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Standardization of Portable Culture Device for Detection of Listeria Monocytogenes from Food Samples

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Abstract

Pathogens emerge and spread from a variety of sources including food, water, air, and clinical samples, resulting in disease epidemics in the community. The early detection of pathogens in food and water samples can help prevent illness transmission. Therefore, detection of harmful bacteria is vital in this setting. Food and water-borne illnesses are more common in underdeveloped and emerging countries than in affluent countries. Because of the lack of well-equipped centralized laboratory facilities, many cases are unreported, resulting in an underestimation of the bioburden of the disease. To address the limitations of culture-based approaches, infections are discovered using advanced methods such as nucleic acid or immunological methods, which are costly and require extensive sample preparation. This study focused on the development of a paper-based Portable Culture Device (PCD) capable of detecting Listeria monocytogenes. The device, optimized at a substrate concentration of 3 gL^{-1} and a temperature range of 28°C-37°C, can detect cell counts of up to10^6 CFU ml^{-1}. The development of paper-based microfluidic detection devices has the potential to overcome the limitations of current organism-detection technologies. These devices adhere to ASSURED standards, which require them to be inexpensive, sensitive, user-friendly, quick, resilient, equipment-free, and supplied to the needy. This could help solve many of the issues associated with centralized testing facilities and simplify on-site microbe detection.

Keywords: On-site detection, foodborne pathogens, *Listeria monocytogenes*, Point of care testing (POCT), Portable Culture Device (PCD)

1. Introduction:

Foodborne diseases (FBD) are a major global health concern [1]. Developing and underdeveloped countries witness more cases because, food is exposed to contaminated environments during production, transportation, and storage at retail outlets [2]. Food safety risks are high in India owing to poor food safety controls, lack of monitoring and detection systems, and inability to handle food safety-related issues and disasters [3]. The Integrated Disease Surveillance Programme (ISDP) of the Ministry of Health, Government of India, estimated that 6921 foodborne infections were observed between 2008-17. Places

such as parties, hostel mess, army unit, marriages, etc., where food is cooked in bulk, are a major source of FBD. India spends almost Rs.1,78,100 crore annually, which is around 0.5% of the country's gross domestic product (GDP) for the management of FBD [3]. Most reported cases are caused by *Listeria monocytogenes, Salmonella typhi, Bacillus cereus, Vibrio cholerae, and Escherichia coli* [4].

Listeria is a facultative anaerobic, gram-positive bacterium [5]. It has proven to be a threat because of its high fatality rate (20-30%) [6]. It accounts for 3.8% of foodborne hospitalizations and 27.6% of the deaths worldwide. It is among the top five pathogens responsible for foodborne illnesses [7]. *Listeria monocytogenes* is the most common species in this genus. It is an opportunistic pathogen that is especially important in food because it can survive even in stressful food environments, such as pH, high salt concentration (40% w/v), and temperature [2]. Listeriosis is a sporadic foodborne infection caused by contaminated food sources. The disease can exist as a mild self-limiting infection or as a more severe cause of complications, such as meningitis, septicemia, and abortion in susceptible people [8]. The most common foods reported to be contaminated with *Listeria* are vegetables, meat, sausages, dairy products, smoked fish, salads, refrigerated products, and ready-to-eat products [9]. *Listeria* contamination is more common in vegetables and meat than dairy products.

Conventional culture-based methods involve two basic steps: isolation of the organism on a selective medium and identification based on biochemical characterization [10]. The chromogenic medium utilizes synthetic chromogenic enzyme substrates to aid microbial detection. There are several commercially available chromogenic media. These media function on the basis of enzyme production and sugar fermentation and may also be supplemented with antimicrobial agents to increase selectivity. Phosphatidylinositol-phospholipase C (PIPL-C) is widely used to detect *Listeria* spp [11]. Cultural methods require longer detection times of approximately five–seven days [12]. However, these methods are still used because of their high selectivity and specificity [13]. Molecular detection methods are highly specific because they can detect specific nucleic acid sequences in the target organism by hybridization with short synthetic oligonucleotides. These include polymerase chain reaction (PCR) and loop mediated isothermal amplification (LAMP) [14]. These detection methods are rapid and provide results within 4-24 hours. Nucleic acid-based methods have been used to detect *Vibrio cholerae, Bacillus cereus, Escherichia coli, Staphylococcus aureus, Bacillus anthracis, Salmonella spp., Pseudomonas aeruginosa, Escherichia coli, and Listeria monocytogenes* [15]. Immunological methods for detection include enzyme-linked immunosorbent assay (ELISA), oligonucleotide DNA microarray, immunomagnetic separation-based methods, and immunochromatographic strips [16]. These methods have been used to detect pathogens, such as *Salmonella typhi, Listeria monocytogenes, Campylobacter jejuni, Bacillus cereus, Shigella flexneri, Escherichia coli, and Staphylococcus aureus* [17]. The detection time of these tests can vary from minutes to hours, which is an advantage over traditional culture-based methods [18]. The disadvantages of the process, such as being expensive and dependent on skilled labor and infrastructure, are inherent to the process [19].

Portable culture devices (PCD) can be used for point-of-care (POC) diagnosis and pathogen detection in resource-limited settings [20]. They employ the principles of molecular markers or culture-based detection systems [21]. These devices follow the ASSURED criteria, which means that they should be affordable, sensitive, specific, user-friendly, rapid, robust, equipment-free, and delivered to the needy [22][23]. In addition to being inexpensive for mass production, it is also useful on-site for low-skilled personnel [24]. Funes-Huacca *et al.* designed a culture device consisting of a medium impregnated on paper sheets, a

PDMS layer, and tape, that can be used to cultivate and enumerate *E. coli* in remote resource-limited settings [25] [26].

2. Materials and Methods

1. **Fabrication of PCD:** The devices were fabricated using patterned Whatmann filter paper no. 1, cotton pad as a media reservoir, and masking tape [27][28][29].
2. **Preparation of test culture:** 18 hours old saline suspension of *Listeria monocytogenes* MTCC 657, *Bacillus cereus* MTCC 430, *Salmonella typhi* MTCC 3223, and *E coli* MTCC 4040 was used. The optical density of the culture was adjusted to 1 at wavelength of 600 nm. Twenty microliters of each culture were loaded onto the devices.
3. **Loading the devices and recording results:** The devices were loaded with 15 µL of 5-bromo-4-chloro-3-indolyl-myo-inositol-1-phosphate (X-IP) (SRL Chemicals, Mumbai), 180 µL of Brain Heart infusion medium (supplemented with 1.5% lithium chloride), and 20 µL of saline suspension of the test organism with an optical density of 1 at 600 nm. After incubation at 37°C, the devices were visualized for color development and the intensity was measured using ImageJ® software.
4. **Optimization of detection using a Portable Culture Device:** The fabricated PCD was tested for its ability to detect *L. monocytogenes* using a chromogenic substrate. Standardization was performed with respect to the following points.
   a. **Substrate specificity and concentration**
      The substrate X-IP specific for the detection of *L. monocytogenes* was tested in mixtures and pure cultures against *B. cereus, S. typhi,* and *E. coli* to check specificity. The mixtures were prepared in the following ratios *S. typhi: L. monocytogenes: B. cereus* 1:1:0.5, 1:0.5:1, 0.5:1:1, 1:1:2, 1:2:1, 2:1:1 and 1:1:1. The devices were loaded with 1–5 gL^{-1} of substrate to determine the optimum substrate concentration. All devices were incubated at 37°C for 18 h, and the results were recorded.
   b. **Incubation temperature and time**
      The devices were inoculated and incubated at 37 and 25°C to determine the optimal incubation temperature. Saline suspensions of varying numbers of *L. monocytogenes* were prepared by serial dilution. Saline suspensions were inoculated onto the PCD. The time required for color development was recorded for 24 h at an interval of two hours. Cell numbers in the suspension were determined using Miles and Misra technique.
5. **Proof of concept studies for detection of *Listeria monocytogenes* from spiked food using PCD**
   Dairy products such as milk and ice cream, were used in the analysis. Food samples (50 g) were homogenized, autoclaved, and spiked with 10 ml of saline suspension of *L. monocytogenes*. The devices were inoculated with spiked samples, incubated, and the results were recorded. The same samples were inoculated onto Modified Oxford Agar medium (Himedia, Mumbai). The plates were observed for development of small greyish colonies surrounded by a halo.

3. Results and Discussions

a. Substrate specificity

All devices inoculated with *L. monocytogenes*, either pure or mixed cultures, showed a blue color, as shown in Figures 1 and 2. Results were recorded after 18 h of incubation at 37°C. This indicated that the detection system was specific to *L. monocytogenes.*

Figure 1- Substrate Specificity (from Top to Bottom: Devices inoculated with *S. typhi, L.monocytogenes, E. coli,* and *B. cereus.* Results recorded after 24 h of incubation)

Figure 2 Devices inoculated with mixtures of *S. typhi, L. monocytogenes, E. coli, and B. cereus* in varying proportions. *{S. typhi: L. monocytogenes: B. cereus*- 1:1:0.5 (Row-1), 1:0.5:1 (Row-2), 0.5:1:1 (Row-3), 1:1:2 (Row-4), 1:2:1 (Row-5), 2:1:1 (Row-6), 1:1:1 (Row-7)}. Results are recorded after 18 h of incubation.

b. Optimisation of Substrate concentration

The devices with 1 gL^{-1} and 2 gL^{-1} of X-IP appeared colorless, while those with 3 gL^{-1} to 5 gL^{-1} of X-IP appeared blue after incubation for 24 h (Figure 3). The devices exhibited a gradation in color intensity. The mean grey value for 3 gL^{-1}, 4 gL^{-1} and 5 gL^{-1} was found to be 183.60 ±0.532, 208.63 ±5.92, 216.84 ±0.66 respectively. However, considering the ASSURED criteria 3 gL^{-1} of substrate was used in further experiments.

Figure 3- Optimization of substrate concentration (from Top to Bottom: Devices inoculated with substrate concentrations ranging from to 1-5 gL^{-1}). Results were recorded after 24 h of incubation.

c. Incubation Temperature

To determine the optimum temperature, the devices were incubated at 37 and 25°C. Blue color was observed in all devices; however, ImageJ analysis did not show any significant difference in the intensity of the color. The mean grey value for the devices incubated at 37°C was 71.59 ± 0.92 and at 25°C was 75.94 ± 0.78. The observed results are in accordance with the proposed application of the potential for on-site detection, as it functions equally efficiently at both temperatures.

d. Detection time and limit of detection

The results (Table 1 and Figure 4) indicate that the intensity of the color was directly proportional to the cell number and inversely proportional to the incubation time. The minimum time required for color development was 4 h, and the minimum number of cells that could be detected was 10^6 CFU ml^{-1}. The infectious dose for *L. monocytogenes* is 10^7–10^8 CFU ml^{-1} which is higher than the detection limit of the device. Thus, this device is suitable for the detection of *L. monocytogenes.*

Figure 4: Determination of limit of detection. From top to bottom: Devices inoculated with cell numbers 10^{13} 10^{11}, 10^9 to 10^6 CFU mL^{-1}. Results were recorded after 20 h of incubation

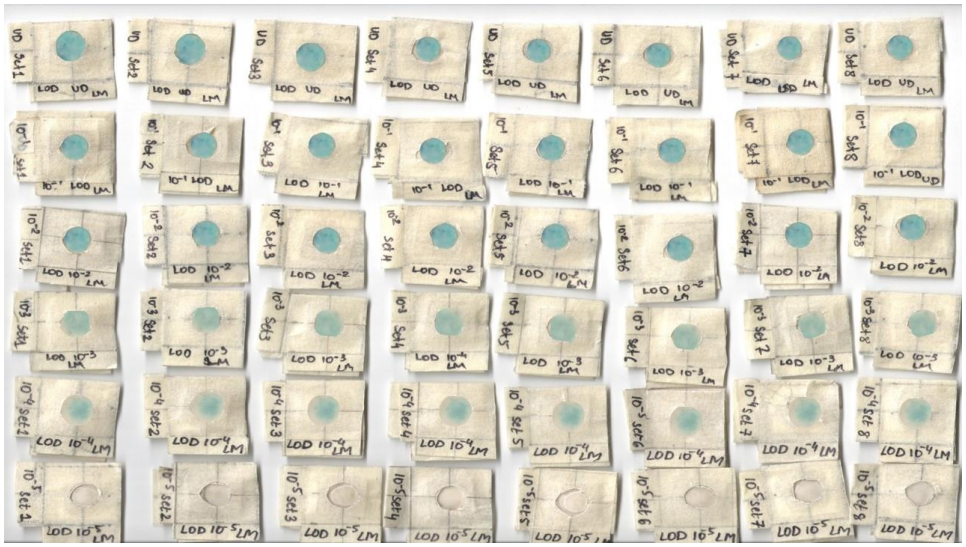

Table 1: Mean gray value measured by ImageJ for devices inoculated with cell numbers ranging from 10^{13} to 10^{6} cells mL^{-1} incubated for 24 h. (Standard deviation = ± 0.15 to 2.23)

Time (h) / Cell number (CFU ml^{-1})	2	4	6	8	10	12	14	16	18	20	22	24
10^{13}	0	76.95	77.41	79.89	79.95	91.32	92.36	92.84	92.95	93.03	93.36	93.65
10^{11}	0	0	0	77.98	78.65	79.41	80.16	91.35	91.57	92.58	92.64	93.48
10^{9}	0	0	0	0	0	74.95	77.47	79.35	79.77	90.43	91.66	91.72
10^{8}	0	0	0	0	0	0	0	75.59	76.85	79.25	79.98	90.65
10^{7}	0	0	0	0	0	0	0	0	0	75.68	76.53	79.02
10^{6}	0	0	0	0	0	0	0	0	0	0	0	76.74

5. Proof of concept studies for detection of *L. monocytogenes* from spiked food using PCD

Food samples and spiked samples (with *L. monocytogenes)* were tested on conventional medium and PCD with a chromogenic substrate for milk and milk-based samples, such as icecream, butter, and cheese. The samples were selected based on literature [30]. Spiked samples showed positive results, whereas samples without spiking were negative for both the conventional growth medium and PCD, as depicted in Figure 5 and Table 2. Hence, a proof-of-concept is established for this device.

Figure 5: Results of samples using PCD for the detection of *Listeria monocytogenes*. Results reported after 24 h.

Table 2: Detection of *L. monocytogenes* in samples using PCD and conventional methods.

Samples	Observations			
	Conventional Method		On PCD	
	Spiked	Sample	Spiked	Sample
Milk	+	-	+	-
Icecream	+	-	+	-
Butter	+	-	+	-
Cheese	+	-	+	-
Key: '+' = Blue colour/Typical colony, '-' = No colour/ No growth				

4. Conclusion

A user-friendly method for detecting of *L. monocytogenes* was developed using a chromogenic substrate on a Portable Culture Device. The device was standardized for substrate concentration, incubation time, and temperature, and the optimum concentration was determined to be 3 gL^{-1}. The optimal time and temperature was determined to be 4 h and 37°C, respectively. This demonstrated that *L. monocytogenes* can be detected using PCD over a period of 4 h. This proof-of-concept was established by detecting *L. monocytogenes* in spiked food samples, proving that the device can be used to detect pathogens in food samples. This concept can also be used to detect other organisms.

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