

Validation of direct plating of a stool sample as a method for *Listeria monocytogenes* detection

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Abstract

The aim of current studies was to validate the direct plating of a stool sample for *Listeria monocytogenes* detection, using selective medium Palcam agar with Palcam selective supplement. Validation was performed using stool samples collected from healthy humans inoculated with *Listeria* sp. strains. Stool samples were frozen to determine the influence of freezing on method robustness. The presented research defines the *Listeria monocytogenes* limit of detection (LOD) as 10³ cfu/g of stools for fresh and frozen samples. Repeatability and reproducibility of the method has been confirmed using statistical methods. We show the effectiveness of direct plating of stool samples on Palcam agar with Palcam selective supplement collected for *Listeria monocytogenes* detection. This method could be useful for this pathogen detection in stool samples collected from patients with diarrhoea.

Keywords

Listeria monocytogenes, faeces, stool seeding, Palcam agar, method validation

INTRODUCTION

According to the obligatory definition of cases in the countries of the European Union (EU), listeriosis is an infection caused by *Listeria monocytogenes*, which may produce any of several clinical syndromes, including stillbirth, listeriosis of the newborn, meningitis, bacteraemia or localised infections. Laboratory criterion for diagnosis is isolation of *L. monocytogenes* from a normally sterile site (e.g. blood or cerebrospinal fluid or, less commonly, joint, pleural or pericardial fluid) [1]. Another form of this disease, which is not included in the EU case definition is intestinal listeriosis, which occurs as sporadic cases or epidemic outbreaks of listeriosis.

For a long time, the role of *Listeria monocytogenes* as a cause of sporadic cases of intestinal listeriosis has not been recognized, especially because this bacterium has been detected in intestinal flora in less than 1% of healthy people [2]. Research performed in Canada in 2002-2004 demonstrated, that this pathogen was detected in 0.21% of 7,775 sporadic cases of diarrhoea. Those results proved that *L. monocytogenes* is not an etiological agent of intestinal listeriosis sporadic cases [2]. Similar results were obtained in studies performed by Müller [3], who examined 1,000 stool samples collected from diarrhoea sporadic cases, and 2,000 of healthy food handlers. *Listeria monocytogenes* was detected respectively in 2.3% and 2.8% of subjects. In the author's opinion, almost the same isolation rate of *L. monocytogenes* in both groups is the result of high exposure of healthy food handlers to these bacteria present in food.

Other situations are foodborne outbreaks. Each year, at least one foodborne outbreak is notified in the EU, where *Listeria monocytogenes* is recognized as an etiological agent. According to European Food Safety Authority (EFSA) data in 2006, 9 outbreaks with 120 cases were notified, of which 74.2% were hospitalised [4]. In 2007, and also in 2008, one outbreak was notified with 21 and 14 cases, respectively [5, 6]. *Listeria*-caused gastroenteritis outbreaks have also been observed in other, non-EU countries [7,8].

To date in Poland, no *Listeria*-caused gastroenteritis outbreak has been notified. In 2010, a gastroenteritis outbreak was reported where *Listeria monocytogenes* was a possible etiological agent. Genetic analysis of the *L. monocytogenes* strains isolated from food samples collected during the outbreak was performed at the National Institute of Public Health – National Institute of Hygiene (NIZP-PZH) in Warsaw. The results of these analyses showed a diversity of isolated strains. Also, detection of *L. monocytogenes* in stool samples collected from patients was performed. All stool samples were negative for *Listeria*. These results showed that the presence of this pathogen in food was a coincidence, not the cause of the outbreak (data not published). Finally, this outbreak was reported as non-recognized.

Also, in the case of immuno-compromised patients and pregnant women, intestinal listeriosis is notified – often as the start of systemic infection [9]. For that reason, all diarrhoea cases in such groups should be tested for listeriosis.

In all mentioned situations, stool samples from at-risk outbreaks participants and other patients have been tested for *Listeria monocytogenes* and other enteric pathogens. In contrast with ISO procedure for *Listeria monocytogenes* detection and enumeration in food samples, there is no standard procedure for isolation of *Listeria* from stool samples [10]. At least 2 isolation procedures for these bacteria

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from stool samples are described in publications. The first is a 'cold-enrichment procedure' which uses the long-term incubation of samples at 4°C in non-selective media. The second method is stool samples seeding on selective media. Doyle and Shoeni compared both procedures and showed the superiority of selective media [11]. Erdogan *et al.* described cold-enrichment as a procedure for *Listeria monocytogenes* isolation from animal faeces. However, that procedure has a serious disadvantage – an extremely long period of incubation [12]. Erdogan *et al.* determined 7 weeks as the optimal incubation period. A long time is acceptable for scientific research; however, in the case of *L. monocytogenes* detection in stool sample collected from persons with diarrhoea, it is unacceptable. Erdogan *et al.* also used selective enrichment in Listeria Selective Enrichment Broth (LSEB) combined with culturing on Listeria Selective Agar (LSA). They obtained high sensitivity and low limit of detection in a short time [12]. Schlech *et al.* [2] used UVM broth containing nalidixic acid and acriflavin hydrochloride for double stool samples for pre-enriching, combined with plating on the selective-enriched media Palcam and Oxford. After 1,000 stool specimens had been processed, they eliminated the pre-enriching step because it did not provide additional sensitivity.

Because of the lack of a properly validated method of direct plating a stool sample for *Listeria monocytogenes* detection, we decided to test Palcam agar with Palcam selective supplement. The aim of presented study was to validate this method for use as a routine procedure for *Listeria monocytogenes* detection in stool samples collected from people with diarrhoea.

MATERIAL AND METHODS

Strains. In the present study, 7 strains were used:

- *Listeria monocytogenes* CLIP 74910
- *Listeria innocua* CLIP 74915
- *Listeria monocytogenes* ATCC 19111
- *Listeria monocytogenes* PZH 1/03 – strain isolated from blood (NIZP-PZH)
- *Escherichia coli* ATCC 25922
- *Enterococcus faecalis* ATCC 29212
- *Staphylococcus aureus* ATCC 25923.

The strains were used for media productivity and selectivity evaluation, according to ISO/TS 11133-1,2 technical specification and procedure validation.

Culture media. For validation of the method for *Listeria monocytogenes* detection in stool samples, the media listed below were used:

- Palcam agar with selective supplement, based on the formulation described by Van Netten *et al.* [13] in versions:
 - ready-to-use medium (marked as 'PALCAM G');
 - home-made media prepared from dehydrated medium – from a freshly opened package (marked as 'PALCAM N');
 - home-made media prepared from dehydrated medium – from a package with a close expiry date (marked as 'PALCAM S');
- Agar Brilliance™ *Listeria*-prepared media on plates (Oxoid);
- Oxford agar prepared media on plates (Oxoid);
- Tryptic Soya Agar (TSA) and Brain-Heart Infusion (BHI) agar from Oxoid, as reference media for media evaluation.

To evaluate the aerobic intestinal flora in stool samples, standard 'home-made' media were used: MacConkey, SS agar, and Hektoen agar.

Establishing inoculum for research. All strains were stored at -70°C in BHI broth with 20% glycerol. 18 h *Listeria* cultures collected from BHI agar plates were used to prepare 2 McFarland standard (2 McF) suspension in 0.85% saline, and diluted up to 10⁻⁷. From 3 dilutions: 10⁻⁵, 10⁻⁶, 10⁻⁷ 0.1 ml of suspension were seeded on BHI agar to calculate the number of colony forming units per 1 ml (cfu/ml) of undiluted suspension.

Evaluation of media used for studies. Evaluation of media used for studies was performed according to ISO/TS 11133-1, 2 norm [14]. Media quality control included estimation of general features (media pH, appearance, colour, surface smoothness) and microbiological features (selectivity, productivity, stability).

Inoculum of *L. monocytogenes* and *L. innocua* for media evaluation was 100 cfu/ml in 0.1 ml of bacterial suspension. For media selectivity evaluation, *E. coli*, *E. faecalis* and *S. aureus* undiluted suspensions were used. Inoculated media were incubated according to ISO/TS 11133-1, 2 and PN-EN ISO 11290-1,2 norms and in the case of PALCAM agar – manufacturer's recommendation [10, 14, 15].

After 18 h of incubation, Productivity Ratio (P_r) and Selectivity Factor (S_f) were calculated for each media. Media specificity for reference strains was tested, defined as presence/absence or intensity level of characteristic biochemical or physiological reactions. Stability of different Palcam agar batches with a different time, was evaluated after first package opening. This feature was defined as in compliance with quality criteria: productivity, selectivity and specificity. To determine the conformity of obtained results (interlaboratory repeatability), 2 series of tests were performed.

Stool samples used for studies. In the presented study, stool samples collected from a healthy child were used. Before inoculation, the samples were tested for the presence of *Listeria* and aerobic intestinal pathogens.

Seeding of stool samples contaminated with *L. monocytogenes* and *L. innocua* suspensions. The aim of this research stage was to designate the limit of detection, repeatability and reproducibility of *Listeria monocytogenes* detection in the stool sample. The limit of detection (LOD) is defined as the minimum number of *L. monocytogenes* cells in 1 g of stools, when at least 1 colony was observed on evaluated media. The repeatability of the method was understood as the accordance of independent results obtained from tests of different samples, performed in the same laboratory, with the same method, but at different time points. The method reproducibility is the accordance of independent results obtained from tests of the same sample, using the same method, performed in the same laboratory, by different analysts.

Listeria strains used for samples inoculation were cultured on BHI agar plates. Bacterial suspension (2 McF) in saline was prepared, where the number of bacteria was determined (in cfu/ml). Stool sample used for this study was divided into 24 portions (6 of each from 4 strains) in sterile Eppendorf tubes. The net weight of stool portions was determined. To

each of them, 0.1 ml of 2McF bacteria suspension were added from 3 dilutions: 10^{-4} , 10^{-5} , 10^{-6} . Inoculated stool samples were plated on Palcam agar. After 48 h of incubation in capnophylic conditions, the number of characteristic for *Listeria* colonies was determined. One or 2 of them were reseeded on BHI agar and identified according to previously published procedure [16, 17]. To determine the repeatability and reproducibility, each portion was plated by 2 operators, and the whole procedure was tripled at time intervals. To assess the quality of aerobic intestinal flora, the inoculated samples were seeded on typical media: McConkey, SS and Hektoen agar.

Seeding of frozen stool samples. Stool sample was divided as above, in 4 repeats. The first part of the samples was plated according to the procedure described above. Then, all samples were frozen at $-23 \pm 3^\circ\text{C}$. Once each week, 1 portion of the samples was thawed, carefully mixed, and plated according to the procedure described above. Once each week, the samples were seeded on media: McConkey, SS and Hektoen agar, to check the quality of aerobic intestinal flora.

Statistical analysis of data. Conformity of results obtained by the 2 operators was assessed by calculation of similar results percentage. The percentage of similar results was assessed by McNemary test. Calculations were performed using SPSS 12.0 software.

RESULTS

During validation, we compared Palcam agar, Oxford agar and Brilliance™ *Listeria* agar as media for *Listeria monocytogenes* detection by direct stool plating. The results of microbiological control of the media with the reference strains are presented in Tables 1 and 2. All media passed the quality criteria of general features (media pH, appearance, colour, surface smoothness) and microbiological features (selectivity, productivity, stability).

Productivity of tested medias was in accordance with ISO/TS 11133-1,2 norm [14]. For BHI agar P_R ranged from 0.96-1.04; for Palcam agar – 89-1.28, and for Brilliance™

Table 2. Selectivity factor (S_F) for medias used for research according to TSA.

Media	<i>E. coli</i> ATCC 25922		<i>E. faecalis</i> ATCC 29212		<i>S. aureus</i> ATCC 25923	
	Cfu*	S_F	cfu*	S_F	cfu*	S_F
TSA	6.9×10^8	N	7.7×10^8	N	7.6×10^8	N
PALCAM G	0	8	0	8	0	8
PALCAM N	0	8	0	8	0	8
PALCAM S	0	8	0	8	0	8
Brilliance™ <i>Listeria</i>	0	8	0	8	0	8
Oxford	0	8	0	8	0	8

* inoculum per plate: average cfu number from two repeats.
N – not used

Listeria – 0.87-1.33. The lowest P_R were obtained for Oxford agar – 0.69-1.04.

The presented research confirmed the suitability of BHI agar as an unselective medium for growing *Listeria*. This media showed almost the same productivity as the reference media – TSA, recommended by PN-EN ISO 1,1290 norm [10]. However, observations made during this research showed the superiority of BHI, as revealed by bigger and easier to isolate colonies.

All *Listeria* strains used for the presented research showed typical *Listeria* morphology. Colonies of *L. monocytogenes* and *L. innocua* on Brilliance™ *Listeria* chromogenic agar were easily distinguishable.

The *E. coli*, *E. faecalis* and *S. aureus* strains growth was totally inhibited on selective media, and rich on TSA and BHI agar (P_R for these strains for BHI agar according to TSA was: 1.07, 0.9 and 1.07, respectively). The selectivity factor for tested media with these strains was 8, where $S_F > 2$ is the correct value.

During whole research, when inoculated stool samples were plated on Palcam agar, very good selectivity of tested media was observed. After 48 h of incubation, only colonies with morphology characteristic for *Listeria* were observed or no colonies at all. All tested samples contain normal aerobic intestinal flora, which was observed on McConkey, SS and Hektoen agar.

The numbers of colonies with morphology characteristic for *Listeria*, re-isolated from samples by each analyst, are presented in Table 3.

Table 1. Productivity ratio (P_R) of assessed media.

Media	Productivity ratio (P_R) criteria	<i>L. monocytogenes</i> CLIP 74910		<i>L. monocytogenes</i> ATCC 19111		<i>L. monocytogenes</i> PZH 1/03		<i>L. innocua</i> CLIP 74915		Researched strain growth description on media
		cfu*	P_R	Cfu*	P_R	cfu*	P_R	cfu*	P_R	
TSA	$P_R \geq 0.7$	71	N	53	N	45	N	27	N	Rich growth, round, smooth colonies. <i>L. m.</i> and <i>L. i.</i> – undistinguishable.
BHI agar	$P_R \geq 0.7$	71	1.00	55	1.04	47	1.04	26	0.96	Rich growth, round, smooth colonies. <i>L. m.</i> and <i>L. i.</i> – undistinguishable. Colonies are bigger compared to TSA.
PALCAM G	$P_R \geq 0.5$	70	0.99	67	1.26	42	0.93	26	0.96	Good growth. Colonies 1-2 mm in diameter, colour from green-grey to black with black halo. <i>L. m.</i> and <i>L. i.</i> – undistinguishable.
PALCAM N	$P_R \geq 0.5$	65	0.92	68	1.28	46	1.02	26	0.96	
PALCAM S	$P_R \geq 0.5$	63	0.89	57	1.08	43	0.96	29	1.07	
Brilliance™ <i>Listeria</i>	$P_R \geq 0.5$	62	0.87	67	1.26	40	0.89	36	1.33	Good growth. <i>L. m.</i> colonies – turquoise colour with opaque white halo around the colony. <i>L. i.</i> colonies – turquoise colour without halo around the colony.
Oxford	$P_R \geq 0.5$	59	0.83	46	0.87	31	0.69	28	1.04	Good growth. Colonies 1-2 mm in diameter, black-grey with small dip in the middle and black halo around the colonies. <i>L. m.</i> and <i>L. i.</i> – undistinguishable.

* inoculum per plate: average cfu number from two repeats

N – not used

L. m. – *Listeria monocytogenes*

L. i. – *Listeria innocua*

Table 3. Limit of detection (LOD) of *Listeria* in 3 repeats of experiment (PALCAM medium).

Listeria strain	1 st plating				2 nd plating				3 rd plating				
	Inoculum (cfu)	cfu / g of stool	No of colonies		Inoculum (cfu)	cfu / g of stool	No of colonies		Inoculum (cfu)	cfu / g of stool	No. of colonies		
			A1	A2			A1	A2			PALCAM	Oxford	
L. monocytogenes PZH 1/03	2.4×10 ⁴	5.4×10 ³	20	14	4.5×10 ⁴	1.2×10 ⁴	13	20	4.9×10 ⁴	2.0×10 ⁴	40	40	12
	2.4×10 ³	6.4×10 ²	12	0	4.5×10 ³	1.9×10 ³	1	3	4.9×10 ³	3.3×10 ³	2	1	0
	2.4×10 ²	5.3×10 ¹	0	1	4.5×10 ²	1.3×10 ²	0	4	4.9×10 ²	2.2×10 ²	0	0	0
	LOD		6.4×10 ²	5.3×10 ¹	LOD		1.9×10 ³	1.3×10 ²	LOD		3.3×10 ³	3.3×10 ³	2.0×10 ⁴
L. monocytogenes CLIP 74910	2.8×10 ⁴	9.3×10 ³	25	16	3.8×10 ⁴	2.9×10 ⁴	6	3	3.1×10 ⁴	1.5×10 ⁴	4	40	7
	2.8×10 ³	6.4×10 ²	8	1	3.8×10 ³	1.2×10 ³	0	3	3.1×10 ³	1.4×10 ³	4	2	0
	2.8×10 ²	8.0×10 ¹	1	1	3.8×10 ²	1.9×10 ²	1	3	3.1×10 ²	1.3×10 ²	0	0	0
	LOD		8.0×10 ¹	8.0×10 ¹	LOD		1.9×10 ²	1.9×10 ²	LOD		1.4×10 ³	1.4×10 ³	1.5×10 ⁴
L. monocytogenes ATCC 19111	2.4×10 ⁴	7.4×10 ³	20	11	5×10 ⁴	1.1×10 ⁴	54	61	4.6×10 ⁴	9.6×10 ⁴	50	30	1
	2.4×10 ³	6.8×10 ²	2	0	5×10 ³	1.3×10 ³	1	4	4.6×10 ³	2.9×10 ³	0	4	0
	2.4×10 ²	9.2×10 ¹	0	0	5×10 ²	1.6×10 ²	1	2	4.55×10 ²	1.8×10 ²	0	0	0
	LOD		6.8×10 ²	7.4×10 ³	LOD		1.6×10 ²	1.6×10 ²	LOD		9.6×10 ⁴	2.9×10 ³	9.6×10 ⁴
L. innocua CLIP 74915	3×10 ⁴	9.0×10 ³	20	11	4.3×10 ⁴	1.5×10 ⁴	19	15	4.9×10 ⁴	2.3×10 ⁴	5	24	9
	3×10 ³	1.2×10 ²	2	0	4.3×10 ⁴	1.5×10 ³	5	4	4.9×10 ⁴	2.9×10 ³	3	0	1
	3×10 ²	8.1×10 ¹	0	0	4.3×10 ⁴	1.4×10 ²	1	0	4.9×10 ⁴	2.5 ×10 ²	0	0	0
	LOD		1.2×10 ²	9.0×10 ³	LOD		1.4×10 ²	1.5×10 ³	LOD		2.9×10 ³	2.3×10 ⁴	2.9×10 ³

A1, A2 – analyst 1, analyst 2.

In the case of first strain – *L. monocytogenes* PZH 1/03, LOD ranged from 5.3×10¹ cfu/g - 3.3×10³ cfu/g; for *L. monocytogenes* CLIP 74910, LOD ranged from 1.9×10² cfu/g - 1.4×10³ cfu/g; for *L. monocytogenes* ATCC 19111, LOD ranged from 1.6×10² cfu/g - 9.6×10⁴ cfu/g; and for *L. innocua* CLIP 74915, LOD ranged from 1.2×10² cfu/g - 2.3×10⁴ cfu/g.

In order to compare Palcam agar with Oxford agar for *Listeria monocytogenes* detection in stool samples, the same sample was seeded onto both media. On Oxford agar, the growth of incidental flora was observed as large, white-grey, irregular colonies. They were recognized

in Gram staining as bacilli. Those colonies often cause a problem with the re-isolation of colonies with morphology characteristic for *Listeria*. The LOD values for Oxford agar are presented in Table 3.

For *L. monocytogenes* PZH 1/03 LOD was 2.0×10⁴ cfu/g, for *L. monocytogenes* CLIP 74910 – 1.5×10⁴ cfu/g, for *L. monocytogenes* ATCC 19111 – 9.6×10⁴ cfu/g and for *L. innocua* CLIP 74915 – 2.9×10³ cfu/g.

Robustness of the method was determined by defining the influence of freezing stool samples on LOD value. Detailed results are presented in Table 4.

Table 4. Limit of detection (LOD) of *Listeria*, in successive weeks of stool samples frozen-storage (PALCAM medium)

<i>Listeria</i> strain	Inoculum (cfu)	cfu / g of stool	No. of observed colonies									
			0 week *		1 week		2 weeks		3 weeks		4 weeks	
			A1	A2	A1	A2	A1	A2	A1	A2	A1	A2
<i>L. monocytogenes</i> PZH 1/03	4.5×10 ⁴	1.2×10 ⁴	13	20	50	43	11	20	42	30	3	12
	4.5×10 ³	1.9×10 ³	1	3	4	2	8	1	8	1	0	1
	4.5×10 ²	1.3×10 ²	0	4	0	0	0	0	0	0	0	0
	LOD		1.9×10 ³	1.3×10 ²	1.9×10 ³	1.9×10 ³	1.9×10 ³	1.9×10 ³	1.9×10 ³	1.9×10 ³	1.2×10 ⁴	1.9×10 ³
<i>L. monocytogenes</i> CLIP 74910	3.8×10 ⁴	2.9×10 ⁴	6	3	26	32	5	4	18	15	6	4
	3.8×10 ³	1.2×10 ³	0	3	5	2	0	0	1	5	1	0
	3.8×10 ²	1.9×10 ²	1	3	1	0	0	0	0	1	0	0
	LOD		1.9×10 ²	1.9×10 ²	1.9×10 ²	1.2×10 ³	2.9×10 ⁴	2.9×10 ⁴	1.2×10 ³	1.9×10 ²	1.2×10 ³	2.9×10 ⁴
<i>L. monocytogenes</i> ATCC 19111	5×10 ⁴	1.1×10 ⁴	54	61	24	31	1	1	18	49	28	12
	5×10 ³	1.3×10 ³	1	4	3	4	4	8	2	7	1	4
	5×10 ²	1.6×10 ²	1	2	0	0	0	0	0	0	0	0
	LOD		1.6×10 ²	1.6×10 ²	1.3×10 ³	1.3×10 ³	1.3×10 ³	1.3×10 ³	1.3×10 ³	1.3×10 ³	1.3×10 ³	1.3×10 ³
<i>L. innocua</i> CLIP 74915	4.3×10 ⁴	1.5×10 ⁴	19	15	22	42	0	0	18	36	0	10
	4.3×10 ⁴	1.5×10 ³	5	4	10	0	0	0	3	3	0	0
	4.3×10 ⁴	1.4×10 ²	1	0	0	0	0	0	0	0	0	0
	LOD		1.4×10 ²	1.5×10 ³	1.5×10 ³	1.5×10 ⁴	>10 ⁴	>10 ⁴	1.5×10 ³	1.5×10 ³	>10 ⁴	1.5×10 ⁴
Quality of aerobic intestinal flora			+++		++		++		++		+	

* – Samples from 2nd repeat used for freezing; they were also 0 week seeding.

+++ – normal intestinal flora (rich growth).

++ – reduced intestinal flora.

+ – significantly reduced intestinal flora (10-20 colonies only in direct seeding zone)

A1, A2 – analyst 1, analyst 2.

During frozen-storage of samples, the LOD for *L. monocytogenes* PZH 1/03 was determined as 1.9×10^3 cfu/g (at only one time LOD was 1.2×10^4 cfu/g). During the 4-week freezing of samples inoculated with *L. monocytogenes* CLIP 74910, the LOD ranged from 1.9×10^3 cfu/g - 2.9×10^4 cfu/g. For *L. monocytogenes* ATCC 19111 the LOD was constant and amounted to 1.3×10^3 cfu/g. For *L. innocua* CLIP 74915, the LOD values varied within a range from 1.24×10^3 cfu/g - 1.5×10^4 cfu/g.

A significant reduction of aerobic intestinal flora was observed in the frozen samples. This effect was especially significant after 4 weeks of freezing, when only 10- 20 colonies were observed per plate.

When the 2 analysts seeded the samples contaminated with 10^4 cfu of *Listeria*, statistical analysis of their results showed an almost 100% similarity. In the case of 10^3 inoculum, the p value in McNemary test was 0.687, which means that no differences in the percentage of positive values were observed. For 10^2 inoculum, the p value in McNemary test was 1, which means that also in this case no differences in the percentage of positive values was observed.

DISCUSSION

The aim of the presented research was to validate the stool samples direct plating on Palcam agar with Palcam selective supplement for *Listeria monocytogenes* detection. Fresh and frozen stool samples inoculated with *Listeria* strains were used. Similar to the present research, Jensen [18] also inoculated stool samples with *Listeria monocytogenes*, and plated them on PALCAM medium with PALCAM selective supplement. Jensen also studied the influence of pre-enrichment in L-PALCAMY liquid medium on the method sensitivity, and the influence of environmental conditions (atmosphere composition or incubation temperature) on results of *L. monocytogenes* detection in stool samples [18]. However, this author did not determine precisely the method's limit of detection (LOD), and did not determine the method repeatability and reproducibility, which was carried out in the presented research. These features are important factors in any diagnostic method evaluation. Statistical analysis of the obtained results confirmed the high repeatability and reproducibility of the validated method. In the presented study, lower LOD values were determined at the 10^4 level, and higher. The extremely high and detached LOD value obtained for *L. innocua* CLIP 74915 in the 3rd seeding repeat (Tab. 1) was classified as a fatal error, and not used in the analysis of results. The median LOD value in the presented study obtained for all strains was 1.04×10^3 cfu/g. This value is one time higher on the logarithmic scale than in Jensen's results (100 cfu/ml). However, the results obtained by Jensen should not be interpreted as an accurate LOD. Jensen inoculated 11 stool samples with constant numbers of *L. monocytogenes* (100 cfu), and from them determined the recovery of *Listeria*. Jensen did not test smaller or lower inoculum to determine the LOD value. Moreover, the methods used by Jensen to evaluate Palcam agar are not in accordance with routine stool plating methodology, and contrast with present research.

Erdogan *et al.* determined the limit of detection of *Listeria* in stool samples as 7 organisms per 1 gram, which is extremely low [12]. The difference between this value and

LOD in our study stems from the different methods used in both researches. Erdogan *et al.* used a procedure with a selective enrichment stage, which was absent in the presented research. In their experiments, the authors mentioned used autoclaved stool samples, *ipso facto* they removed such elements as: physiological flora, various inhibiting factors, e.g. colicins and bacteriophages, which are normally present in stools. All of them could significantly influence *L. monocytogenes* surveillance in stools, and finally on the results of *L. monocytogenes* recovery. This was visible in the present research when bacilli were present on Oxford agar. Moreover, the aim of present study was to validate the procedure, which could be used routinely in the laboratory. It should reflect elements of routine laboratory practice, e.g. pathogens detection in fresh or frozen stool samples.

It is difficult to discuss the minimal limit of detection, which should characterise the method of *Listeria monocytogenes* detection in stool samples. *L. monocytogenes* is a facultative pathogen, which could be present in the stools of healthy people [2,3]. This means that a good LOD method should be more than the number of *L. monocytogenes* in healthy human stool samples which, unfortunately, is unknown. The LOD determined by Erdogan *et al.* is probably lower than the number of *L. monocytogenes* in a healthy human stool sample. This could be useful for scientific purposes, but not for clinical methods. Also not known is the number of *L. monocytogenes* cfu per one gram of stool sample collected from humans with diarrhoea caused by this pathogen. This is important information because diarrhoea is the main recommendation for examination of stool samples for the presence of *L. monocytogenes*. According to information about other enteric pathogens, e.g. *Salmonella* – present in diarrhoeal stool at the 10^8 level, the LOD determined in the presented study – 10^3 – seems to be reliable.

Results obtained during the plating of inoculated stool samples showed a lower selectivity of Oxford medium. Apart from colonies with morphology characteristic for *Listeria*, many others were observed. These observations are contrary to results obtained during the media control, when both media had the same selectivity factor (Tab. 2) in relation to standard strains of *E. coli*, *E. faecalis* i *S. aureus*. However, colonies observed on Oxford medium were recognized as bacilli. This group of bacteria was not used for media evaluation according to manufacturer recommendations [15]. These differences probably do not result from distinct selective supplement formula in Oxford and Palcam media. It could be the result of different atmosphere during incubation: Palcam-capnophilic conditions, Oxford-oxygen conditions [15]. The selectivity of Palcam medium in relation to aerobic flora was enhanced, not only by the selective supplement, but also by culture conditions. Palcam medium, apart from polymyxin B (present in both media), contains third-generation cephalosporin – ceftazidime in the amount of 20 mg per 1 g. This is an effective inhibitory dose for the majority of enteric rods. However, Oxford agar selective factors are colistin and fosfomycin, which are also efficient for enteric rods. Both media contain the same amounts of lithium chloride. The antimicrobial activity of this compound towards aerobic flora has been confirmed many times [19,20]. Despite the similar activity of both media selective supplements, non-characteristic colonies were observed only on Oxford agar. This suggests the effect of incubation conditions on better selectivity of Palcam agar.

Another part of our research was determination of the robustness of the method. We demonstrated that the freezing of stool samples is an efficient method of storing them for *Listeria monocytogenes* detection. During 4 weeks of storage of *L. monocytogenes*, the LOD value in frozen samples was 10^3 cfu per 1g of stools. This level is comparable with plating fresh stool samples, the amount of which was also around 10^3 . The possibility of using frozen stool samples for *Listeria monocytogenes* detection is important for the elaboration of epidemic outbreaks, where enteric rods and some viruses are routinely searched for. The procedures for detection of these pathogens do not allow a search for *L. monocytogenes*. However, the freezing of stool samples has been used as a method of preservation for virusology tests. When typical bacterial etiological agents have not been detected in stool samples originating from people from a foodborne outbreak, there is the possibility to test frozen samples for *L. monocytogenes*.

CONCLUSIONS

The presented results show the effectiveness of direct plating of stool samples on Palcam agar with selective Palcam supplement for *Listeria monocytogenes* detection. The method is characterised by a relatively low limit of detection (LOD), interlaboratory repeatability and reproducibility – also for frozen samples. It means that this method could be used for *Listeria monocytogenes* detection in stool samples collected from diarrhoea cases.

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