinositol specific phospholipase C (PIPL-C) is an enzyme that is created uniquely by *L. monocytogenes* and *L. ivanovii* [7] and activity of this enzyme is measured using commercially available chromogenic media (Rapid'L.mono® agar, BCM® chromogenic agar test, CHRO-Magar® *Listeria* test and ALOA® etc.) e.g. *L. monocytogenes* exihibits blue colonies on Rapid'L.mono® agar (BioRad, France). These media offer numerous preferences over other tests. They are basic, financially savvy, simple to decipher, permit an enormous test sample throughput, profoundly sensitive and explicit, and can be acted in a similar time span as ELISA strategies. However, none of these tests separate between *L. monocytogenes* and *L. ivanovii*. Separation of *L. monocytogenes* from *L. ivanovii* is practiced by the fermentation of xylose. Another pathogen enzyme utilized for the recognizable proof of *L. monocytogenes* is alanyl peptidase, which is produced by all *Listeria* spp. But not by *L. monocytogenes*. A basic shading response is utilized in which the substrates DL-alanine-b-naphthylamide and D-alanine-p-nitroanilide are hydrolysed [8].

Serological methods

A number of formats, including ELISA, dot-blot and microagglutination (Gruber-Widal reaction) lack sensitivity as well as specificity because of antigenic cross-reactions with components from other gram-positive organisms [9] and have been largely unsuccessful in diagnosis of culture-proven human listeriosis, even in the absence of immunosuppression. However, interest in the serodiagnosis of listeriosis has now been renewed following the introduction of assays based on the detection of serum antibodies against *Listeria* haaemolysin, listeriolysin O (LLO), a major virulence factor produced by all pathogenic strains of *L. monocytogenes* [10]. Anti-LLO antibodies (ALLO) have been demonstrated to be reliable markers of listeric infection both in people and in animals. Immunoassay strategies have likewise been applied in food testing for a long time and they are well known in view of their straightforwardness, sensitivity, exactness and furthermore on the grounds that testing can be done legitimately from enrichment media without monotonous sample preparation.

One of the commercially available kit based on the enzyme linked fluorescent technology are widely used in the field. The strategy right now being utilized is endorsed by the Association of Official Agricultural Chemist (AOAC) and Safe Food Alliance is presently attempting to complete approval of the LMX VIDAS technique which takes less than 24-hour time to give the end results.

Molecular methods

Identification of *Listeria* spp. also, especially *L. monocytogenes* utilizing molecular strategies is turning out to be progressively well known in light of the fact that these methods are very precise, sensitive and specific. A portion of the routinely utilized molecular methods for recognizable proof of *Listeria* spp. are: DNA hybridization.

It is the simplest non-amplified, molecular method used primarily for the detection and differentiation of *L. monocytogenes* from other *Listeria* spp. in foods by targeting probes to virulence factor genes e.g. Probe target is Internalin A (*inlA*), *inlB* and *inlC* for specific detection of *L. monocytogenes* whereas Internalin D (*inlD*) for detection of *L. ivanovii*. Some of the commercially available DNA hybridization tests are: *Accuprobe (Gen-Probe Inc., USA) and VIT®:* (vermicon identification technology).

In-vitro amplification methods

PCR is presently settled as a reliable and reproducible strategy for recognizable proof of *Listeria* spp. And, more importantly, for the differentiation of *L. monocytogenes* from other *Listeria* spp. using primers targeting genes of virulence factors or RNA sub-unit genes (e.g. 16 S RNA, phospholipase A/B, *inl*A/B, *sig*B, *hly*, *fbp*, *iap*, *mpl*, *dth*, *prf*A etc.). It has been observed that direct testing of samples using PCR without prior enrichment gives unreliable results [11] so detection is carried out after selectively enriching samples for 24 - 48 hours. But, there is a significant deterrent for the utilization of PCR in environmental and food samples straightforwardly after enrichment. The enrichment broths and food matrix contain inhibitors of PCR which offer ascent to bogus negative outcomes. Although, different methodologies, for example, specific treatment of samples, utilization of magnetic beads, plunge sticks or filters during isolation can be taken to expel target DNA from response inhibiting test sample matrices [11].

Multiplex PCR permits the synchronous identification of more than one pathogen in a same test sample, for example, *Listeria* and *Salmonella* [12]; *L. monocytogenes* and other *Listeria* spp [13,14] and even differentiation of serovars 1/2a and 4b from other serovars of *L. monocytogenes* by targeting 2 virulence genes at a one time [14]. This approach is very attractive for food analysis, due to reduction in reagents, labour costs and testing time.

Since, only pathogenic living *Listeria* cells can cause the disease so testing of food/environmental samples should only target living organisms. DNA based tests, for example, PCR have been censured in light of the fact that dead organisms can give positive outcomes because of the generally high stability of DNA molecules. The choice of RNA or mRNA as a target for food pathogen testing has been appreciated since the presence of mRNA is an indication of the living state of the cell and mRNA being a labile molecule, readily gets degraded after cell death by RNases and environmental factors such as heat [15]. Another favorable reason of testing for mRNA is that different duplicates of the target gene are available, which thus improves the sensitivity of the test. The RNA based amplifications methods include Reverse Transcription (RT)-PCR, Real-time quantitative PCR, and Nucleic acid sequence-based amplification (NASBA).

Diagnostic techniques for molecular epidemiology

Identification of *Listeria* in food, environmental and clinical examples utilizing regular, biochemical, serological just as some molecular strategies, for the most part recognizes the contaminating *Listeria* to the species level. *L. monocytogenes* is viewed as a significant human pathogen and just three serotypes of it are involved in significant foodborne listeriosis episodes. Hence, epidemiological examinations must utilize phenotypic and additionally genetic subtyping strategies that are equipped for perceiving closely related *L. monocytogenes*

strains/subtypes so as to improve our comprehension of the biology, ecology and the study of disease transmission of *L. monocytogenes*. Hence, subtyping strategies has massively expanded or capacity to affirm source of outbreaks, set up patterns of transmission, and monitor reservoirs of epidemic strains. Additionally, typing frameworks must have the ability to effectively characterize all epidemiologically related isolates from an outbreak as indicated by clonal connections (epidemiological concordance).

Typing of *L. monocytogenes* isolates depend on phenotypic markers, for example, somatic 'O' and flagellar 'H' factors for serotyping, phage receptors for phage typing or proteins/enzymes (Multilocus Enzyme Electrophoresis-MLEE), or they depend on molecular varieties inside the microorganisms genome (molecular typing techniques).

In comparison to other molecular subtyping methods, serotyping shows poor discriminatory power for many foodborne pathogens including *L. monocytogenes* [16]. While that a significant drawback of the phage typing method is that not all *L. monocytogenes* isolates are typable. Specifically, *L. monocytogenes* serotype 1/2 isolates have a low typability contrasted with different serotypes [17]. Since, *L. monocytogenes* 1/2 isolates are usual food contaminants and one of the serotypes liable for listeriosis episodes; this is a genuine disadvantage in utilizing phage-typing of *L. monocytogenes* isolates for epidemiological studies. On the other hand, MLEE is a typing technique that has been widely used for studies on the population genetics of many bacterial pathogens, including *L. monocytogenes.* This method is difficult to standardize between laboratories but it usually provides 100% typability.

Molecular subtyping strategies depend on DNA hybridization, PCR, RE (Restriction Enzyme) analysis or direct DNA sequencing. Albeit, direct DNA sequencing is almost the exact method for assessing hereditary connections (contrasts or likenesses) of organisms yet it is likewise the most costly and tedious technique. Another technique which allows unambiguous typing of any strain is Multi Locus Sequence Typing (MLST). MLST often refers to a molecular subtyping approach that uses DNA sequencing of multiple housekeeping genes to differentiate bacterial subtypes and to determine the genetic relatedness of isolates. Additional virulence-associated genes (e.g. *inlA*) not linked to this virulence island have also been identified. These virulence genes are unique to *L. monocytogenes*, thus providing ideal targets for the development of DNA sequencing-based subtyping methods [16].

When ribosomal RNA/DNA probes are utilized, just the specific restriction fragments related with the chromosomal loci for rRNA are identified. This strategy is known as ribotyping and it has been generally utilized for subtyping *L. monocytogenes*, mostly using the restriction endonuclease EcoRI. Ribotyping of *Listeria* isolates includes the RE digestion of chromosomal DNA followed by DNA hybridization utilizing a rRNA probe. Resulting banding patters are in this manner dependent on just those DNA fragments that contain the rRNA genes. These DNA banding patterns are utilized to sort *Listeria* isolates into ribotypes and set up the relatedness of these isolates.

L. monocytogenes subjected to the restriction endonucleases (RE analysis) in a WHO Multicentre study revealed that HaeIII, HhaI and CfoI were the most helpful [18]. In any case, in light of a possibly huge number of enzyme recognition locales in the bacterial genome, now and again complex fingerprints develop, with overlaping or inadequately resolved groups that are hard to decipher. The procedure is subsequently not sufficient for contrasting countless isolate patterns or for building any kind of databases [18].

PFGE (Pulsed-Field Gel Electrophoresis) is particularly useful for subtyping serotype 4b isolates, which are not satisfactorily subtyped by most other subtyping methods [19]. The primary drawbacks of PFGE are that it requires time period of approximately 2-3 days to finish the procedure, the enormous amounts of costly REs required, and the requirement for particular and costly hardware. However, the mix of macro-restriction digest with PFGE is viewed as the best typing technique due to its effortlessness, time and cost effectiveness. Due to high level of sensitivity for discrimination of *L. monocytogenes* strains, PFGE is often considered the current gold standard for discriminatory ability.

Approach that randomly amplifies DNA fragments include Random Amplification of Polymorphic DNA (RAPD). This strategy has a few favorable points over other molecular methods viz. simplicity of the test, the speed with which *Listeria* strains can be typed to a sub-spe-

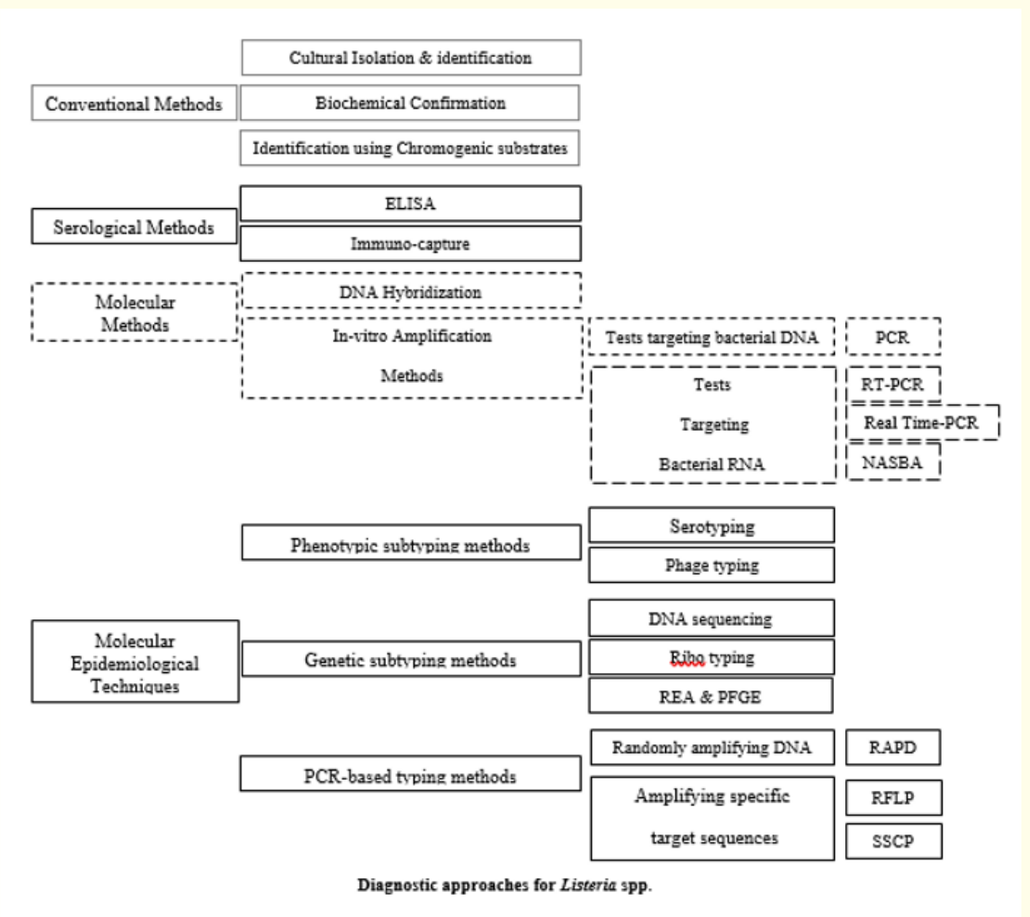
cies level [20], ability to screen large number of samples etc. RAPD typing is likewise a great technique for epidemiological surveillances and has been widely used to connect *L. monocytogenes* isolates separated from listeriosis cases to food that were involved in outbreaks.

RFLP (Restriction Fragment Length Polymorphism) and SSCP (Single-strand Conformation Polymorphism) are the two methodologies that enhance explicit objective sequences and examine PCR amplified products or their RE digests, by looking at lengths of DNA fragments (RFLP) or conformational varieties (SSCP) inside these PCR products. Although, PCR-RFLP approach is rapid, easy to use and interpret, however, its use in epidemiological studies is limited due to its low discriminatory power. On the other hand, SSCP-CE (SSCP-Capillary Electrophoresis) is the most commonly applied gel electrophoresis in investigations using SSCP typing and has also been explored for typing of *L. monocytogenes* [21]. SSCP-CE is a financially savvy choice to direct DNA sequencing, the only other technique fit for identifying SNPs (single nucleotide polymorphisms).

All in all molecular typing techniques are better than phenotypic typing techniques. Although, since inter-laboratory standardization of individual molecular test parameters are frequently missing, there is additionally an absence of expository information for comparative studies. Consequently, current epidemiological studies include the utilization of a few strategies put together both with respect to phenotypic and molecular procedures to effectively characterize involved *Listeria* isolates.

Futuristic approach

DNA microarrays based on DNA or RNA hybridization are existing new technologies which can be used for microbial evolution and epidemiological investigations. They can also serve as a diagnostic tool for environmental, clinical or food testing. There are two principle microarray groups, the first one depends on sequence specific oligonucleotides and the second one utilizes specific PCR end products. The most alluring component of this innovation is the capacity of simultaneous identification and typing of *Listeria* isolates in a single test, an amazing element that none of other tests offer. Notwithstanding, the disservices are that high measures of target DNA or RNA are required to play out the test and high-throughput testing is cost restrictive. However, with the latest development in the diagnostics, it is now conceived that the continuing innovations such as microarrays, biosensors, and next generation sequencing are surely going to offer promise to further improve the rapidity, sensitivity and specificity of laboratory characterization of *Listeria* genus, species, lineages, serovars and epidemic clones.



Diagnostic approaches for *Listeria* spp.

Figure

Conclusion

Historically, for a considerable period, *Listeria* spp. remained unnoticed as a major food pathogen which can be credited to its challenging isolation from food or other samples. But now *L. monocytogenes* is an established food-borne pathogen of public health significance and is widely present in food, environmental and clinical samples. Distinguishing proof customarily included isolation of microorganisms utilizing culture techniques with characterization dependent on morphology of colonies, fermentation of wide range of sugars and haemolytic effects. These are the best quality levels; however they are tedious and may not be appropriate for testing of short-lived food items. Therefore, progressively quick and sensitive serological (ELISA) and molecular (RT-PCR or DNA hybridization) tests were created. These tests give a proportion of cell viability as well as be utilized for quantitative studies. Moreover, an assortment of tests can be utilized for sub-species characterization, which are especially helpful in epidemiological studies. Typing strategies of past separated isolates dependent on phenotypic markers, for example, MLEE, phage typing and serotyping. These phenotypic typing strategies are being supplanted by molecular techniques, which reflect genetic connections among isolates and are progressively exact. These new strategies are at present for the most part utilized in research yet their impressive potential for routine testing later on can't be disregarded.

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