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Original Research

Performance Estimation of Nested PCR-Based Assays for Direct Detection of *Listeria monocytogenes* in Artificially Contaminated Materials

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Abstract

Our study evaluated the sensitivity and repeatability of nested PCR-based assays for directly detecting *L. monocytogenes* in artificially contaminated human serum, pasteurized milk and physiological saline samples. The detection of the *hlyA* (267bp) and *iap* (371bp) gene fragments was compared. The logistic regression (logit model) was used to evaluate the probability of detection of *L. monocytogenes* at various contamination levels and to calculate the number of test repetitions required to reach necessary detection limits (e.g. 50%, 80%, 90%, and 95%). The reliable limit of detection for both genetic markers, ensuring ≥95% probability of detection, was established at 10^2 CFU/100μL.

Keywords: *L. monocytogenes*, nested PCR, limit of detection, logistic regression, artificially contaminated specimens

Introduction

Listeria monocytogenes is an environmental gram-positive bacterium and a common inhabitant of the animal intestinal tract [1]. However, it is also an important animal and human pathogen responsible for focal and systemic infections, known as listeriosis [1, 2]. In 2005, listeriosis was the fifth most common zoonotic infection in Europe, after *Campylobacter*, *Salmonella*, *Yersinia*, and VTEC infections [3]. Listeriosis is a predominantly food- or feedborne disease (so-called *silage sickness*), occurring worldwide in both sporadic and epidemic forms. Virtually all domestic animals are susceptible to infection, although outbreaks most frequently have been observed in sheep, cattle and goats [1]. In humans, an invasive listeriosis is primarily an opportunistic infection affecting persons with depressed cell-mediated immunity, such as the young, old, pregnant, immunocompromised individuals (so-called YOPI group), and usually occurs as meningitis, blood, fetoplacental and neonatal infections [2, 4]. In contrast, non-invasive listeriosis (or listerial gastroenteritis) is observed in people with no predisposing conditions. Therefore, according to the ICMSF (*The International Commission on Microbiological Specifications for Foods*) guidelines *L. monocytogenes* is classified in two out of the four categories of foodborne pathogens: category 2 (serious hazard, incapacitating but not life-threatening) and category 4 (3B) (severe hazard for restricted populations, life-threatening) [5].

Although listeriosis is still a relatively rare disease, *L. monocytogenes* is an important health concern in humans for several reasons. Firstly, the ubiquitous presence of these

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bacteria in various environments (e.g. soil, water, decaying plants and sewage) contributes to relatively frequent contamination of a wide range of raw (e.g. vegetables, meat, milk) and processed foods [1, 4, 6]. In addition, *L. monocytogenes* can multiply and reach high numbers even in refrigerated foods. Secondly, unlike infection with other common foodborne pathogens, invasive listeriosis is associated with a high mortality rate of 30-40% [4]. Finally, the conventional culture and identification methods, which are "gold standard" in the detection of *L. monocytogenes,* require a long time period (4-8 days or more) [7].

PCR-based methods, so-called diagnostic PCRs, have a great potential for rapid detection of pathogens in various samples [8]. Unfortunately, the lack of uniform nucleic acid isolation procedures, problems with elimination of PCR inhibitors from samples, and the great variety of PCR techniques, to mention only a few, impede the standardization of these methods [9]. Moreover, some uncertainties relating to the PCR sensitivity (a limit of detection) and methods of its evaluation have frequently caused controversy over results [10]. In consequence, the performance of PCR-based assays has not been evaluated systematically, and these assays often remain poorly estimated statistically. However, knowledge about the detection probability of target pathogens at various concentrations by PCR-based assays is essential for their application as routine diagnostic tests [9, 11-13].

Therefore, in the present study, the probability of *L. monocytogenes* detection in artificially contaminated human serum, pasteurized milk, and physiological saline samples at various contamination levels by nested PCR-based assays was assessed using the logistic regression (logit model) and/or the Spearman-Kärber method [14, 15]. In addition, the performance of two nested PCRs for detecting *hlyA* and *iap* gene fragments specific for *L. monocytogenes* was compared.

Experimental Procedures

Bacterial Strains and Materials

A total number of 20 *L. monocytogenes* strains (serotypes 1/2a, 1/2b, 1/2c, 3a, 4b, 4c, and 7) from ATCC and CCM collections, including 7 strains of a clinical origin isolated in the Department of Microbiology of the Medical University of Białystok, were used in the study. In addition, *L. ivanovii* ATCC 19119 and *L. innocua* ATCC 30090, as well as 15 strains representing 8 species of Gram-positive (*S. aureus* ATCC 29213 and 25923, *S. epidermidis* ATCC 12228, *E. faecalis* ATCC 29212 and 49477, *E. faecium* ATCC 49474, *S. pneumoniae* ATCC 49150, *B. subtilis* ATCC 6633, *S. pyogenes* ATCC 19615, and *Corynebacterium* spp.) and 4 species of Gram-negative bacteria (*E. coli* ATCC 10536 and 25922, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 700603, and *K. oxytoca*) were included. The following materials artificially contaminated by *L. monocytogenes* serotype 1/2a and/or other bacteria were used: human serum (O Rh- obtained from healthy donors; Regional Blood and Hemotherapy Center in Białystok), pasteurized milk and sterile physiological saline.

Cultivation and Enumeration of Bacteria

L. monocytogenes strains were cultured on tryptose agar (*Difco*) at room temperature for 24h. Appropriate bacterial concentrations from 10^{-1} to 10^{-6} CFU/mL were obtained via serial 10-fold dilutions in sterile physiological saline. According to the experiment design, suspensions containing only *L. monocytogenes* or in combination with other Gram-positive and Gram-negative bacteria, were prepared and used to contaminate materials. To isolate bacterial DNA, 100 µL of contaminated materials was used.

The number of *L. monocytogenes* in samples was enumerated by the colony-counting technique using a calibrated loop (10 µL). The concentration of *L. monocytogenes* in pasteurized milk samples artificially contaminated was counted on the selective agar plates.

DNA Isolation

Bacterial genomic DNA was isolated using the Genomic DNA PrepPlus kit (A&A Biotechnology) based on the property of DNA to adsorb to the silica surface at a high concentration of chaotropic salts. The DNA isolation was preceded by incubation of samples with lysozyme (Fluka) (20 mg/mL in TE buffer pH 8.3) at 37ºC for 45 min. and performed according to the manufacturer's instructions. Briefly,

(i) bacterial cells were lysed using a special lysing solution and Proteinase K;
(ii) loaded onto a spin column with a silica filter;
(iii) after several washing steps contaminants were removed;
(iv) and finally pure DNA was eluted in 100µL of Tris buffer (10 mM Tris-HCl; pH 8.5).

PCR Conditions

Both rounds of nested PCR were performed in 50 µL of PCR mixture containing of 1x PCR buffer (10 mM Tris-HCl pH 7.5; 50 mM KCl; 1.5 mM $MgCl_2$) in deionized water; 200 mM of dNTP mix (dATP, dGTP, dCTP, dTTP); 1U of Taq polymerase (MBI Fermentas); and the appropriate concentration of each primer (see below). For the first PCR round, 10 µL of the isolated DNA was added and 5 µL of the first PCR mixture after amplification was used as a template to perform the nested PCR round. Amplification was performed in the Mastercycler® gradient thermocycler (Eppendorf). The PCR primer pairs were selected from the following publications: for the *hlyA* [16] and *iap* gene [17].

DNA of *L. monocytogenes* serotype 1/2a was used as a positive control. For each PCR reaction, two negative controls were included:

(i) for DNA isolation, and
(ii) PCR mixture preparation processes.

In addition, all non-contaminated pasteurized milk samples were also analyzed by both nested PCRs.

Table 1. The relationship between the log concentration of *L. monocytogenes* (log CFU/100 µL) and the number of *hly*A and *iap* nested PCR-based repetitions required us to obtain the specified limit of detection.

log CFU/100 µL	Logit probability for the *hlyA/iap* nested PCR			The number of nested PCR-based repetitions required to achive the specified limit of detection (*hlyA/iap*)											
	Material			Serum				Milk				NaCl			
	Serum	Milk	NaCl	50%	80%	90%	95%	50%	80%	90%	95%	50%	80%	90%	95%
0.5	0.12/0.1	0.1/0.03	0.13/0.13	5/7	13/15	18/22	23/28	7/23	15/53	22/76	28/98	5/5	12/12	17/17	22/22
1	0.47/0.49	0.36/0.26	0.56/0.54	1/1	3/2	4/3	5/5	2/2	4/5	5/8	7/10	1/1	2/2	3/3	4/4
1.5	0.84/0.88	0.75/0.78	0.91/0.90	1/1	1/1	1/1	2/1	1/1	1/1	2/2	2/2	1/1	1/1	1/1	1/1
2	0.97/0.98	0.95/0.97	0.99/0.99	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1

hlyA Nested PCR

In the first round of PCR, primers LM1 (5'-CCTAAGACGCCAATCGAA-3') and LM2 (5'-AAGCGCTTGCAACTGCTC-3') at a final concentration of 0.1 µM were used to amplify a 702 bp fragment of the *hlyA* gene. The following amplification conditions were used: an initial denaturation at 95˚C for 3 min., followed by 30 cycles consisting of denaturation at 94˚C for 1 min., primer annealing at 60˚C for 1 min., and DNA extension at 72˚C for 1 min. The reaction was completed by a final extension at 72˚C for 5 min. and cooling PCR products at 4˚C. The same conditions, except for the primer annealing temperature 55˚C, were used in the nested PCR round with internal primers LL5 (5'-AACCTATCCAGGTGCTC-3') and LL6 (5'-CTGTAAGCAATTTCGTC-3') for amplifying a 267bp fragment of the *hlyA* gene.

iap Nested PCR

In the first round of PCR, primers UniLisA (5'-GCTACAGCTGGGATTGCGGT-3') and LisB (5'-TTATACGCGACCGAAGCCAA-3') at a final concentration of 0.15 µM were used to amplify a 1456 bp fragment of the *iap* gene. After initial denaturation at 95˚C for 3 min., the reaction was carried out in 30 cycles consisting of denaturation at 94˚C for 1 min., primer annealing at 57˚C for 1 min. and DNA extension at 72˚C for 1 min., followed by a final extension at 72˚C for 5 min. in the last cycle and cooling PCR products at 4˚C. The same conditions, except for the primer annealing temperature of 56˚C and DNA extension time of 45 s, were used in the nested PCR round with internal primers MonoA (5'-CAAACTGCTAACACAGCTACT-3') and MonoB (5'-GCACTTGAATTGCTGTTATTG-3') at a final concentration of 0.17 µM for amplifying a 371bp fragment of the *iap* gene.

The PCR products were detected by agarose gel electrophoresis (stained with ethidium bromide) and visualized under UV transilluminator (Gel Doc 2000; Bio-Rad). The DNA band size was estimated using the DNA size marker (GeneRuler 100bp DNA Ladder Plus; MBI Fermentas) and Quantity One software (Version 4.2.2; Bio-Rad).

The Detection Probability of the Nested PCR-Based Assays

In the present study, the sensitivity of nested PCR-based assays described above was examined in two integrated parts. During the first part a broad range of dilutions in the study materials, between $<10^1$ to 10^6 CFU/µL (Fig. 1), was used to establish lower and upper concentrations of *L. monocytogenes,* for which nested PCR gives repeatable negative and positive results. Three such concentrations, between $<10^1$ to 10^3 CFU/µL, were determined based on 7 and 3 independent nested PCR-based experiments for the detection of the *hlyA* and *iap* gene, respectively. In the second part, 10 repetitions of these three dilutions were tested in 3 independent experiments (a total of 30 repetitions for each dilution). In the final statistical analysis, the data generated from these 37 and 33 experiments (Table 1) were used to assess the probability of detection at various concentrations of *L. monocytogenes* in the study samples, using the logistic regression (logit model).

Since the results of qualitative PCR can be expressed simply as positive or negative, statistical methods of modeling the probability of binary responses, such as logistic regression, can be used to describe the relationship between the proportion of PCR-positive results (the probability of detection) and the corresponding log concentrations of target DNA/cells in the sample. For example, the probability of *L. monocytogenes* detection by a single nested PCR-based experiment (the dependent response variable) with regard to its log concentration in the sample – log CFU/100 µL (the independent predictor variable) can be expressed by the following equation:

$$p(x) = [\exp(\alpha + \beta x)]/[1+\exp(\alpha + \beta x)] \quad (1)$$

...where x is the log concentration of *L. monocytogenes* in a sample (log CFU/100 µL) and α, β are regression coefficients, which were estimated using the maximum likelihood method using Statistica 8 software.

Furthermore, the calculated logit probability values of a positive response at a given log concentration of *L. monocytogenes* were used to estimate the number of nested PCR

experiment repetitions required to obtain certain levels of sensitivity (or limits of detection; LOD) (e.g. 50%, 80%, 90%, and 95%), according to the following formula:

$$probability\ of\ at\ least\ one\ positive\ result\ in\ n\ repetitions = 1 - (1\text{-}logit)^n \quad (2)$$

In addition, the values of $LOD_{50\%}$ (50% limit of detection; or 50% probability of a false negative results) were independently assessed by the Spearman-Kärber method [14].

The log concentrations of *L. monocytogenes* that can be detected with the probability of ≥90% (LOD_{90}) were taken to define the practical/diagnostic sensitivity of nested PCR-based assays tested in the study.

Results

The 37 *hlyA* nested PCR-based experiments showed no significant differences in the proportion of positive results in detecting *L. monocytogenes* at concentrations of 10^1 and 10^2 CFU/100 µL (1 and 2 log CFU/100 µL) in the study materials. In contrast, the 33 experiments for detection of the *iap* gene revealed the significantly lower proportion of positive PCR responses for pasteurized milk samples versus human serum (χ^2; p=0.0439) and physiological saline solutions (χ^2; p=0.0128) in detecting *L. monocytogenes* at a concentration of 10^1 CFU/100 µL (1 log/100 µL). Differences between detection of the *hlyA* and *iap* gene were found only for pasteurized milk and physiological saline solutions (χ^2; p=0.0068). Fig. 2 shows the relationship between probability of detecting with regard to *L. monocytogenes* log concentration in all study materials by the *hlyA* and *iap* nested PCR-based assays generated from the logistic regression.

The detection limit of both nested PCR-based assays was estimated at 10^1 CFU/100 µL (1 log/100 µL) based on the experimental data. However, from the logit model, the detection probability at this concentration was assessed by a single *hlyA* and *iap* nested PCR experiment to: 47% and 49% – human serum, 36% and 26% – pasteurized milk, 56% and 54% – 0.9% NaCl, respectively (Table 2). Therefore, this concentration level should not be treated as practical/diagnostic sensitivity of *hlyA/iap* nested PCR-based assays as several repetitions are required to produce a satisfactory limit of detection, e.g. 4/3 – human serum, 5/8 – pasteurized milk, and 3/3 – 0.9% NaCl to have 90% limit of detection (LOD_{90}).

The logistic regression model employed in the study showed that intermediate concentration of 1.5 log/100 µL ensured ≥95% probability of detecting *L. monocytogenes* in human serum and pasteurized milk samples, but only under the condition of two repetitions. The detection probability for this concentration, for example in pasteurized milk by the *iap* nested PCR-based assay, could be read from the graph (Fig. 2) or more precisely calculated from the following equation:

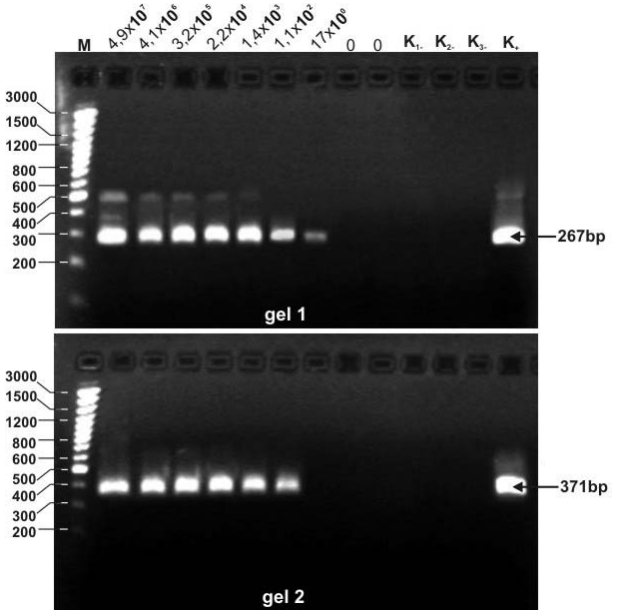

Fig. 1. Results of nested PCR for detecting a 267bp fragment of the *hlyA* (gel 1) and a 371bp fragment of the *iap* gene (gel 2) in artificially contaminated by *L. monocytogenes* serotype 1/2a pasteurized milk samples. K_1 – milk control, K_2 – negative control for DNA isolation process, K_3 – negative control for PCR mixture preparation process, K+ positive control, M – DNA size marker.

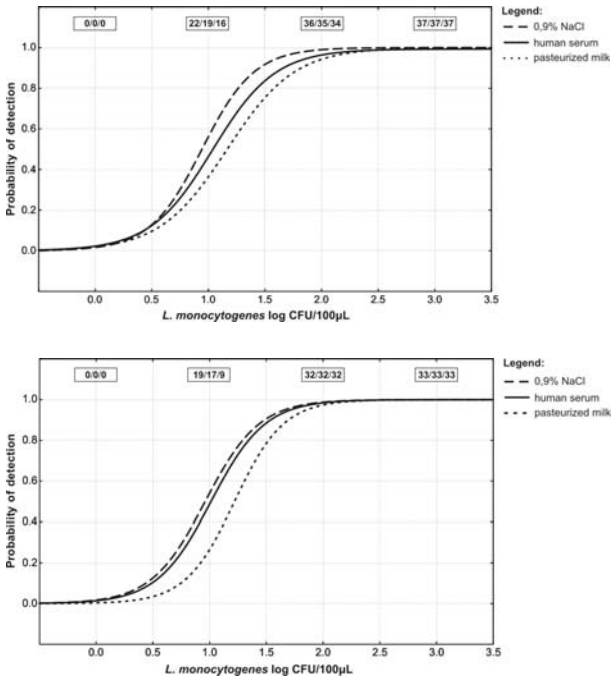

Fig. 2. The probability of *L. monocytogenes* (log CFU/100 µL) detection in various artificially contaminated specimens by nested PCR-based assays for detecting the *hlyA* (the top graph) and *iap* gene (the bottom graph).
The probability was evaluated using the logit model, based on 37 and 33 experiments, for the *hlyA* and *iap* gene, respectively. The numbers in rectangles along the top of the graph indicate the number of positive PCR results.

Table 2. Comparison of LOD_{50} values calculated from the logistic regression (logit model) and the Spearman-Kärber method.

LOD_{50}	Results of nested PCR					
	hlyA			*iap*		
	human serum	pasteurized milk	0.9% NaCl	human serum	pasteurized milk	0.9% NaCl
Logit model	1.03	1.14	0.94	1.01	1.22	0.96
Spearman- Kärber	1.04	1.17	0.90	1.01	1.25	0.95

$$y = \exp(-5.6485 + (4.63069)\cdot x) / (1+\exp(-5.6485+(4.63069))$$

...to be 78%.

In conclusion, from a diagnostic point of view, the sensitivity of both nested PCR-based assays ensuring 90% probability of *L. monocytogenes* detection in human serum and pasteurized milk samples should be assessed at 1.5 log/100 µL; if the assay is repeated twice the probability can increase to at least 95% (Table 1). So, at only a concentration of 2 log/100 µL, a single experiment is enough to obtain ≥95% detection probability in both human serum and milk samples.

The values of LOD_{50} calculated from the logit model for the *hlyA* and *iap* nested PCR were as follows: 1.03 and 1.01 log CFU/100 µL – human serum; 1.14 and 1.22 log CFU/100 µL – pasteurized milk; 0.94 and 0.96 log CFU/100 µL – 0.9% NaCl. Similar LOD_{50} values were obtained by the Spearman-Kärber method (Table 2).

The presence of bacteria other than *L. monocytogenes* in the study samples at a concentration of 10^5 CFU/100 µL had no influence on the sensitivity of nested PCR used in the study (data not shown).

Discussion

Once *L. monocytogenes* was identified as a foodborne pathogen in the 1980s, quantitative limits (e.g. <100 CFU/g) or even "zero tolerance" policies were introduced for its presence in certain food products [6]. Unfortunately, the isolation of *L. monocytogenes* at such low concentrations from samples often containing a high level of background microflora remains a huge diagnostic dilemma [7]. Since the limit of *L. monocytogenes* detection by direct sampling on selective agar media is >100 CFU/g [18], an enrichment step in broth-selective media (e.g. Fraser broth, LEB) with the following subculturing to agar media is essential for *L. monocytogenes* recovery [7, 18]. Therefore, at least 3 days are required to get a negative result and at least 4 additional days for biochemical and/or serological identification to confirm a positive result [18]. PCR as a highly sensitive technique is a promising alternative for such conventional diagnostic methods, ensuring simultaneous detection and identification of pathogens in less than a few hours. Indeed, the use of PCR-based assays to detect pathogens in food, environmental and clinical samples has become widely adopted during the last two decades [8].

It should be emphasized that amplification of the target DNA sequence by PCR is only a part of a complex procedure in PCR-based assays, which includes a pre-PCR step (e.g. sample preparation and DNA isolation) and a post-PCR step of detection of PCR products (e.g. by gel electrophoresis, hybridization, etc.), both critical for the overall assay sensitivity. Therefore, the sensitivity of diagnostic PCR should reflect the whole procedure and be distinguished from the theoretical detection limit of PCR (e.g. one target DNA sequence per PCR tube). The sensitivity is often defined as the minimum number of target DNA/cells that can be detected, but such an approach implies that the probability of detection is 1.0 at or above the detection limit and zero below it, which may not be true [10]. A small target DNA sample volume (e.g. 1-10 µL) in contrast to a large volume of the sample analyzed (e.g. 1-25 mL) is an important limiting factor for a diagnostic PCR, because it significantly decreases the probability of detection. For example, the detection probability of 1 CFU/mL is 50% in a single experiment, excluding the possibility of loss DNA during isolation, if only 5 µL of isolated DNA is used for amplification (without a prior DNA concentration). Additionally, DNA polymerase can be inhibited by many substances, which is the Achilles' heel of PCR [8]. In conclusion, the sensitivity ensuring repeatable results for the example mentioned above should be assumed as $\geq 10^3$ CFU/mL. Thus, to improve the reliability of PCR-based assays, their sensitivity should be associated with the detection probability of target DNA/cells at certain concentrations.

In order to calculate such probabilities, an endpoint limit of a detection procedure can be used (a sample containing the known concentration of the target DNA/cells is serially diluted by a constant value to produce dilutions for which a defined limit of detection can be obtained). The LOD_{50} method is usually assessed because it normalizes the results of such studies by estimating the concentration of the target DNA/cells, which would correspond to 50% of positive results. Although the probability of detection can be directly estimated from experimental counts and such an approach is relatively simple, it leaves little possibility of interpolation to find probabilities at intermediate concentrations, and typically requires a relatively high number of repetitions at each concentration to provide the reliable probability. Statistical modeling of the probability binary responses, such as logistic regression, offers a useful alternative. Using these methods, the sensitivity of an assay can

be characterized by an S-shaped curve showing the probability of a positive result as a function of the number of target DNA/cell molecules in a sample.

The limit of detection for the *hlyA* and *iap* nested PCR-based assays used for detecting *L. monocytogenes* in artificially contaminated specimens was determined as 1 log/100 μL or 10^1 CFU/100 μL (10^0 CFU/PCR). However, the frequency of positive results was unsatisfactory (generally below 60%) at such a low concentration and the number of PCR repetitions required for 95% LOD were: 5/5 – human serum, 7/10 – pasteurized milk and 4/4 – 0.9% NaCl, for the *hlyA* and *iap* gene, respectively. Therefore, the sensitivity that should be accepted as useful for diagnostic purposes (≥ 95% probability of detection) was determined at 2 log/100 μL or 10^2 CFU/100 μL (10^1 CFU/PCR). *L. monocytogenes* can also be detected with ≥95% probability at the concentration of 1.5 log/100 μL, but two repetitions are required. Smieja et al. [13] suggested that such duplications should be a "reference standard" for PCR, until DNA isolation and detection techniques were improved to the level where single PCR would provide similar sensitivity. These values were comparable with the results obtained by other authors. For example, Herman et al. [16], using nested PCR to detect the *hlyA* gene in artificially contaminated milk samples, determined the sensitivity at 10 CFU/25mL (100% of positive results) and 5 CFU/25mL (60% of positive results). Similarly, Jaton et al. [19] determined the limit of detection at 200 CFU/mL (4 CFU/PCR), detecting the *iap* gene in artificially contaminated cerebrospinal fluid samples by nested PCR. Similarly, the authors, employing other genes specific for *L. monocytogenes* than the *hlyA* and *iap* in nested PCR, obtained comparable limits of detection. Simon et al. [20], using primers for the *prfA* gene, was able to detect 100 CFU/g of *L. monocytogenes* in a smoked salmon. Interestingly, the literature includes examples of large discrepancies in the sensitivity of nested PCRs, in spite using identical primers and amplification conditions. For example, Chen et al. [21], detecting *L. monocytogenes* in intestinal mucosal biopsies from patients with inflammatory bowel disease, obtained the detection limit of 10fg DNA/PCR (about 2 CFU/PCR). On the contrary, in similar studies, Chiba et al. [22] detected as many as 9pg DNA (~1.8×10^3 CFU/PCR).

Moreover, the interpolation of the number of target DNA molecules directly from CFUs counting can be responsible for an underestimation of the "real" sensitivity. Hein et al. [23] using real-time PCR proved that the number of *L. monocytogenes* genome copies was 100 times higher than the counted number of CFUs. Such discrepancies may result from bacteria aggregation or amplification of target DNA from dead cells.

Since the PCR sensitivity is associated with the nature of primers via their capability of binding to the target sequence, and even primer pairs for the same gene can exhibit up to 1,000-fold differences in the sensitivity, it is important to test the efficiency of various primers and/or genetic markers to optimize pathogen detection [8]. The values of LOD_{50} calculated from both logistic regression and the Spearman-Kärber methods were fully comparable for the *hly*A and *iap* nested PCR-based assays. So, they both can be used interchangeably, but different genetic markers can be useful for the detection of unusual *Listeria* strains [24]. However, significant differences were found in the probability of *L. monocytogenes* detection using the *iap* nested PCR in pasteurized milk at a concentration of 1 log CFU/100 μL when compared to human serum and physiological saline samples. In fact, the detection probability by the *hlyA*-nested PCR was also the lowest in pasteurized milk samples, but the differences were not statistically significant. It could be partially explained by the influence of calcium ions, which are well documented inhibitors of PCR [25]. However, it still remains unclear why only detection of the *iap* gene was so strongly affected.

Conclusion

In conclusion, it is essential to understand the detection probability for a single PCR-based experiment, since this knowledge can be helpful in deciding optimal replicate numbers required to obtain a specific LOD (e.g. 95%) and in choosing minimum concentrations to achieve reasonable confidence of detection and/or avoid detection failures. Therefore, the application of statistical methods to evaluate the sensitivity of PCR-based assays, especially in detecting the small number of *L. monocytogenes*, can help to evaluate the usefulness of these assays as potentially routine diagnostic tests.

References

1. BOROWSKI J., FUROWICZ J., KĘDZIA A., TOMASZEWSKI R., ZAREMBA M.L. Listeriosis. Wydawnictwo Lekarskie PZWL. Warszawa **1974** [In Polish].
2. ZAREMBA M.L., BOROWSKI J. Medical Microbiology, 3rd Ed. Wydawnictwo Lekarskie PZWL. Warsaw **2001** [In Polish].
3. ANONYMOUS. The Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents, Antimicrobial resistance and Foodborne outbreaks in the European Union in **2006**.
4. FARBER J.M., PETERKIN P.I. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. **55**, 476, **1991**.
5. FORSYTHE S.J. Microbiology of safe foods. Blackwell Science Ltd. **2000**.
6. DONNELLY C.W. *Listeria monocytogenes*: a continuing challenge. Nutr. Rev. **59**, 183, **2001**.
7. BEUMER R.R., HAZELEGER W.C. *Listeria monocytogenes*: diagnostic problems. FEMS Immunol. Med. Microbiol. **35**, 191, **2003**.
8. SACHSE K., FREY J. PCR detection of microbial pathogens. Methods in Molecular Biology, volume **216**, Humana Press Inc, **2003**.
9. MALORNY B., TASSIOS P.T., RÅDSTRÖM P., COOK N., WAGNER M., HOORFAR J. Standardization of diagnostic PCR for the detection of foodborne pathogens. Int. J. Food. Microbiol. **83**, 39, **2003**.
10. HUGHES J.P., TOTTEN P. Estimating the accuracy of polymerase chain reaction-based tests using endpoint dilution. Biometrics **59**, 505, **2003**.

11. KNUTSSON R., BLIXT Y., GRAGE H., BORCH E., BORCH E., RÅDSTRÖM P. Evaluation of selective enrichment PCR procedures for *Yersinia enterocolitica*. Int. J. Food. Microbiol. **73**, 35, **2002**.
12. LOFSTROM C., KNUTSSON R., AXELSSON C.E., RÅDSTRÖM P. Rapid and specific detection of *Salmonella* spp. in animal feed samples by PCR after culture enrichment. Appl. Environ. Microbiol. **70**, 69, **2004**.
13. SMIEJA M., MAHONY J.B., GOLDSMITH C.H., CHONG S., PETRICH A, CHERNESKY M. Replicate PCR testing and probit analysis for detection and quantitation of *Chlamydia pneumoniae* in clinical specimens. J. Clin. Microbiol. **39**, 1796, **2001**.
14. HITCHINS A. AOAC Excel worksheet: Limit of Detection Program for Qualitative Microbiology Methods (LOD50% Spearman-Kärber), version 09/11/2002, available at www.aoac.org/accreditation/DEMO.xls (2 October **2009**).
15. KOTZ S., BALAKRISHNAN N., READ C., VIDAKOVIC B, JOHNSON NL (Eds.). Kärber Method. In Encyclopedia of Statistical Sciences, 2nd ed., John Wiley & Sons, Inc., Hoboken, New Jersey, **6**, 3820, **2006**.
16. HERMAN L.M., De BLOCK J.H., MOERMANS R.J. Direct detection of *Listeria monocytogenes* in 25 milliliters of raw milk by a two-step PCR with nested primers Appl. Environ. Microbiol.; **61**, 817, **1995**.
17. BUBERT A., RIEBE J., SCHNITZLER N., SCHÖNBERG A., GOEBEL W., SCHUBERT P. Isolation of catalase-negative *Listeria monocytogenes* strains from listeriosis patients and their rapid identification by anti-p60 antibodies and/or PCR. J. Clin. Microbiol. **35**, 179, **1997**.
18. ANONYMOUS. Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of *Listeria monocytogenes* - Part 1: Detection method ISO 11290-1:**1996.**
19. JATON K., SAHLI R., BILLE J. Development of polymerase chain reaction assays for detection of *Listeria monocytogenes* in clinical cerebrospinal fluid samples. J. Clin. Microbiol. **30**, 1931, **1992**.
20. SIMON M.C., GRAY D.L., COOK N. DNA extraction methods for the detection of *Listeria monocytogenes* in cold-smoked salmon. Appl. Environ. Microbiol. **62**, 822, **1996**.
21. CHEN W., LI D., PAULUS B., WILSON I., CHADWICK V.S. Detection of *Listeria monocytogenes* by polymerase chain reaction in intestinal mucosal biopsies from patients with inflammatory bowel disease and controls. J. Gastroenterol. Hepatol. **15**, 1145, **2000**.
22. CHIBA M., FUKUSHIMA T., INOUE S., HORIE Y., IIZUKA M., MASAMUNE O. *Listeria monocytogenes* in Crohn's disease. Scand. J. Gastroenterol. **33**, 430, **1998**.
23. HEIN I., KLEIN D., LEHNER A., BUBERT A., BRANDL E., WAGNER M. Detection and quantification of the *iap* gene of *Listeria monocytogenes* and *Listeria innocua* by a new real-time quantitative PCR assay. Res. Microbiol. **152**, 37, **2001**.
24. JOHNSON J., JINNEMAN K., STELMA G., SMITH B.G., LYE D., MESSER J., ULASZEK J., EVSEN L., GENDEL S., BENNETT R.W., SWAMINATHAN B., PRUCKLER J., STEIGERWALT A., KATHARIOU S., YILDIRIM S., VOLOKHOV D., RASOOLY A., CHIZHIKOV V., WIEDMANN M., FORTES E., DUVALL R.E., HITCHINS A.D. Natural atypical *Listeria innocua* strains with *Listeria monocytogenes* pathogenicity island 1 genes. Appl. Environ. Microbiol. **70**, 4256, **2004**.
25. BICKLEY J., SHORT J.K., MCDOWELL D.G., PARKES H.C. Polymerase chain reaction (PCR) detection of *Listeria monocytogenes* in diluted milk and reversal of PCR inhibition caused by calcium ions. Lett. Appl. Microbiol. **22**, 153, **1996**.