doi: 10.7251/ZSAN2302289D **Original scientific paper**

THE IMPACT OF LEVEL OF DETECTION ON POSITIVE FINDING OF *LISTERIA MONOCYTOGENES* IN MILK

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Abstract: *The bacterium Listeria monocytogenes ingested with food causes the so-called listeriosis, to which groups of the population (pregnant women, children, oncology patients, etc.) are susceptible. The symptoms of listeriosis are very diverse, they can last from days to several weeks, and in some cases, lead to death. The presence of listeria in food is usually associated with ready-to-eat foods, unpasteurized milk, dairy products made from unpasteurized milk, etc. To determine the limit of detection (LOD) of the bacterium L. monocytogenes, four levels of artificial contamination of the initial dilution of ultra-high temperature processed milk (UHT milk) available on the market were prepared. Pre-enrichment and enrichment until streaking on the two solid media were performed according to the modified method ISO 11290-1:2017. Streaking on the ALOA medium was performed only after the enrichment, and the additional streaking on Blood agar plates was performed in parallel. Positive findings, the number of contaminated samples, and the contamination level were processed using the calculation program PODLOD_ver11.xls EXCEL to estimate the POD function and the LOD of a qualitative microbiological measurement method by Wilrich and Wilrich. The obtained results show comprehensible matrix effect statistics, the LOD50 is 0,403 [0,181; 0,899] CFU/initial sample dilution and the LOD95 is 1,742 [0,781; 3,885] CFU/initial sample dilution. The number of positive findings on the ALOA medium is similar to the findings on the Blood agar plates, and consequently, the obtained LOD refers to the method of isolating Listeria from milk for each of the media used. The obtained results contribute to a better understanding of the low level of contamination and the need to test at least five units of the same sample for the detection of L. monocytogenes.*

Key words: *Listeria monocytogenes, milk, the limit of detection (LOD)*

Introduction

Listeria spp. are gram-positive, facultative anaerobes that are widespread in nature and have been isolated from soil, water, plant materials, animals, and their environments [1]. Only seven species of the genus Listeria have been identified until 2010 and recently have been reported that the genus Listeria consists of 26 species [2]. Only *L. monocytogenes* and *L. ivanovii* are of primary concern to humans and ruminants [3].

L. monocytogenes and *L. ivanovii* share many virulence factors and mechanisms of pathogenicity. *L. ivanovii* shows host tropism for small ruminants and rodents and much lower virulence for humans compared to *L. monocytogenes* [4]. However, *L. monocytogenes* is still a major food-borne pathogen responsible for a disease called listeriosis, which is potentially lethal in immunocompromised individuals [5]. The history of listeriosis dates back to the first suspected oral infection with the bacteria observed in animals in 1924 by Murray [6]. In 1981 the first outbreak of listeriosis in Canada was linked to a contaminated food source [7]. The presence of *L. monocytogenes* in food is usually associated with ready-to-eat (RTE) foods including raw vegetables and has been responsible for large-scale food outbreaks worldwide [8,9]. Listeriosis in humans usually manifests as one of three clinical syndromes febrile gastroenteritis, maternal-fetal/neonatal listeriosis, or bacteriemia with or without cerebral infections such as meningitis, meningoencephalitis, rhombencephalitis or brain abscess [10].

Currently, the standard culture method ISO 11290-1 [11] is used to detect *L. monocytogenes* in food. Although this method is far from 100% effective [12], a primary requirement for an alternative, the faster method should be to achieve equivalence with the current standard method [13]. In microbiological methods, accurate determination of the absolute level of contamination is often not possible [14]. Theoretically, one visible cell of the test organisms per tested portion is the limit of detection (LOD), and homogenization of the sample plays a very important role in detecting a particular microorganism.

The experimental design of this work has a goal to investigate and clarify the LOD of the method based on part of ISO 11290-1 [11] (pre-enrichment, enrichment) and isolation performed on ALOA agar required by the standard and additionally on Blood agar in parallel. In addition, the influence of the LOD on the positive finding of *L. monocytogenes* in milk depending on the number of tested unit samples will be explained.

Material and methods

Ultra-high temperature processed milk (UHT milk) (25 ml) was homogenized in 225 ml of Half Fraser enrichment broth (HiMedia) to determine the LOD. An overnight culture of L. monocytogenes (serotype 4b) ATCC 13932 grown on Tryptic Soy Agar (TSA, HiMedia) was used to prepare the suspension for artificial contamination of milk. Material of one separately grown colony is suspended in physiological saline to obtain the density of 0,7 McF corresponding approximately to bacterial suspension of 10^8 CFU/ml. For enumeration of listeria cells, 0,1 ml of dilutions 10^{-5} and 10^{-6} of the bacterial suspension were plated on TSA in duplicate and incubated at 37±1 ºC for 48 h. The obtained result was used to clarify the number of cells at the inoculation levels of samples. UHT milk (nominal volume 250 ml; milk fat 3,2%) was purchased from at local supermarket in Belgrade, Serbia. For the determination of the LOD a test portion of 25 ml was added to the selective primary enrichment medium half-Fraser broth, and homogenized. Four contamination levels

I, II, III, and IV were prepared by adding 1 ml of dilution 10^{-8}, 2 ml of dilution 10^{-8}, 1 ml of dilution 10^{-9}, and 2 ml of dilution 10^{-9}, respectively. Five samples were contaminated, for each level, in total 20 samples. Five samples were not contaminated and were analyzed to prove the absence of L. monocytogenes in the sample.

The primary enrichment medium was incubated for 25 h±1 h at 30 °C. After incubation, 0,1 ml of the initial suspension was transferred to 10 ml of the second enrichment broth (Fraser broth) and incubated for 24±2 h at 37 °C. After incubation, the Fraser broth was loop inoculated onto the surface of ALOA agar (HiMedia) and Blood agar (Torlak). The inoculated plates were incubated for a total of 48±2 h at 37 °C. A characteristic growth on ALOA agar blue-green colonies surrounded by an opaque halo and on Blood agar beige colonies with hemolysis was recorded as a positive finding. Only one colony per inoculation level was tested (microscopic observation, beta-hemolysis, L- rhamnose, and D- xylose) to confirm the presence of the strain.

Sensitivity is calculated as the number of samples found positive divided by the number of samples tested at a given level of contamination. Specificity is expressed as the number of samples found negative divided by the number of samples tested at a given level of contamination.

The LOD50 is the concentration (CFU/test portion) for which the probability of detection is 50%. The LOD95 is the concentration (CFU/test portion) for which the probability of detection is 95%. Excel program PODLOD_ver11.xls by Wilrich and Wilrich [15] was used to calculate LOD50 and LOD95.

Results and discussion

A bacterial suspension of *L. monocytogenes* was used for artificial contamination of the milk sample, and the colonial growth of listeria on TSA was counted and calculated as the number of viable cells (Table 1). The result was used to determine the number of listeria cells present in inoculation levels of the contaminated portion (Table 2).

Table 1. Suspension of *L. monocytogenes* – colony count on TSA

Dilutions	Colony count	
	Petri plate 1	Petri plate 2
0,1 ml of dilution 10^{-5}	187 CFU/plate	175 CFU/plate
0,1 ml of dilution 10^{-6}	16 CFU/plate	19 CFU/plate
Cell number in inoculum	$1{,}8 \times 10^{8}$ CFU/ml	

In the case of *L.monocytogenes*, a safe negative result is available after 96 h (4 days). Positive results are available after 96–144 h (4–6 days), depending on the growth of the bacteria [16]. In the experimental design with artificial contamination analysis, *only one colony per inoculation level is tested (microscopic observation of gram-positive rods, beta-hemolysis +, L- rhamnose +, and D- xylose -) and the presence of the strain is confirmed, the control sample showed no growth of microbes.*

Enrichment in half-Fraser broth followed by subculture in Fraser broth according to ISO 11290-1 [11] was used. The performed two-step enrichment procedure is finished as streaking enriched material on ALOA or Blood agar by a loop. The characteristic morphology of *L. monocytogens* is presented in Figure 1.

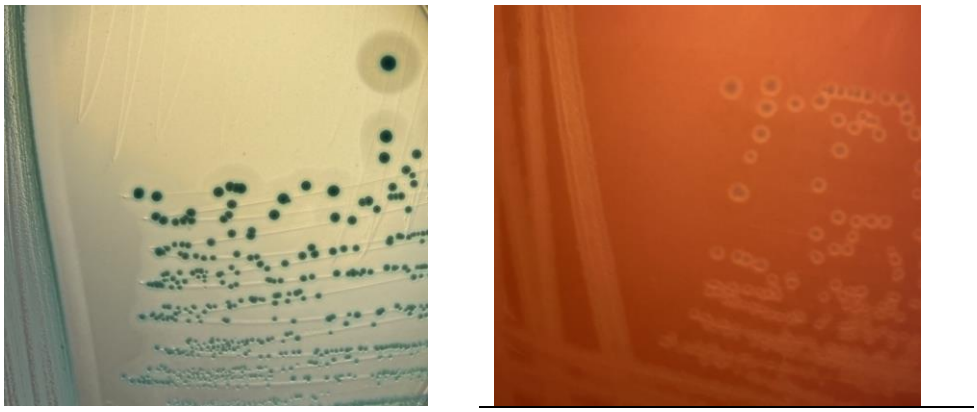

Figure 1. Colonia growth of *L. monocytogenes* on ALOA agar (left) and on Blood agar (right)

The finding of a characteristic colony is noted as a positive finding and as information for expressing sensitivity and specificity. The sensitivity and specificity presented in Table 2 were the same for isolation on ALOA agar and isolation on Blood agar. A sensitivity of 100% was achieved at an inoculation level of 1,8 CFU/250 ml initial suspension.

Scotter et al. [17] reported that sensitivity increased to 100% with the higher inoculum level (50–100 CFU *L. monocytogenes* per 25 g). For reference materials containing approximately 23 CFU, pure culture of *L. monocytogenes*, a slightly higher sensitivity of 89,5% was obtained.

Table 2. Results of *L. monocytogenes* detection

Inoculation level	Viable cells of *L. monocytogenes*/ initial suspension (CFU/250ml)	Number of inoculated samples	ALOA agar			BLOOD agar		
			Positive finding	Sensitivity	Specificity	Positive finding	Sensitivity	Specificity
0	0	0	0	-	-	0	-	-
I	0,18	5	1	20%	80%	1	20%	80%
II	0,36	5	2	40%	60%	2	40%	60%
III	1,8	5	5	100%	0%	5	100%	0%
IV	3,6	5	5	100%	0%	5	100%	0%

However, the sensitivity of the method, when challenged with egg powder, was poor, with only 53,7% of samples correctly identified as positive at the lower

inoculum level and 88,4% at the higher level. The reasons for this disparity in this study are uncertain.

Table 3. Results of the POD LOD calculations

Matrix effect Fi	Log matrix effect fi	SD of log matrix effect sfi	LOD50			LOD95			Test statistic matrix efect Iz_iI
			Detection limit $d_{0,50,I}$	Lower conf. limit $d_{0,50,I,L}$	Upper conf. limit $d_{0,50,I,U}$	Detection limit $d_{0,95,I}$	Lower conf. limit $d_{0,95,I,L}$	Upper conf. limit $d_{0,95,I,U}$	
1,719	0,542	0,401	0,403	0,181	0,899	1,742	0,781	3,885	1,469

Based on our results of positive findings on ALOA and Blood agar, the calculations of POD LOD are similar for both media. Since the matrix statistic was $Iz_iI<2$, according to Wirlich and Wirlich [15], the null hypothesis that there is no matrix effect is accepted.

In general, artificial contamination with the *L. monocytogenes* strain and without background microbes show LOD50 of 0,181 and LOD95 of 1,742 CFU/initial sample dilution (Table 3). In the presented case it is equivalent to a sample of 25 ml in 225 ml broth. The estimated probability of detection according to Wilrich and Wilrich [15] is presented in Figure 3. The results are within in 95% confidence band (Figure 3) which is also a confirmation of their validity.

Besse et al. [18] reported that mean LOD50 values ranged from 0,5 to 1 CFU/25 g, depending on the matrix type. These values are satisfactory for a method expected to detect at least one cell in 25 g, which is the regulatory criterion in EC Regulation 2073/200519 and in other regulatory texts [20,21]. Additionally, the presence of background microbes can make problems in the isolation of *L. monocytogenes* leading to false negative results. Identification of *L. monocytogenes* on chromogenic medium using the standard procedure was impossible if *L. innocua* was able to overgrow *L. monocytogenes* by more than three orders of magnitude after enrichment in model samples. These results were confirmed using naturally contaminated food samples. In conclusion, the standard microbiological method is insufficient for the reliable detection of 10^0 CFU *L. monocytogenes* in the presence of more than 10^0 CFU *L. innocua* per sample [22].

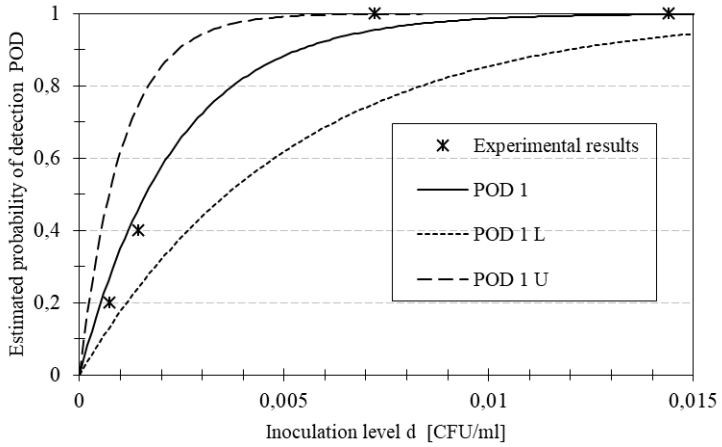

Figure 3. Estimated POD curve and 95% confidence band

The human pathogen *L. monocytogenes* is widespread with infections resulting in a high mortality rate of more than 20% [23] and analysis of the presence of *L. monocytogenes* in food requires special attention. *L. monocytogenes* is widely distributed in soil, rotting vegetation, and animal feces so it can be transferred from the environment to vegetables, including leafy greens, during growth, and wetting by rain and irrigation [24]. It is very important to note that *L. monocytogenes* is an example of a pathogen that can grow and adapt to refrigerator temperatures. Prolonged exposure to low temperatures leads to the selection of genetically stable variants of *L. monocytogenes* at low temperatures [25,26].

Taking into account the above warning as well as presented results, analysis of more than one sample portion is required to test for the presence of *L. monocytogenes*, and current regulations [19,20,21] require testing of five sample units to ensure confidence in the detection of this pathogen.

Conclusion

Methods for the detection of viable *L. monocytogenes* in food are essential for the health safety evaluation of food and timely detection of contaminated food. Based on the results of artificial sample contaminations, the modified ISO 11290-1 showed the same sensitivity and specificity for the isolation of *L. monocytogenes* on ALOA and Blood agar. The distribution of microorganisms in food and the difficult homogenization of samples can contribute to the accuracy of the test results. The performed tests for the presence *L. monocytogenes* on monocultural inoculated and well-homogenized initial sample dilution of UHT milk with no background microbial load indicating that the representative sample must have more than one tested sample unit. It is well known that the cost of analysis increases with the increase in the number of sample units that are tested, but the costs and adverse health effects due to not detecting pathogenic microorganisms in food and making the wrong decision are more expensive due to their consequences.

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UTICAJ GRANICE ODREĐIVANJA NA POZITIVAN NALAZ *LISTERIA MONOCYTOGENES* U UZORCIMA MLIJEKA

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Sažetak: *Bakterija Listeria monocytogenes unesena hranom izaziva bolest listeriozu, na koju su osetljive visoko rizične grupe populacije (trudnice, djeca, onkološki bolesnici,...). Simptomi listerioze su vrlo raznoliki, mogu da traju od nekoliko dana do nekoliko nedjelja, a u nekim slučajevima se završavaju smrtnim ishodom. Prisustvo listerije u hrani obično se povezuje za hranom spremnom za konzumaciju (RTE), nepasterizovanim mlijekom, mliječnim proizvodima od nepasterizovanog mlijeka, itd. Za određivanje granice detekcije (LOD) bakterije Listeria monocytogenes pripremljena su četiri nivoa vještačke kontaminacije inicijalnog razređenja uzoraka sterilizovanog viskokom temperaturom (UHT) mlijeka, dostupnog na tržištu. Predbogaćenje i bogaćenje do presijavanja na dve čvrste podloge provedeno je prema modifikovanoj metodi ISO 11290-1:2017. Presijavanje na ALOA podlogu provedeno je samo nakon bogaćenja, a paralelno je provedeno i dodatno presijavanje na krvne ploče. Pozitivni nalazi, broj kontaminiranih uzoraka i nivo kontaminacije obrađeni su u PODLOD_ver11.xls EXCEL programu za procjenu POD funkcije i LOD kvalitativnih metoda za mikrobiološka ispitivanja, od Wilrich i Wilrich. Dobijeni rezultati pokazuju prihvatljivu statistiku efekta matriksa, LOD50% je 0,403 [0,181; 0,899] cfu/inicijalnom razređenju uzorka, odnosno LOD95% je 1,742 [0,781; 3,885] cfu/inicijalnog razređenja uzorka. Pozitivni nalazi na ALOA podlozi u potpunosti su ispraćeni nalazima na krvnim pločama, te se dobijene granice određivanja odnose na metodu izolacije listerije iz mlijeka za svaku od podloga. Dobijeni rezultati su također doprinos boljem razumijevanju niskog nivoa kontaminacije i neophodnosti ispitivanja najmanje pet jedinica istog uzorka zbog otkrivanja Listeria monocytogenes.*

Ključne rječi: *Listeria monocytogenes, mlijeko, granica određivanja (LOD)*