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Evaluation of raw and pasteurized milk samples for prevalence of *Listeria monocytogenes*

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Abstract
The present study was set out to determine the frequency of *Listeria monocytogenes* in milk. "Two stage enzyme assay for detection of *L. monocytogenes* in milk and milk products" developed at ICAR-NDRI, Karnal was used to analyse a total of 70 samples, 35 of which were each of raw and pasteurized milk. For further assurance, ISO 11290-1:2017 was also used to validate the results. One raw milk sample tested positive for *L. monocytogenes* during the assay examination, a finding that was corroborated by the traditional approach as well. The principle of the assay is colorimetric reaction due to enzyme substrate interaction. The LOD of the developed assay is 1.0 ±0.5 log cfu/25 ml for milk. The detection time taken by developed assay for *L. monocytogenes* and *Listeria* spp. is 4.30±0.15 hours and 2.30±0.15 hours respectively after pre enrichment for 24±1 hours. The determining duration of the developed assay is much less compared to the traditional method which takes 5 days to a week for validation. The results obtained by the developed assay were rapid and had the same accuracy as that of conventional method thus, indicating the wide scope for its application in dairy and food products.

Keywords: *Listeria monocytogenes*, two stage enzyme assay for detection of *L. monocytogenes* in milk and milk products, ISO 11290-1:2017, enzyme substrate reaction

1. Introduction
L. monocytogenes is an opportunistic and may infiltrate food-related items either during or following preparation. Customers might be really at danger from this because fatality rate of listeriosis, which is a disease caused by it is 20-30%. This is significantly greater than that of infections caused by other pathogens (Olaimat *et al.,* 2018) [6].
L. monocytogenes has been linked to dairy products and milk exposure as one of its main causes of spread. The primary reasons of contamination include animals, environment and most notably post-processing contamination (Ulusoy and Chirkena, 2019) [7].
34 of the 2801 samples of milk-related items that the FSSAI recently examined were found to be infected with *L. monocytogenes*. This constitutes about 1.2% of the total samples. Highest contamination was found in the samples collected from Sikkim and it was followed by Rajasthan and Bihar (FSSAI, 2020) [1]. A number of cases reporting outbreaks of listeriosis from developed as well as developing countries throws a light on the urgent need to curb entry of this deadly pathogen in the food chain. As a result, stringent procedures must be implemented to prevent its introduction in diet (Muthulakshmi *et al.,* 2018) [5].
There is now a wide range of detection approaches in use, such as immunological tests, conventional methods and molecular methods. Even though these techniques are frequently employed, they have drawbacks of their own, such as high costs, the need for specialized labor, and infrastructure requirements. As per ISO 11290-1:2017 [2], identifying *Listeria* requires about 5-7 days which includes a series of steps including primary and secondary enrichment followed by isolation on selective agar plates and confirmation by biochemical tests which is very time consuming and labor intensive (Välimaa *et al.,* 2015) [8]. Therefore, the development of assays that are affordable, simple to use, quick, and user-friendly is urgently needed.
Recently enzyme substrate methods for detection have gained a lot of attention. Based on this idea, a "Two stage enzyme assay for detection of *L. monocytogenes* in milk and milk products" was developed at ICAR-NDRI, Karnal. This patented technology is a highly sensitive, rapid and cost effective technique (Kumar *et al.,* 2013, Indian Patent No. 410633) [4].
Therefore, this developed assay was used for the evaluation of the samples taken under study. The results obtained by this method were also validated by ISO 11290-1:2017.

2. Materials and Methods

2.1 Examining samples by "Two stage enzyme assay for detection of *L. monocytogenes* in milk and milk products"

All the samples of milk were procured and kept under refrigeration conditions before testing. No further processing of the samples were done. The samples were mixed well prior to the analysis and the protocol mentioned in (Fig-1) was followed to evaluate the samples. Listeria selective enrichment medium (LSEM) was employed for primary enrichment of samples for 24±1 hours which constitutes the stage 1 of the assay. This medium is highly selective for *Listeria* spp. For assessment, 225 ml of LSEM was filled with 25 ml milk sample, and it was then kept at 37 °C for 24±1 hours until the appearance of black color which indicated the presumptive confirmation of *Listeria* spp. in the sample. All the steps further were done in duplicates and this formed the stage 2 of the assay. To obtain the cell pellet, 6 ml of this blackened LSEM was spun in a centrifuge for 10 minutes at 5,000 rpm. The resultant cell pellet was washed three to four times in 2.0 ml of a buffered phosphate solution (10.0 mM). The pH of buffer used was 6.80. This was done to completely reduce the black components of broth until it became milky white in appearance. 250±50 µl of this buffer containing obtained cell suspension was inoculated in tubes containing 50±2 µl enzyme substrate mixture (ESM). This ESM tubes contains the substrate highly specific for marker enzymes produced by *L. monocytogenes* and *Listeria*. A colorimetric reaction takes place when substrate reacts with targeted marker enzyme secreted by this pathogen. ESM tubes 1 and 2 are designed specifically to recognize *L. monocytogenes* and species of *Listeria* respectively and were incubated at 37 °C. Tube 1 was incubated for 4.30±0.15 hours and tube 2 was incubated for 2.30±0.15 hours. Color change in the tubes were noted after the incubation at specified conditions (Kumar *et al.*, 2013, Indian Patent No. 410633) [4].

2.2 Analysis by ISO 11290-1:2017

The samples found positive by the used assay were further confirmed by the conventional method i.e. ISO 11290-1:2017 [2]. All the suspected samples were subjected to the tests mentioned in the method protocol. The primary enrichment was done initially followed by the secondary enrichment of the sample. Plating of the on selective media and biochemical test helped in validating the obtained outcomes.

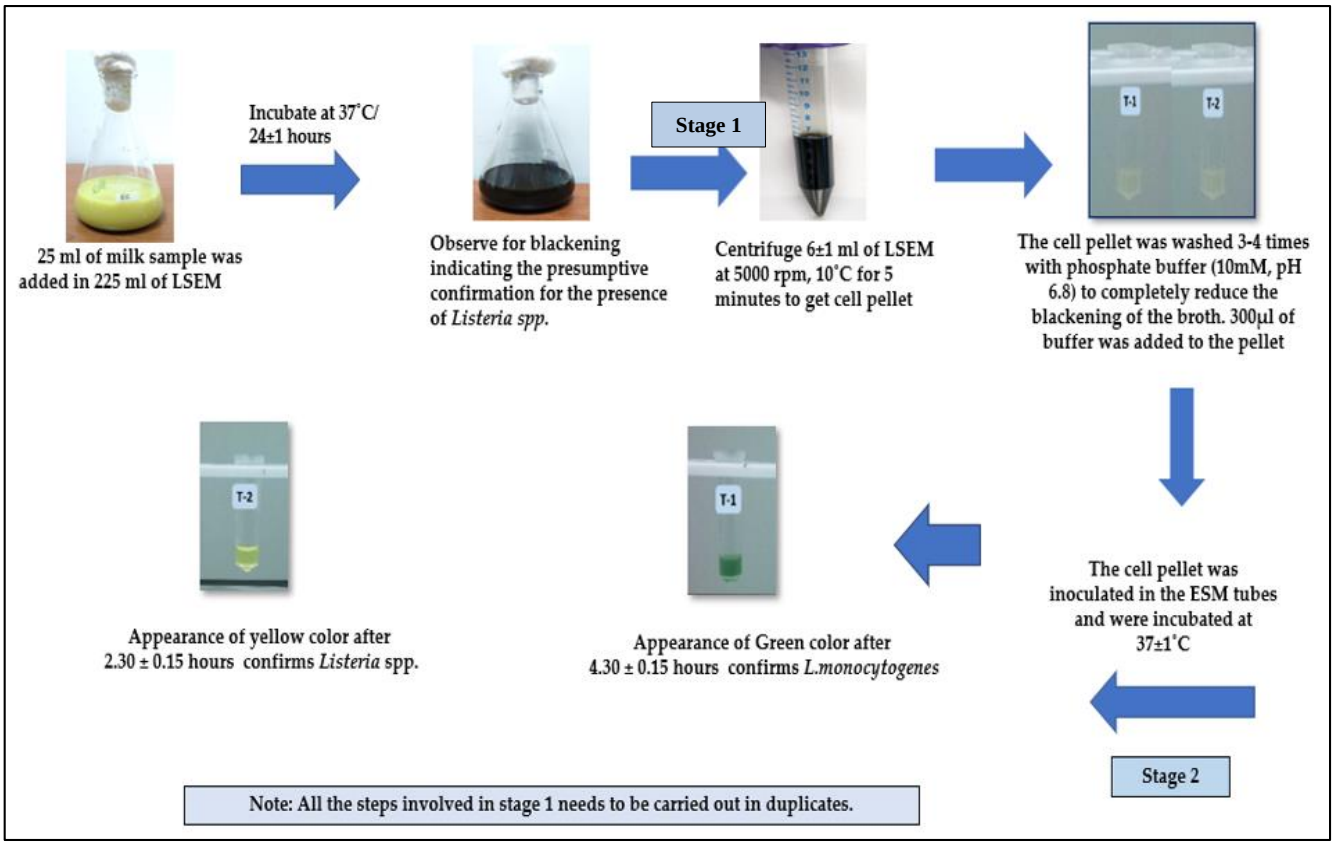

Fig 1: Protocol for "Two stage enzyme assay for detection of *L. monocytogenes* in milk and milk products"

3. Results and Discussion

3.1 Evaluation of the samples by "Two stage enzyme assay for detection of *L. monocytogenes* in milk and milk products"

One raw milk sample, among the total 70, displayed blackening at stage 1 of the analysis, indicating the probable confirmation of *Listeria* spp. presence. Notably, neither of the pasteurized milk samples exhibited blackening during the initial stage, and as a result, they were not subjected to additional analysis. In stage 2, more analysis was done on the raw milk sample that tested positive in stage 1. After incubation at 37 °C green color development was observed in tube 1 after 4 hours and yellow color development was seen after 2.30 hours. In targeted milk sample the formation of yellow color in tube 2 indicated the existence of *Listeria* spp., while the development of green color in tube 1 indicated the inclusion of *L. monocytogenes*.

3.2 Evaluation of the sample by ISO 11290-1:2017

The raw milk sample that was found to be containing *L. monocytogenes* by the developed assay was further evaluated by ISO 11290-1:2017 [2]. Following that, the sample was put through biochemical testing, which verified that *L.*

monocytogenes was present in the sample. On selective Listeria Ottaviani and Agosti Agar (ALOA), the isolated strain displayed blue-green colonies with halo zones and were Gram positive short rods that fermented L-rhamnose and were negative for D-xylose. The isolated strain showed light narrow and clear haemolysis on plates. According to the findings, the strain identified was isolated from *L. monocytogenes*. There have also been reports of *L. monocytogenes* contamination of raw milk, with 16 out of 40 samples (40%) testing positive for this pathogen (Zafar *et al.*, 2020) [9]. While this pathogen was found to be present in raw milk samples at a high rate of 25% in Tamil Nadu, no pasteurized milk samples tested positive for the bacterium (Muthulakshmi *et al.*, 2018) [5]. A further investigation revealed a 5.1% frequency in raw cow milk from Central Indian districts (Kalorey *et al.*, 2008) [3].

4. Conclusions

The results of the current research demonstrated that raw milk was contaminated with *L. monocytogenes*. Since pasteurization effectively lowers the danger of milk contamination with this lethal bacterium, none of the pasteurized milk samples tested positive for its contamination. It is totally undesirable for dairy products to be contaminated with this microbe causing listeriosis due to the high fatality rate. When compared to the conventional method, which is quite time-consuming and difficult, the proposed assay takes substantially less time to identify *L. monocytogenes*. The designed assays results also correlated with those of the traditional approach, proving the assays effective application in food industry.

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