Advanced Diagnostic Techniques for Listeriosis

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ABSTRACT

Listeria monocytogenes is the significant food-borne microbe causing a yearly flare-up of food contamination on the planet. Babies, pregnant moms, and immune-compromised individuals are at high hazard. Due to the epithelial grasp (by E-cadherin restricting), it can smother safe cells and flourish in the gastrointestinal lot till the cerebrum through blood stream. Identification generally elaborate culture techniques in view of specific advancement and plating followed by the portrayal of Listeria spp. in view of morphology, sugar aging and haemolytic properties. These techniques are the highest quality level; yet they are extended and may not be appropriate for testing of food varieties with short time spans of usability. Thus more quick tests were created in light of antibodies (ELISA) or molecular techniques (PCR or DNA hybridization). While these tests have equivalent responsiveness, they are quick and permit testing to be finished within 48 h. All the more as of late, molecular techniques were formed that target RNA instead of DNA, like RT-PCR, or nucleic acid based sequence amplification (NASBA). These tests give a proportion of cell feasibility as well as be utilized for quantitative examination. Furthermore, different tests are accessible for sub-species characterization, which are especially valuable in epidemiological studies. Differential test used were phenotypic markers comprised of multilocus enzyme electrophoresis and serotyping. At present phenotyping techniques are replaced by molecular methods which are more precise and rapid. These new techniques are presently principally utilized in research however their extensive potential for routine testing in the future can't be disregarded.

Key words: Listeriosis, Serology, Polymerase Chain Reaction, Electrophoresis, phenotypic techniques

CITATION

Munir MZ, Zaman SH, Sarfraz-ur-Rahman, Khan JA, Jabbar A, Khan S, Younas M, Yaqoob M, Ullah MR and Hussain I, 2023. Advanced diagnostic techniques for listeriosis. In: Altaf S, Khan A and Abbas RZ (eds), Zoonosis, Unique Scientific Publishers, Faisalabad, Pakistan, Vol 4: 520-530. https://doi.org/10.47278/book.zoon/2023.176

CHAPTER HISTORY Received: 20-March-2023 Revised: 12-May-2023 Accepted: 9-Aug-2023

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1. INTRODUCTION

Listeria is a Gram-positive bacterium that proliferates within cells. It causes food-originated diseases in humans and animals. Spoiled silage frequently leads to this disease in animals, but it is very difficult to identify the root cause of the disease because it is prevalent in the natural environment and farm premises (Orsi et al. 2016). Carrier animals may continuously shed Listeria in their milk and feces (Dehkordi et al. 2013). Listeriosis is an emerging food-borne zoonotic disease that is transmitted through contaminated milk, meat, its products, water, ready-to-eat food, salads, etc. (Meyer-Broseta et al. 2003). Due to changes in life style of human beings, people prefer to take ready-to-eat food so there is a high chance of carrying the Listeria spp. Especially, the immune-compromised people such as old aged, neonates, and pregnant women are affected by this disease. The significant symptoms include septicemia, encephalitis, and abortion (Hunt et al. 2012).

L. monocytogenes is the causative agent of Listeriosis that can persist and reproduce in different climatic circumstances i.e. decreased temperature, high saline concentrations, and low pH (Sleator et al. 2003). The main source of contagion is spoiled fodder, meat, milk, etc. with Listeria spp. Refrigerated products are more susceptible to listeria contamination. With the recent advancement in science, it has been revealed that the mortality due to Listeriosis is higher than all the other food-borne diseases such as Salmonella, Campylobacter, and Vibrio (Behravesh et al. 2011).

Various pathogenic factors are linked with the *L. monocytogenes*. Among these Haemolysin and Lysin O are the most important factors and assist in the escape from the phagocytic defense mechanism of the mammalian cell. Haemolysin factor is encoded by the hlyA gene (Camejo et al. 2011). The iap gene is also essential for invasion into the intestine of the host and this gene is specific for the host to target. For the molecular affirmation of pathogenic factors of *L. monocytogenes* it is essential to target both these genes (hlyA and iap). There is a huge number of *L. monocytogenes* in clinical samples, but it is very difficult to detect in food samples owing to the limited numbers in food items. The food authority in the US has devised a zero-tolerance for Listeria in ready-to-eat (RTE) foods. Therefore, a single bacterium in the RTE is critical and dangerous for consumers and only the PCR techniques can detect this very low level of pathogen in food items (Luber et al. 2011).

Accurate diagnosis of Listeriosis can be made by isolation and identification of bacterium, but it is tiresome and laborious for cultural growth and biochemical characterizations. Different sero-diagnostic tests have been devised to detect listeriosis, but the chances of false positive results are higher. Consequently, now-a-days, ELISA and advanced molecular techniques are preferred as compared to conventional cultural methods so it is the need of the hour to appraise different advanced diagnostic tools for the identification and detection of Listeria spp. in food items and other clinical samples, and precautionary measures should be adopted to prevent the spread of this disease globally. The detail of detection and typing methods mostly used for listeriosis is described in Fig. 1.

A 100 CFU per gram of Listeria in foodstuff is required to be infectious for animals or humans. Because of non-indicated side effects, it is hard to distinguish at the beginning phase. It was observed that the 10 CFU in 25 grams of packed food items caused this disease and 100 CFU per mL led to the reappearance. In this way, researchers fostered a few methods to satisfy the requirement for a vigorous and delicate strategy to distinguish *L. monocytogenes*. The pertinent and accessible techniques for the detection of Listeriosis are mentioned below:

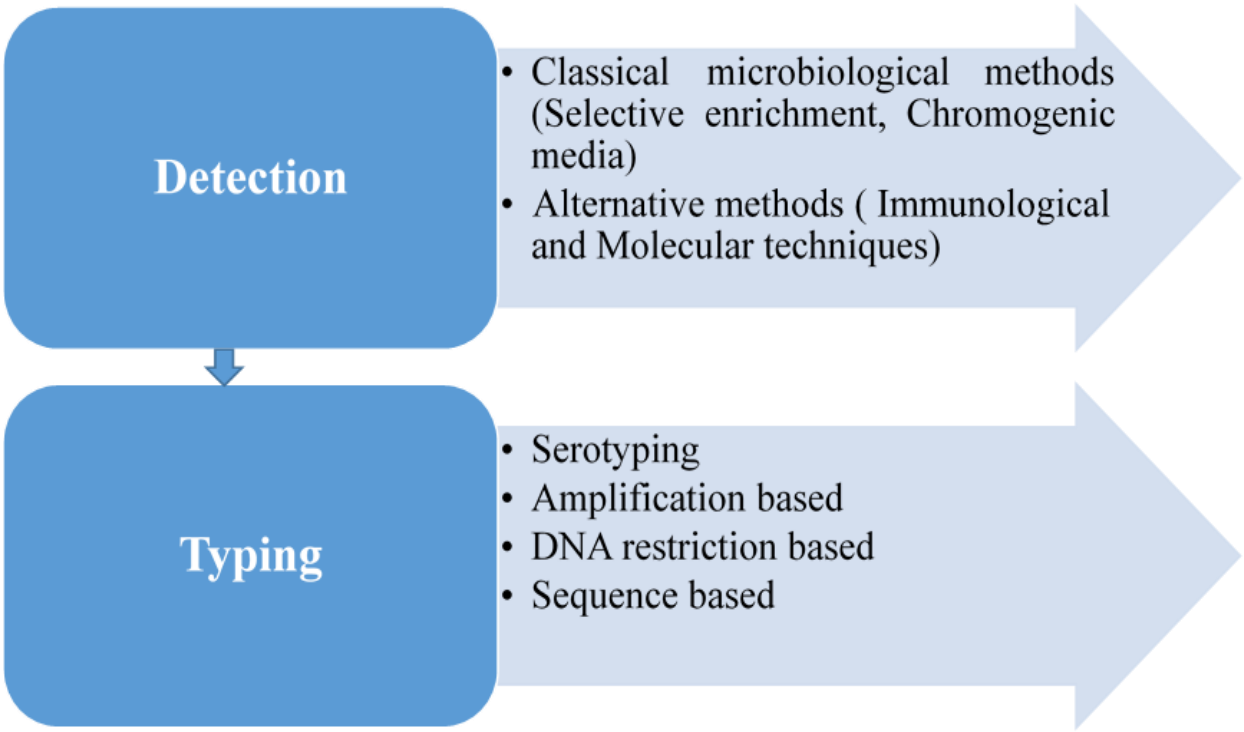

Fig. 1: The detection and typing methods for Listeriosis

1. CULTURE-BASED TECHNIQUES

The tedious yet exact cold advancement technique was made during the 1990s (Lorber 2007). The Food and drug authority (FDA) proposes to use chromogenic medium for the distinguishing proof of Listeria species (Janzten et al. 2006). Lecithin was hydrolyzed, and the blue/green settlements showed up because of the separation of the substrate by a compound β-D-glucosidase. Subsequent to the affirmation of Listeria, it was re-culture in non-particular agar and ready for 5 days in length biochemical analysis. Furthermore, there may be chances of false positive results, a requirement for a few synthetic compounds, media, and reagents, as well as a necessity of time and energy (Jadhav et al. 2012). According to the FDA procedure for isolation of Listeria in milk and fish samples, the Limit of detection (LOD) should be below 1 CFU/mL (Hitchins and Jinneman 2013; Valimaa et al. 2015). The results of Valimaa et al. were identical to ISO 11290-1 technique created in 2004. Afterward, it was revealed that the LOD was 1 CFU per gram through the USDA-FSIS technique (Valimaa et al. 2013). The MPN method was more delicate than a chromogenic media (Dwivedi and Jaykus 2011). To distinguish proof of Listeria, the PCR method was more reassuring than past methodologies i.e., cultural and chromogenic methods (Law et al. 2015).

2. IMMUNOLOGICAL TECHNIQUES

Antigen-neutralizer test was revealed to be capable of identification of listeriosis. As reported the immunological method is more sensitive as compared to conventional techniques, which is 105 cells/mL. However, the preparation of antibody for the immunological reaction is time time-consuming process (Diaz-Amigo et al. 2013).

3. ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

In this assay, the antibody is immobilize to a microtitre plate for capturing antigen, and then a secondary antibody labeled with enzyme was added to identify the antigen. This is a very rapid method for the detection of Listeria spp. mostly for food samples. A recent advancement of the Listeria test facility from food and environment samples within 30 h of receipt. This test is very accurate and delicate, and equally sensitive to cultural methods.

Traditionally, serological methods have been used for the detection of listeriosis but they have been mostly untrustworthy and deficient in precision. Substantial cross-reactivity with other Gram-positive bacteria has been noticed. *L. monocytogenes* is widespread in the environment, and humans and animals are frequently exposed to this bacterium. In humans about 53% of serum antibody against *L. monocytogenes* have been described. A similar pattern has been stated in animals but with minor alterations in different species (Dhama et al. 2015).

Haemolysin and listeriolysin O are the main pathogenic factors. These factors can stimulate an antibody response. Indirect ELISA was used for the detection of anti-LLO in listeriosis but cross-reactivity of LLO with cytolysins has been reported. This is the hurdle in the development of a dependable ELISA test for the detection of listeriosis (Hara et al. 2008).

The Sandwich ELISA technique was better than traditional methods to distinguish Listeria in foodstuff tests (Bell and Kyriakides 2005). The LOD was 105-106 CFU per mL while the counter antigen was utilized to focus on Listeria. Enzyme-linked fluorescence assay (ELFA) is used for the detection of listeria spp. in food samples (Ueda and Kuwabara 2010). Depending upon the sample's acidity and alkalinity, an LOD of 105–106 CFU/mL is established to be precise. For detection of listeriosis, the sera samples were diluted at 1 ratio 200 and used in indirect ELISA. The positive negative ratio was fixed to more than 2 (Malla et al. 2021).

4. IMMUNO-MAGNETIC SEPARATION

In this method concentrated bacterial cells are combined with a magnetic field by using nanoparticles. This technique was used to increase the reliability of detection (Amagliani et al. 2006). The immune-based technique uses anti-Listeria with immune-magnetic nanoparticle coated beads for identification of gene (hlyA) in milk sample. The LOD was found to be less than 102 CFU/0.5 mL (Yang et al. 2007). Additionally, magnetic beads coated with endolysin were used for the detection of Listeria from contaminated raw milk. The LOD range is 102 - 103 CFU/mL (Walcher et al. 2010).

5. MOLECULAR METHODS OF DETECTION

5.1. DNA MICROARRAYS

The bacterium genes plcA, plcB, clpE and inlB can be manipulated for DNA microarray (Volokhov et al. 2002). Volokhov and colleagues described that the detection of Listeriosis was positive through this technique. The scientists explored serotype-explicit test separation by consolidating 585 genomic DNA (10 samples) blended tests and observed that it was effective for 29 tests (Borucki and Call 2003). From that point forward, it was used as a corroborative strategy to take a look at the particularity of polymorphism and PCR enhancement. With an identified breaking point of 8 logs CFU/mL (Brehm-Stecher and Johnson 2007), it was revealed that 9/16 of microarray practiced to experiment falsely tainted milk were disease positive. This method is precise and authentic. However, it needs tolerance and can cross-hybridize, which may lead to a false result (Bang et al. 2013).

5.2. DNA HYBRIDIZATION

It is the simplest technique to identify Listeria spp. in foodstuff. The occurrence of an objective succession is recognized using an oligonucleotide test of correlative grouping to the objective DNA arrangement which encompasses a name for identification. Radioactive elements integrated into an oligonucleotide arrangement were recently used as marks for recognition. biotinylated tests, tests integrating digoxygenin permit identification of target arrangements with identical aversions to radioactive tests, lacking the dangers related to radioactivity. Hybridization in a microtitre plate is a helpful and exceptionally delicate and explicit methodology for the location of Listeriosis in a high quantity (Paniel, N et al. 2013). This test point fundamentally for the separation of different Listeria species by focusing on the qualities of the degenerative factors. Industrially accessible hybridization tests are regularly utilized for the testing of food sources and are widely established for their responsiveness and exactness. Accuprobe is a hybridization of labeled probes to pathogenic factor mRNA, therein only viable cells are identified. This test was established based on in-situ hybridization of labeled probes to target RNA (Umesha et al. 2018). This test has been used so far just for the recognizable proof of Listeria in sewage (Stephan et al. 2003).

5.3. RIBOTYPING

It is based on diversities in ribosomal proteins. This strategy was primarily practiced to lay out phylogenetic relations to organize prokaryotes. Relations of organic entities can be matched by determining how closely DNA sequences resemble a particular feature. The utmost valuable feature for the valuation of phylogenetic relation is the feature coding for ribosomal RNA on the point that ribosomal qualities exists in all organic entities. Frequent copies across the genome and ribosome capability have been dared to be stable over lengthy transformative spans. Ribotyping of Listeria isolates comprises the constrained chemical processing of chromosomal DNA followed by hybridization using an rRNA test. Regarding banding designs, these are utilized to sort Listeria into ribotypes and lay out the likeness of secludes. Ribotyping has been broadly dispensed in epidemiological investigations and computerization has permitted the variation of this strategy for routine investigation. Although this strategy is helpful and provides great reproducibility with the force of separation for *L. monocytogenes*, it does not exactly match with other molecular procedures (Louie et al. 1996).

5.4. RESTRICTION ENZYME ANALYSIS

In this technique, the specific DNA components were sliced through enzymes. The separation of DNA creates pieces of bands with different sizes. These DNA band sizes and quantities were analyzed and visualized through gel electrophoresis. The chromosomal bacterial DNA is referred as restriction enzyme and the exhibition of this technique is fundamentally upgraded by blending with Pulse Field Gel Electrophoresis. Consequently, DNA particles flows under the electrical field. DNA components can be isolated by their sizes. The larger body mass particle travel less whereas the less weight particles move faster in the electric field. Utilizing regular electrophoresis, DNA particles of up to 20 k base may also be isolated by Pulse Field Gel Electrophoresis (Maule et al. 1998). With just slight changes, this procedure can be useful to specify any bacterium. This method is more specific as compared to other composing techniques (Jadhav et al. 2012).

5.5. PCR METHODS

This molecular technique is used for the detection of microbes in samples of the tiniest quantity. Intensity cycles in PCR requisite a bunch of particular introductions for amplification of target region/ gene of interest. The different stages in PCR reaction include denaturation, annealing and extension of DNA (Fig. 2).

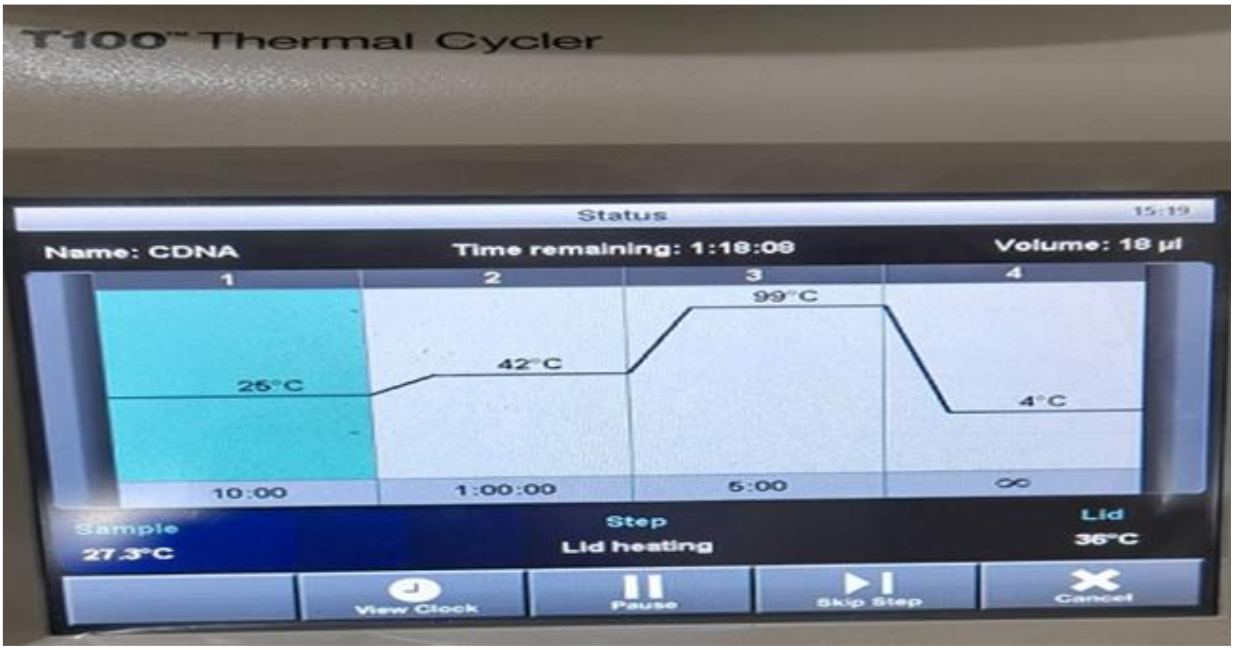

Fig. 2: Thermocycler indicated different cyclic conditions of a PCR reaction

The outcomes are then separated by electrophoresis. The different PCR techniques are used to distinguish Listeria as under:

5.5.1. CONVENTIONAL PCR

Polymerase chain reaction (PCR) is used to amplify the segment of DNA and a very minute amount of target DNA is required for detection. Recently PCR technique is considered to be the most sensitive and reliable for the detection of Listeria spp. The differences among different Listeria *spp* and the primers targeting the specific genes of pathogenic factors have been established. Before proceeding to PCR, it is recommended to selectively enrich the food items as the foodstuff contains the inhibitors.

The result obtained from PCR was affirmative for 56 out of 217 cases in normally spoiled samples. The scientists used a basis intended to focus on the genes coding for pathogenic and different proteins of Listeria for the detection (Aznar and Alarcón 2003).

In non-reasonable DNA enhancement, the PCR strategy proved a misleading positive value (Klein and Juneja 1997). Invert transcriptase PCR was used as mRNA that has a short lifetime and quickly breaks down after cell demise, it also focuses on the feature (hly and PrfA) records as opposed to DNA. To approve the procedure, they utilized cooked meat that was deliberately contaminated. They observed that the diagnosis was delicate to 1 CFU per gram. Skillet and Breidt used constant PCR and made progress with ethidium monoazide to enhance lifeless cells, contending contrary to it as a proficient technique for distinguishing microscopic organisms in low quantities (Pan and Breidt 2007).

5.5.2. DNA SEQUENCING

Sequences are defined as the method for determining the sequence of nucleotide bases in DNA. The nucleotide sequence codes the genetic information that cells use to grow and function. It is essential for assessing the function of genes.

DNA sequencing is the most precise technique for assessing genetic relationships of Listeria spp. Multilocus sequence typing (MLST) has been engaged for sequencing the other genes. This technique is developed for targeting the genes (fla, hly, actA, iap, inl, mpl and prfA) and typing of *L. monocytogenes*. In Navsari Gujarat, a total of 200 samples of food were analyzed and 18 samples were found positive for Listeria spp. The highest prevalence was observed in milk samples (8 Nos.). *L. seeligeri*, *L. innocua*, *L. welshimeri* and *L. monocytogenes* were detected in food items of animal origin. Further, *L. monocytogenes* was tested for pathogenic factors (iap, actA and hly) which showed that high chance of transmission of listeriosis through the consumption of raw milk (Nayak et al. 2015). Various *L. monocytogenes* qualities and their capabilities were clarified and there was truly expanding succession information aggregated in data sets like the data set of the Public Community for Biotechnology which is accessible for observations. The data that the total sequence of *L. monocytogenes* genome is essential and the effect of which is enormous. A business sequencing pack focusing on 16S RNA qualities is accessible from Applied Biosystems (Allerberger 2003).

Epidemiological studies on a worldwide level are significant to research the hazards linked with *L. monocytogenes* genes in food samples. Then, the World Health Organization has found a method to evaluate the hazards that are connected with Listeria in food items. The pattern of epidemiological testing is focused on molecular strategies and in this manner measures should be taken to optimize these tests. In light of the fact that numerous labs use different response conditions or limitation catalysts as well as various test boundaries (Rocourt et al. 2003).

5.5.2.1. MULTIPLEX PCR

Multiplex PCR is used for the detection of many pathogens in the same isolate. This technique is frequently used for food samples as it reduces costs and labor. In the Nested PCR technique, many primers target the same gene. This increases the reliability and sensitivity for the detection of Listeria spp. in clinical isolates of milk and environment.

Multiplex PCR is as a solid, productive, and efficient strategy to diagnose disease in suspected samples (Alarcón et al. 2004). This technique is mostly used to recognize six normal food-originated microbes in ready-to-eat food (Lei et al. 2008). The Multiplex PCR (MPCR) technique focuses on the haemolysin gene of *L. monocytogenes*, the nuc gene of *S. aureus* and the invA gene of *S. enterica*, with a breaking point of 1 CFU per mL (Zhang et al. 2009). The MPCR is vague for the comparative estimated amplicon and advancement (Mustapha and Li 2006).

5.5.3. RT-PCR

In this technique, in the first step mRNA is converted to cDNA through reverse transcriptase enzyme. In the second step, the cDNA is amplified by using target-specific primers and DNA polymerase. Listeria spp. can be detected in meat and waste samples via PT-PCR.

The SYBR green is used for binding dye with DNA. The light is emitted on excitement. The light enhances with the intensity of PCR products. SYBR green is the simplest and most cost-effective dye for use in RT-PCR. The PCR-based examination was created with recognition the breaking point of Colony-forming Unit per 25 grams of food, which is comparable to the ISO procedure (11290-1) for Listeria identification. LOD acquired was 1 × 104 CFU/mL (Kaclíková et al. 2003). It was revealed that the absolute viable count was identified in broccoli (Bhagwat 2003). A hly, PCR examination to identify Listeria was made and used various fixations to spike the sample, and as far as still up in the air to be 8 (Rodriguez-Lazaro et al. 2005). To grow the extent of the procedure, Reverse transcriptase PCR to evaluate the fluorescence transmitted by samples. The got Limit of detection was 10-105 CFU per mL (Berrada et al. 2006). An identification

S. no.	Techniques	Samples	Description	LOD	References
.	Cultural	Foodstuff	Blue/Green colonies of *Listeria*	4 days	Ottaviani et al. 1997
.	BAM	Dairy	Specific for *Listeria*	1 Colony forming unit per mL	Valimaa et al. 2015
.	ELISA	Foodstuff	*Listeria* and other microbes	10^5–10^6 CFU per mL	Bell and Kyriakides 2005
.	Immuno-magnetic tech	Dairy samples	For the detect hlyA gene of Listeria	10^4 CFU per milliliter	Yang et al. 2007
.	Microarrays	Dairy samples	An antigen based probe was used	10^8 CFU per milliliter	Brehm-Stecher and Johnson 2007
.	PCR	Clinical Sample	Primers to target *specific genes*	101 CFU per milliliter	Aznar and Alarcón 2003
.	MP- PCR	Human	Food originated pathogens	7.9×10^1 CFU per milliliter of *Listeria*	Alarcón et al. 2004
.	T- PCR	Foodstuff	For detect *Listeria* from food.	10^4 CFU per milliliter	Kaclíková et al. 2003
.	RT-PCR	Foodstuff	*ssrA* gene amplication	One to five CFU per milliliter	O'Grady et al. 2008
.	qRT-PCR	Meat samples	Reverse transcriptase-PCR	10^2 CFU per milliliter	Suo et al. 2010
.	Biosensor	Antigen of bacteria	immobilize to polyclonal antibodies	tiny detection	Leonard et al. 2004
.	Plasmon resonance	Antigen of bacteria	immobilizing Au-labeled secondary antibodies	10^2 CFU per milliliter	Poltronieri et al. 2009
.	Immuno- sensor	B-lymphocyte	B-lymphocyte cell fused in collagen.	10^2–10^4 CFU per mL	Banerjee and Bhunia 2010
.	Paper multi-biocatalyst	Bacteria	For identification *L. monocytogenes* by using various biomarkers	10^4 CFU per mL	Zhang et al. 2022

technique utilizing SYBR indicates in occurrence of non-specific DNA and dimer development (Fairchild et al. 2006). The focus on the ssrA quality in normally and misleadingly polluted food varieties (milk items, meat, and veggies) brought about an identification cutoff of 1-5 CFU per 25 grams (O'Grady et al. 2008). Subsequently, he decided that it was a shrewd procedure for the specific sample. It was revealed the consequence of a PCR examination with the discovery of furthest reaches of 18 CFU per g on normally and misleadingly defiled ground hamburger and chicken (Suo et al. 2010).

6. BIOSENSOR-BASED TECHNIQUES:

It is a natural sample analyzer utilizing a sample as an item and an electrochemical setup creating comprehensible information. An antibody passed over a chip (biosensor) halted on polyclonal anti-rabbit antibodies (Fab) to identify *L. monocytogenes* (Leonard et al. 2004). For the affirmation of *L. monocytogenes*, the surface plasmon resonance is used and it has LOD is 102 CFU/mL (Poltronieri et al. 2009). In this stage, Au-marked optional antibodies were utilized. On additional progression, it was revealed the utilization of B-lymphocyte converged in collagen lattice as a detecting stage to identify lysin O of Listeria from spoiled foodstuff with a detection cutoff value of 102-104 CFU per gram (Banerjee and Bhunia 2010).

A miniature fluidic gadget that identifies DNA amplicons based on hybridization responses with a immobilized test and biotin signal DNA strands, and catalyzed by a horseradish peroxidase (Lui et al. 2015). In this technique, CL signs created utilizing HRP-luminol framework were uplifted with p-iodine and recognized with CCD framework. The incorporating location of 3 markers was effectively made by two

altered changed working cathodes on a multi-biocatalyst stage. The touchy and solid recognizable proof of *L. monocytogenes* was accomplished by utilizing the versatile multi-biocatalyst stage with a more extensive identification sort and lower limit (Du et al. 2022). Likewise, in 2022 to additional development, the detecting innovation Du et al. fostered a fluorescence-based double acknowledgment gathering utilizing Fe3O4. The direct scope of the discovery of unadulterated culture went from 1.4 × 101 to 107 CFU per mL. Among previously described techniques, the culture methods are normally favored owning to their accessibility, responsiveness, practicality and the highest quality levels contrasted and different techniques that are approved. To sum up, the accessibility and advance of Listeria identification techniques are introduced in Table 1.

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