LISTERIA MONOCYTOGENES DETECTION IN FOOD USING AN ELISA-BASED METHOD

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ABSTRACT

As one of the major food-borne pathogens, *Listeria monocytogenes* need to be quickly and easily monitored in the food chain. A new method, called Transia Plate *Listeria monocytogenes*, combining a two-step enrichment done respectively in L-Palcam broth followed by Fraser broth, and an ELISA test, has been developed for the food application.

The ELISA test detected all the different serotypes of *Listeria monocytogenes* (totally 56 strains tested) and did not show any crossreactivity towards the other *Listeria* species (n = 48), such as *Listeria innocua* (n = 29), and towards other bacteria belonging to different genus (n = 36).

The studies of spiked food samples (rillettes, soft cheese, raw milk, smoked trout and courgettes) contaminated by different levels of *Listeria monocytogenes* serotypes (1/2a, 1/2b, 1/2c, 3a and 4b) showed the limit of detection of the method was lower than 10 CFU/25 g of food. Finally, when screening naturally contaminated food samples (329 samples, such as vegetables (n = 80), raw and processed meat products (n = 159), raw and smoked seafood products (n = 90), the Transia Plate *Listeria monocytogenes* method offered equivalent results to the ISO method 11290-1.

INTRODUCTION

The *Listeria* genus involves gram positive, catalase positive, rod shaped bacteria, widely distributed in the environment (soil, water, plants and animals). That genus regroups 6 species (respectively *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi*), being well characterised since the years 80's, with the extensive use of the molecular methods (J. Rocourt, 1996). Only *L. monocytogenes* has been described as a human pathogen. Listeriosis can result in very severe diseases like septicemia and meningitis, especially with elderly people, pregnant women, newborns and immuno-suppressed people.

It has now been clearly established that cases of listeriosis in human are due to foodborne infection (W.F. Schlech, 1996). Raw and processed meat, delicatessen products, seafood, dairy products are the main food vehicles responsible for listeriosis cases. Although the number of outbreaks is yearly very low, the mortality rate is high (till 30%), and major outbreaks were described in various countries for the last 20 years (USA, France, Switzerland, Canada,…).

In order to prevent those *Listeria* outbreaks, the agrofood industries have been screening their food products for *Listeria monocytogenes*. Several methods are currently available for *Listeria* detection in food (traditional cultural methods, immunoassays, genomic tests), but very few are specific for *Listeria monocytogenes* (J.L. Bind *et al*., 1996).

A new microplate ELISA (Enzyme Linked Immunosorbent Assay), based on the detection of the *L. monocytogenes* specific P60 protein has been developed for food analysis. That assay is carried out after a 2-step enrichment protocol (totally 43 hours), combining successively L-Palcam and Fraser broths. The performances of this new method, presented in that work, have been determined with pure strains, then with artificially and naturally contaminated food samples and have been compared to the ISO 11290-1 method.

MATERIAL AND METHOD (1)

➢ Material

Trypticase Soya, Yeast Extract media (TS/YE), L Palcam broth and supplement, Palcam and Oxford agars were purchased from Merck (Darmstadt, Germany). The sheep blood and the API *Listeria* gallery were purchased from bioMérieux (Marcy l'Etoile, France). The Fraser base and supplement were supplied by Biokar (Beauvais, France). The ferric ammoniacal citrate was purchased from Sigma (St Louis, USA). Transia Plate *Listeria monocytogenes* (TPLM) kits were manufactured by Diffchamb (Lyon, France).

➢ Enrichment protocol

The protocols used for both the ISO 11290-1 and the Transia Plate *Listeria monocytogenes* methods are described in figure n°1. Suspected *Listeria monocytogenes* colonies isolated onto both Oxford and Palcam agars were further analysed by β haemolysis onto sheep blood agar plates, then by biochemical identification.

➢ ELISA test:

The different steps are presented on figure n°2.

➢ ELISA interpretation:

The positive threshold was calculated by adding 0.15 to the optical density (OD) mean of both negative controls:

↳ **Positive Threshold (PT) = [(NC1 + NC2)/2]+ 0.15**

Any positive sample was confirmed by streaking of the Fraser broth onto Oxford and Palcam agar plates.

The negative threshold was calculated by multiplying the value of the positive cut-off by 0.9:

↳ **Negative Threshold = PT * 0.9**

If any sample showed an OD between both thresholds, it was declared doubtful, and needed to be confirmed by streaking the Fraser broth onto Oxford and Palcam agar plates.

MATERIAL AND METHOD (2)

➢ Study of the immunoassay specificity :

Fifty six *Listeria monocytogene s*trains (serotypes 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4b, 4c, 4d, 4e), 48 other *Listeria* strains (*L. innocua*, *L. grayi*, *L. ivanovii*, *L. welshimeri* and *L. seeligeri)*, and 36 non *Listeria* strains were tested with the ELISA test.

➢ Limit of detection of the method with artificially contaminated samples

Five food matrix/*Listeria monocytogenes* strain couples were studied: rillettes/*L. monocytogenes* 1/2a, raw milk/*L. monocytogenes* 1/2b, soft cheese/*L. monocytogenes* 1/2c, courgettes/*L. monocytogenes* 3a and smoked trout/ *L. monocytogenes* 4b. All the strains were previously isolated from food products.
About 700g of each food matrix were homogenised in a warring blender, then divided in portions of 25 g. Each food matrix was contaminated at 3 theoretical inoculum levels: 3, 20 and 100 cells/25 g. Any level of contamination was repeated 3 times. Non-contaminated samples were tested in parallel.
The concentration of the contaminating suspension was estimated by enumeration in TSYE agar. The ISO method was performed simultaneously to the Transia Plate *Listeria monocytogenes* method.

➢ Study of natural samples

Totally 329 food products (159 meat products, 90 fishes and 80 vegetables), bought in several supermarkets, were analysed by both the ISO and the TPLM methods.
As naturally contaminated vegetables were not found, artificial contaminations were performed: before being analysed, the whole vegetables were soaked in water contaminated with clods of earth and about 10^4 cells of *Listeria monocytogenes* / ml, during 24h-72h at 2-8°C.
In order to be tested by both methods, the samples were divided in two parts after an homogenisation in a warring blender.

RESULTS AND DISCUSSION (1)

➢ Study of the immunoassay specificity

The overall *Listeria monocytogenes* serotypes were specifically detected by the ELISA (Table I), while any of the other *Listeria* species showed a positive signal. With other bacterial genus, only the *Propionibacterium* strain showed a weak positive signal after culture in TSYE broth, but that signal turned clearly negative when the bacteria was grown in Fraser broth.

With pure strains, the TPLM assay showed a very high specificity for *Listeria monocytogenes*, confirming the P60 protein represented a good target for developing a specific *L. monocytogenes* ELISA, as previously demonstrated by A. Bubert *et al*., 1994.

➢ Limit of detection with artificially contaminated samples

Whatever the levels of contamination, the positive ELISA results were all confirmed after streaking (Table II). One low contaminated soft cheese sample was found negative by ELISA, and confirmed negative after streaking: the lack of contaminating bacteria could explain most probably this negative result. The same behaviour was observed for the ISO method with two low contaminated rillettes and courgette samples.

The background flora of the different food matrices before the spiking was estimated at the following values: 2.10^5 CFU/g in the rillettes, 4.10^5 CFU/g in the raw milk, 3.10^9 CFU/g in the soft cheese, 4.10^6 CFU/g in the courgettes and 6.10^8 CFU/g in the smoked trout. Those different levels of background flora did not affect the immunoassay performances, demonstrating the robustness of the TPLM method.

Both methods showed equivalent performances in recovering the contaminated food samples. For the different food matrices, the Transia Plate *Listeria monocytogenes* method confirmed a limit of detection as low as 1 to 9 CFU/25 g of product, whatever the background flora.

RESULTS AND DISCUSSION (2)

➢ Study of natural samples

For the seafood products (Tables III and IV), both methods gave 6 common positive results. Both methods found supplementary positive samples, respectively 3 for the ISO method and 4 for the TPLM method. One positive ELISA method was not confirmed by streaking onto agar plates.
For the raw and processed meat samples (Tables III and V), both methods showed 40 common positive results, the ISO method finding 9 supplementary positive and the TPLM method 8 more. Three positive ELISA results were not confirmed by streaking onto agar plates, whose one was nevertheless found positive by the ISO method.

As both methods did not use the same broth for the first enrichment step (1/2-Fraser for ISO and L-Palcam for TPLM), the analysis was run two different 25g-fraction of the food sample. Between both methods, the discrepancies observed for the meat and seafood products could be explained by:
a non-homogeneous contamination of the food sample by *L. monocytogenes*, meaning that one sample fraction got contaminated but not the other one,
the use of 2 different enrichment protocols which would stimulate the growth of the *Listeria* in two different manners, according to the background flora, to the food matrix, to the level of the cellular stress, etc…
Such discrepancies were previously observed when both methods were using the same enrichment protocol and so the same sample fraction (Norton *et al*., 2000).
The vegetable contamination was not natural, but was performed in conditions mimicking some situations of those vegetables in the nature. The 34 contaminated samples (Tables III and VI) were found positive by both TPLM and ISO methods, giving a 100% agreement rate. None discrepancy between both methods was expected, as the whole vegetables were soaking in the soil-water solution contaminated by *L. monocytogenes*, ensuring an homogeneous contamination at the vegetable surface.

For the overall food samples (Table III), the global percentage of agreement between both methods was 94%. The pair-wise statistical analysis for those 329 samples using the method of Mac Nemar concluded that both methods were not statistically different.

CONCLUSION

The Transia Plate *Listeria monocytogenes* method, combining a two-step enrichment protocol and a specific *Listeria monocytogenes* immunoassay, was demonstrated specific, effective and reliable for *Listeria monocytogenes* detection in food.

The enrichment protocol was applicable to various kinds of food matrix, and the ELISA using a microplate format could be manipulated manually, or automatically using the immunoanalyser, Transia Elisamatic II.

Such a method can be easily implemented in any food analysis laboratory.

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FIGURE N°1:
ENRICHMENT PROTOCOLS FOR BOTH ISO AND TP *LISTERIA MONOCYTOGENES* METHODS

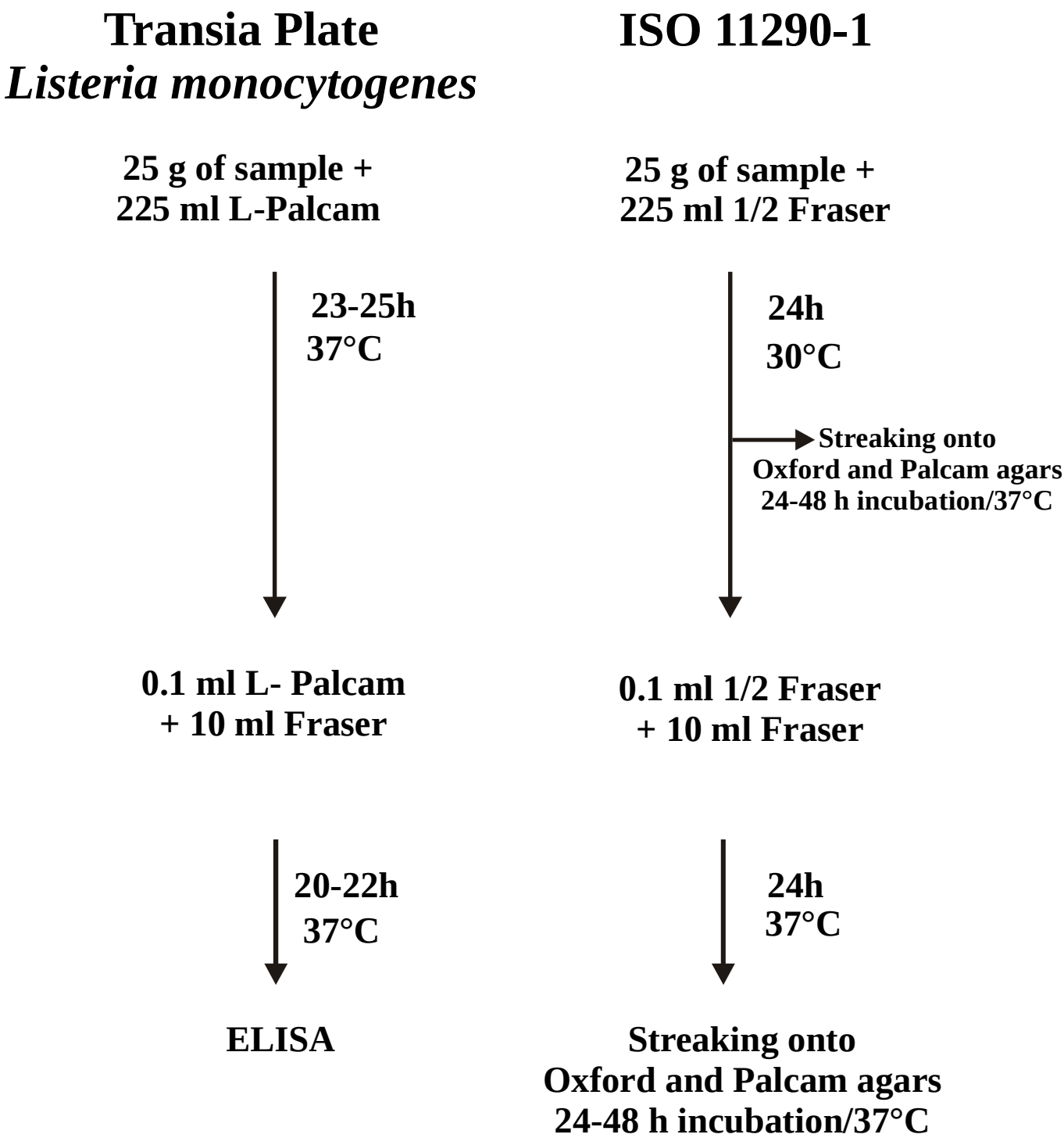

FIGURE N°2 ELISA PROCEDURE

This assay is using breakable 8-well strips coated with antibodies directed against the specific *Listeria monocytogenes* P60 protein.

- **Step 1**

Distribute the controls (negative and positive) and the samples into the wells. Incubate for 1 hour min at 37°C. Then wash the wells 5 times.

- **Step 2.**

Distribute the conjugate (Anti-*Listeria monocytogenes* P60 antibodies labelled with the peroxydase enzyme), and leave for incubation during 1 hour at 37°C. Then wash the wells 5 times.

- **Step 3.**

Distribute the substrate to each well. Incubate 30 min at room temperature.

- **Step 4**

Stop the colorimetric reaction by adding the stop solution.

- **Step 5**

Read the optical densities in a microplate reader at a double wavelength 450/620 nm.

TABLE I
SPECIFICITY OF TRANSIA PLATE LISTERIA MONOCYTOGENES WITH DIFFERENT BACTERIA GENUS.

Strains	Number of strains	Positive Signal
Listeria monocytogenes	56	56
Listeria innocua	29	0
Listeria ivanovii	2	0
Listeria grayi	4	0
Listeria seeligeri	7	0
Listeria welshimeri	6	0
Bacillus cereus	9	0
Streptococcus	6	0
Staphylococcus	8	0
Brochotrix	1	0
Salmonella	2	0
Escherichia coli	2	0
Propionibacterium	1	0[(b)]
Rhodococcus equii	1	0
Erysipelothrix	1	0
Lactobacillus	2	0
Kurthia zopfii	1	0
Jonesia	2	0

[(a)]: The *Listeria monocytogenes* strains were cultured in Fraser broth, overnight at 37°C. All the other bacteria were grown in trypticase soy yeast extract broth, overnight at 37°C.
[(b)]: This strain cultivated in Fraser broth gave a negative signal.

TABLE II:
RESULTS FOR BOTH METHODS WITH THE OVERALL ARTIFICIALLY CONTAMINATED SAMPLES

Levels of contaminations (CFU/25 g)	No. of samples	Transia Plate Listeria monocytogenes		ISO 11290-1
		Positive ELISA	Confirmation	Positive results
0	15	0	0	0
1-9	21	20[(c)]	20[(c)]	19[(d)]
19-44	15	15	15	15
95-132	9	9	9	9
Total No. of positive sample	45	44	44	43

[(c)]: One soft cheese sample was ELISA negative and confirmed negative after streaking onto agar plates.
[(d)]: One rillettes and one courgette samples were found negative.

TABLE III:
RESULTS FOR BOTH METHODS WITH THE OVERALL NATURALLY CONTAMINATED SAMPLES.

Food matrices	No. of samples	Transia Plate *Listeria monocytogenes*		ISO 11290-1
		Positive ELISA	Confirmation	Positive results
Raw and Processed Meat	159	51	48[(e)]	49
Raw and smoked seafood products	90	11	10[(f)]	9
Vegetables	80	34	34	34
Total No. of samples	329	96	92	92

[(e)]: Three positive ELISA samples were not confirmed after streaking onto Oxford and Palcam agar plates.
[(f)]: One positive ELISA sample was not confirmed after streaking onto Oxford and Palcam agar plates.

TABLE IV:
RESULTS FOR BOTH METHODS WITH RAW AND SMOKED SEAFOOD PRODUCTS.

ISO Method **TP *L. monocytogenes***	**Positive**	**Negative**	**Total**
Positive	6	4	10
Negative	3	77	80
Total	9	81	90

TABLE V:
RESULTS FOR BOTH METHODS WITH RAW AND PROCESSED MEAT PRODUCTS.

ISO Method **TP *L. monocytogenes***	**Positive**	**Negative**	**Total**
Positive	40	8	**48**
Negative	9	102	**111**
Total	49	110	**159**

TABLE VI:
RESULTS FOR BOTH METHODS WITH VEGETABLES.

ISO Method **TP L. monocytogenes**	**Positive**	**Negative**	**Total**
Positive	34	0	**34**
Negative	0	46	**46**
Total	34	46	**80**