DEAD LISTERIA MONOCYTOGENES CELLS ARE DETECTED IN COOKED MEAT AND SMOKED FISH WITH A COMMERCIAL PCR-BASED KIT

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ABSTRACT

By using a commercial PCR-based kit (Probelia *Listeria monocytogenes* ^{TM}) for the detection of *Listeria monocytogenes* in cooked meat and smoked fish we found a high proportion of « false positive » results, i.e. : that we were unable to confirm with culture or any other alternative method. We raised the hypothesis that dead cells of *L. monocytogenes* present in the food matrixes were detected by the PCR-based method. To test this hypothesis, cooked meat and smoked fish products that were initially negative for *L. monocytogenes,* both with the reference method (ISO 11290) and the PCR-based method, were spiked with heat inactivated *L. monocytogenes* cells and then retested with both methods. A suspension of *L. monocytogenes* in PBS buffer (about $1.5 \pm 0.5\ 10^9$ CFU/ml) was heat inactivated at 70 °C for 30 minutes and then serially diluted to the tenth. Complete inactivation of the cells was confirmed by culture methods. Food products and PBS buffer were spiked with the inactivated cells in the range of 10^3 to 10^9 cells per 25g or 25 ml respectively. All experiments were done in triplicates, including for unspiked matrixes used as control. While the reference method gave negative results with all spiked and unspiked food products as well as PBS buffer samples, the PCR-based method gave positive results with both the meat and fish matrixes spiked with respectively 10^7 to 10^9 and 10^6 to 10^9 inactivated cells / 25 g but not with unspiked samples. According to the respective enrichment procedures, the lowest dead cell concentration that generated a positive PCR result corresponded to approximately 10 inactivated cells in 5 l of sample used for the PCR assay. The same results were obtained for PBS samples. We demonstrated that dead cells of *L. monocytogenes* generated positive PCR tests in cooked meat and smoked fish products with a threshold of 10^7 and 10^6 cells / 25g respectively.

INTRODUCTION

In a preliminary study, by using a commercial PCR-based kit (Probelia *Listeria monocytogenes* ™ [BIORAD]) for the detection of *Listeria monocytogenes* in cooked meat and smoked fish, we found a high proportion of « false positive » results, i.e. : results that we were unable to confirm either with culture or any other alternative method. We raised the hypothesis that dead cells of *L. monocytogenes* present in the food matrices were detected by the PCR-based method. To test this hypothesis, cooked meat and smoked fish products that were initially tested negative for *L. monocytogenes*, both with the reference method (ISO 11290) and the PCR-based method, were spiked with heat inactivated *L. monocytogenes* cells (calibrated suspensions) and then retested with both methods immediately and after 14 days of conservation at 4 °C.

MATERIAL AND METHODS

Bacterial strain : A suspension of *Listeria monocytogenes* (strain # 598, serotype 4b) ($1.5 \pm 0.5\ 10^9$ CFU/ml) in PBS buffer was heat inactivated at 70°C for 30 minutes and then serially diluted to the tenth. Complete inactivation of the cells was confirmed by culture methods. Food products and PBS buffer (control) were spiked with the inactivated cells in the range of 10^3 to 10^9 cells per 25g or 25 ml respectively. All experiments were done in triplicates, including for unspiked matrices used as control.

Food matrices : Cooked meat product (« Rillettes ») and Smoked fish product (Smoked salmon) were used. First, a total of 9 lots of each product were tested for *L. monocytogenes* both by the reference culture method and by the Probelia method. Second, one lot of each product category negative for L. monocytogenes by the two methods was selected for artificial contamination.

Artificial contamination : For each food matrices 600 g were mixed (stomacher) and 25 g were aliquoted in 24 bags. The inactivated cell suspensions were spiked directly to the product in each bag. Twelve bags were analyzed immediately and 12 were analyzed after 14 days of conservation at 4°C.

Sample preparation and enrichment : According to the manufacturer instructions, 25 g of spiked sample were homogenised in 225 ml of half Faser medium and incubated without shaking for 24 ± 2 hours at 30 °C. For smoked fish, the Probelia test was performed on 1 ml of decanted, enriched food suspension, while in the case of cooked meat 0.1 ml of pre-enriched half Fraser medium was added to 10 ml of Fraser medium for a further 24 ± 2 h enrichment at 30 °C and then 1 ml of that second enrichment medium was tested with Probelia. After lysis and centrifugation 5 µl of supernatant were used for the amplification reaction.

Nucleic acid extraction, amplification and detection : The Probelia test was done according to manufacturers instructions. It is based on a *L. monocytogenes* specific-gene amplification by the polymerase chain reaction (PCR) technique followed by microplate sandwich hybridization (coated capture-probe, peroxidase-labelled detection probe) and colorimetric detection (TMB). Two negative and one positive controls were included in each series of amplification, as well as one DNA extraction control. Internal amplification controls

were run for each sample. The UNG procedure was used to prevent contamination (dUTP was used instead of dTTP in the amplification mix containing Uracyl-N-Glycosylase). Good laboratory practices concerning PCR were followed, three separated rooms were used and each was equipped with pipettes, lab coats, gloves, etc.

Reference culture method : All samples were also tested following the ISO 11290 method, which confirmed in each cases that no viable *L. monocytogenes* was recovered from spiked samples.

RESULTS

- **Dead L. *Monocytogenes* cells are detected in artificially contaminated cooked meat products**
- **Dead *L. monocytogenes* cells are detected in artificially contaminated smoked salmon**
- **Dead *L. monocytogenes* cells are still detected in artificially contaminated cooked meat products after 14 days at 4 °C**
- **Dead *L. monocytogenes* cells are still detected in artificially contaminated smoked salmon after 14 days at 4 °C**

Table I : Results of the PCR tests performed on cooked meat artificially contaminated with different initial concentrations of dead *L. monocytogenes* cells.

Matrice samples and PBS controls	Theoretical Number of dead cells spiked in 25 g (cooked meat) or 25 ml (PBS)								Controls		
	0	10^3	10^4	10^5	10^6	10^7	10^8	10^9	Neg.	Neg.	Pos.
Cooked Meat											
Sample 1	-	-	-	-	-	+	+	+	-	-	+
Sample 2	-	-	-	-	-	-	+	+	-	-	+
Sample 3	-	-	-	-	-	-	+	+	-	-	+
PBS											
Sample 1	-	-	-	-	-	-	+	+	-	-	+
Sample 2	-	-	-	-	-	-	+	+	-	-	+
Sample 3	-	-	-	-	-	+	+	+	-	-	+

Table II : Results of the PCR tests performed on smoked salmon artificially contaminated with different initial concentrations of dead *L. monocytogenes* cells.

Matrice samples and PBS controls	Theoretical Number of dead cells spiked in 25 g (smoked salmon) or 25 ml (PBS)								Controls		
	0	10^3	10^4	10^5	10^6	10^7	10^8	10^9	Neg.	Neg.	Pos.
Smoked salmon											
Sample 1	-	-	-	-	+	+	+	+	-	-	+
Sample 2	-	-	-	+	+	+	+	+	-	-	+
Sample 3	-	-	-	-	+	+	+	+	-	-	+
PBS											
Sample 1	-	-	-	-	+	+	+	+	-	-	+
Sample 2	-	-	-	-	+	+	+	+	-	-	+
Sample 3	-	-	-	-	+	+	+	+	-	-	+

Table III : Results of the PCR tests performed on cooked meat artificially contaminated with different initial concentrations of dead *L. monocytogenes* cells after 0 and 14 days at 4 °C.

Matrice samples and PBS controls	Theoretical Number of dead cells spiked in 25 g (cooked meat) or 25 ml (PBS)				Controls		
	0	10^6	10^7	10^8	Neg.	Neg.	Pos.
Cooked Meat after day 0							
Sample 1	-	-	-	+	-	-	+
Sample 2	-	-	+	+	-	-	+
Cooked Meat after day 14							
Sample 1	-	-	-	+	-	-	+
Sample 2	-	-	+	+	-	-	+
PBS after day 0							
Sample 1	-	-	-	+	-	-	+
Sample 2	-	-	+	+	-	-	+
PBS after day 14							
Sample 1	-	-	-	+	-	-	+
Sample 2	-	-	-	+	-	-	+

Table IV : Results of the PCR tests performed on smoked salmon artificially contaminated with different initial concentrations of dead *L. monocytogenes* cells after 0 and 14 days at 4 °C.

Matrice samples and PBS controls	Theoretical Number of dead cells spiked in 25 g (smoked salmon) or 25 ml (PBS)				Controls		
	0	10^6	10^7	10^8	Neg.	Neg.	Pos.
Smoked salmon after day 0							
Sample 1	-	+	+	+	-	-	+
Sample 2	-	+	+	+	-	-	+
Smoked salmon after day 14							
Sample 1	-	-	+	+	-	-	+
Sample 2	-	-	+	+	-	-	+
PBS after day 0							
Sample 1	-	-	+	+	-	-	+
Sample 2	-	-	+	+	-	-	+
PBS after day 14							
Sample 1	-	+	+	+	-	-	+
Sample 2	-	+	+	+	-	-	+

Table V : Results of the PCR tests performed on cooked meat directly contaminated with DNA of *L. monocytogenes*, at the initial time of addition and after 14 days at 4 °C.

Matrice samples and PBS controls	Theoretical* number of cells equivalent to the DNA added to samples				Controls		
	0	10^6	10^7	10^8	Neg.	Neg.	Pos.
Cooked Meat at day 0							
Sample 1	-	-	-	+	-	-	+
Sample 2	-	-	+	+	-	-	+
Cooked Meat after day 14							
Sample 1	-	-	-	+	-	-	+
Sample 2	-	-	+	+	-	-	+
PBS at day 0							
Sample 1	-	-	-	+	-	-	+
Sample 2	-	-	+	+	-	-	+
PBS after day 14							
Sample 1	-	-	-	+	-	-	+
Sample 2	-	-	-	+	-	-	+

* as deduced from the *L. monocytogenes* genome size

Table VI : Results of the PCR tests performed on smoked salmon directly contaminated with DNA of *L. monocytogenes*, at the initial time of addition and after 14 days at 4 °C.

Matrice samples and PBS controls	Theoretical* number of cells equivalent to the DNA added to samples				Controls		
	0	10^6	10^7	10^8	Neg.	Neg.	Pos.
Smoked salmon at day 0							
Sample 1	-	+	+	+	-	-	+
Sample 2	-	+	+	+	-	-	+
Smoked salmon after day 14							
Sample 1	-	-	+	+	-	-	+
Sample 2	-	-	+	+	-	-	+
PBS at day 0							
Sample 1	-	-	+	+	-	-	+
Sample 2	-	-	+	+	-	-	+
PBS after day 14							
Sample 1	-	+	+	+	-	-	+
Sample 2	-	+	+	+	-	-	+

* as deduced from the *L. monocytogenes* genome size

DISCUSSION

PCR-based diagnostic tests rely on the detection of DNA, not necessarily of viable cells. When no or a very limited culturing of the bacterial cells occur, the question whether such methodology detects dead bacteria has to be examined. Moreover, when processing of the food products involve bactericidal treatment (e.g. pasteurization), the question still need to be adressed even if culturing steps occur for pre-enrichment and enrichment.

In this study we have shown that *L. monocytogenes*, killed by heat treatment, which is of practical significance in the food industry, remain detectable by PCR. Our data showed that dead cells of *L. monocytogenes* must reach 10^7 CFU/25g in meat products and 10^5-10^6 CFU/25g in fish products in order to generate a positive PCR test with the Probelia kit, when pre-enrichment and/or enrichment of the food sample are performed according to manufacturer's instructions. When looking at the dilution factor represented by the different steps of enrichment, these thresholds corresponded both roughly to 10 to 100 cells in the 5 l sample added to the PCR mix, as expected.

Our data also showed that dead cells are still detectable, with a threshold not significantly different, after 14 days in the matrices, which suggested that DNA is not degradated so fast in the food matrices tested. This is under investigation in our lab.

CONCLUSION

While PCR detection after culturing is advisable for monitoring viable *L. monocytogenes* cells in food products, we must consider that the initial concentration of dead *L. monocytogenes* must be under 10^5 and 10^6 CFU/25g for smoked fish and cooked meat, respectively, to avoid false positives. Such levels of contamination before pasteurization for example, are very high and indicate a very bad hygienic status of the product even if no viable *L. monocytogenes* will never be recovered by culture method from the sample. Application of a PCR detection method thus may suffers from the drawback that false positive results can be obtained, when heat-processed food are analyzed. Should this be really construed as a drawback ?

CONCLUSION

While PCR detection after culturing is advisable for monitoring viable *L. monocytogenes* cells in food products, although one must consider that the initial concentration of dead *L. monocytogenes* must be under 10^5 and 10^6 CFU/25g for smoked fish and cooked meat, respectively, to avoid false positives. Such level of contaminations before pasteurization for example, are very high and indicate a very bad hygienic status of the product even if no viable *L. monocytogenes* will never be recovered by culture method from the sample. Application of a PCR detection method thus may suffers from the drawback that false positive results can be obtained, when heat-processed food are analyzed.