Veterinaria Italiana, 2011, **47** (3), 281-290

Development and validation of an antigen capture ELISA based on monoclonal antibodies specific for *Listeria monocytogenes* in food

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Summary

A capture enzyme-linked immunosorbent assay (ELISA) for the identification of *Listeria monocytogenes* in food was standardised and validated. The assay was refined by analysing samples of meat, seafood, dairy products, pasta and flour. The method was found to be 100% specific for *Listeria* spp. tested, with a limit of sensitivity of 6.6×10^3 colony-forming units (cfu)/ml. Comparison of *L. monocytogenes* capture ELISA against the official International Organization for Standardization (ISO) method 11290-1:1996 for the isolation and identification of *L. monocytogenes* in food matrices produced a significant concordance index. The assay was validated on food matrices including meat, seafood and dairy products in line with ISO 16140:2003 concerning qualitative analytical methods. The assay was found to be accurate, specific, sensitive, selective, reproducible and fast, resulting in lower costs and faster turnaround in microbiological screening of foods.

Keywords

Capture ELISA, Enzyme-linked immunosorbent assay, ELISA, Food, Hygiene, International Organization for Standardization, Italy, *Listeria monocytogenes*.

Introduction

The genus *Listeria* comprises six species, namely: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi* and can grow in a wide range of temperatures (from 1°C to 45°C). It has a pH of 5 to 9 and osmolarities (1-10% sodium chloride) and is a common contaminant of fresh and preserved foods (2, 8, 9, 10). It can be detected in poultry meat, dairy products (particularly unpasteurised milk and soft cheeses), seafood, vegetables, sauces and ice creams (2, 3, 5, 7, 22, 24, 25) as well as in food processing environments (13, 16).

L. monocytogenes can cause various clinical syndromes in humans (grouped under the name 'listeriosis'), including diarrhoea, stillbirth and meningitis, sometimes with fatal outcomes, particularly in people with an impaired immune system (cancer, HIV, etc.), the elderly and pregnant women.

In Europe and the United States there are between 2 and 15 cases of listeriosis per million people every year, with a mortality which can exceed 50% (2). In 2008, 2 500 cases of *Listeria* infection were reported in the United States alone, with 500 deaths and over 60% of quarantined food products testing positive for *L. monocytogenes*. Regulation EC 2073/2005 of the European Commission, which stipulates the microbiological criteria to

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be applied in food safety control programmes, requires the total absence of *L. monocytogenes* or the maximum limit of 100 colony-forming units (cfu)/g as the safety requirement for various types of ready-to-eat foods at the end of shelf life.

The most common methods used to detect *L. monocytogenes* are conventional microbiological techniques, involving enrichment with selective broths and media, followed by biochemical identification, polymerase chain reaction (PCR)-based methods and immunological assays (3, 5, 7, 8, 13, 15, 19, 21, 22, 23, 25). The conventional methods are time consuming while PCR-based techniques are more rapid. However, the latter require costly equipment and additional steps, such as electrophoresis or preparation of fluorescent probes which are necessary to identify the amplified product. In addition, this method does not provide an assessment in regard to whether a pathogen is able to induce infection or not (26). Immunological assays, such as enzyme-linked immunosorbent assay (ELISA) and Western blotting, using polyclonal and monoclonal antibodies (MAbs) (27) are the most suitable techniques for rapid and low-cost food screening.

This paper describes the standardisation and validation of a capture ELISA technique for the detection of *L. monocytogenes* in food products. If prepared as a ready-to-use kit, this method could be suitable for use in food hygiene laboratories.

Materials and methods

Monoclonal antibodies

MAbs against *L. monocytogenes* were produced by immunising Balb/c mice with a strain of heat-inactivated, sonicated *L. monocytogenes* (American Type Culture Collection [ATCC] 7644). The cell lysate, with a protein concentration of 50 µg/ml, was emulsified with complete Freund's adjuvant and inoculated intraperitoneally. Three further inoculations of cell lysate were then performed using incomplete Freund's adjuvant emulsified with phosphate-buffered saline (PBS). Splenocytes were subjected to fusion with myeloma cells Sp2/O-Ag-14 from mice. Hybridomas were cultured in the Dulbecco modified Eagle's medium containing 20% foetal calf serum, HAT 50×, antibiotics and antimycotics. Hybridomas secreting anti-*L. monocytogenes* antibodies were cloned by limited dilution (4, 11, 28). Hybridomas were screened by indirect ELISA using microplates activated with the same antigen as that used for immunisation of the laboratory animals (17). The same method was used to check for cross-reactions with different bacterial strains (18). The isotype was established and MAbs with isotype IgG were purified by protein A affinity chromatography and conjugated with peroxidase (1, 20).

Bacterial strains

The bacterial strains used for the standardisation and validation of *L. monocytogenes* capture ELISA were obtained from the ATCC, the *Bundesinstitut für Gesund-heitlichen Verbraucherschutz und Veterinärmedizin* (BGVV) and the laboratory collection of bacterial strains isolated from food samples (Table I). Strains isolated from food samples were identified and typed using biochemical and serological methods. The micro-organisms used in the trial were cultured in brain heart infusion broth at 37°C for 14-16 h. The bacterial suspensions were titred, taken to a working concentration of 2×10^8 cfu/ml and subjected to cell lysis by heat treatment.

Food samples

The dairy product, meat and seafood and pasta and flour samples used in the trial were obtained both commercially and from routine diagnostic activities. A total of 25 g aliquots were taken and used to prepare non-contaminated controls, matrices artificially infected with *L. monocytogenes* and non-target bacterial strains. The food matrices were spiked with 10 ± 3 cfu/ml of each micro-organism, homogenised in a stomacher and processed up to preparation of a culture broth with Fraser broth in accordance with the method described in International Organization for Standardization (ISO) 11290-1:1996 (12). Culture broths underwent heat treatment and were analysed by *L. monocytogenes* capture ELISA.

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Table I
Bacterial strains used for standardisation and validation processes

Bacterium	Origin	Bacterium	Origin
Bacillus cereus (ATCC 11778)*	ATCC	*Listeria ivanovii* (ATCC 19119)*	ATCC
*Bacillus cereus**	Collection	*Listeria ivanovii**	Collection
*Bacillus subtilis**	Collection	*Listeria monocytogenes* (ATCC 7644)	ATCC
Bacillus subtilis (ATCC 6633) *	ATCC	*Listeria monocytogenes*	Collection
Bordetella bronchiseptica	Collection	*Proteus vulgaris* (ATCC 49132)	ATCC
Citrobacter freundii	Collection	*Pseudomonas aeruginosa*	Collection
Enterobacter agglomerans	Collection	*Salmonella bredeney*	Collection
Enterobacter amnigenus	Collection	*Salmonella derby*	Collection
Enterobacter cloacae	Collection	*Salmonella enteritidis* (ATCC 13076)	ATCC
Enterococcus faecalis (ATCC 25923)*	ATCC	*Salmonella enteritidis*	Collection
*Enterococcus faecium**	Collection	*Salmonella hadar*	Collection
*Enterococcus faecium**	Collection	*Salmonella muenchen*	Collection
Escherichia coli (ATCC 25922)*	ATCC	*Salmonella panama*	Collection
*Escherichia coli**	Collection	*Salmonella saint-paul*	Collection
Escherichia coli O14 (BGVV)	BGVV	*Salmonella typhimurium* (ATCC 14028)	ATCC
Escherichia coli O157:H7	Collection	*Shigella flexneri* (ATCC 12022)	ATCC
Escherichia fergussoni	Collection	*Staphylococcus aureus* (ATCC 25923)*	ATCC
*Hafnia alvei**	Collection	*Staphylococcus epidermidis*	Collection
Klebsiella oxytoca (ATCC 49131)	ATCC	*Streptococcus faecalis* (ATCC 29212)	ATCC
Klebsiella pneumoniae	Collection	*Staphylococcus lentis**	Collection
Listeria innocua (ATCC 33090)*	ATCC	*Yersinia enterocolitica* (ATCC 23715)	ATCC
*Listeria innocua**	Collection	*Yersinia enterocolitica*	Collection

* non-target strains used for selectivity testing
ATCC American Type Culture Collection
Collection laboratory collection of bacterial strains
BGVV *Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin*

Listeria monocytogenes capture enzyme-linked immunosorbent assay

The 96-well ELISA microplates were coated with MAb 9B8F7 (anti-*L. monocytogenes*) by dispensing 100 µl/well at a concentration of 20 µg/ml in 50 mM carbonate/bicarbonate buffer at pH 9.6 and incubating at room temperature for 8 h. The microplates were then washed three times with 10 mM PBS containing 0.05%Tween 20. A total of 100 µl of culture sample (S), positive control, negative control and negative culture broth (N) were then dispensed in the microplates and incubated under shaking for 1 h at 37°C. They were then washed as before and 100 µl of monoclonal antibody 6F12C8, conjugated with peroxidase (6F12C8-HRP) at a working dilution of 1:20 000, was dispensed into all wells and incubated for 1 h at 37°C. After washing, 100 µl of chromogen substrate (3,3′, 5,5′-tetramethylbenzidine liquid substrate system for ELISA) was dispensed and after 30 min incubation at room temperature, the enzyme reaction was interrupted by adding 50 µl 0.5 N sulphuric acid. The optical density was measured at 450 nm with a microplate reader. The examined samples were diagnosed positive or negative for the presence of *L. monocytogenes* based on the quotient (S/N) between the optical densities of tested samples (S) and the optical density produced by Fraser broth control (N) negative for *L. monocytogenes*.

The limit of detection (LOD) was determined by titration with serial dilutions of a *L. monocytogenes* culture (ATCC 7644) in Fraser broth. The LOD was calculated by interpolating the mean absorbance of negative Fraser broth plus three times the standard

deviation on the standard curve. The number of units in the *L. monocytogenes* broth was determined by counting in a dish containing Oxford agar.

Validation of *Listeria monocytogenes* capture enzyme-linked immunosorbent assay

The *L. monocytogenes* capture ELISA method was validated as indicated in ISO 16140:2003 for qualitative analytical methods (13). The assay was validated in meats, seafood and dairy products (Table II). Intra-laboratory tests were used to evaluate relative accuracy, relative sensitivity, relative specificity and relative limits of detection and selectivity. Discordant data was analysed using McNemar and Cohen's Kappa statistical tests.

Relative accuracy, sensitivity and specificity

Relative accuracy, sensitivity and specificity were determined by analysing samples that had been infected naturally and artificially with strains of *L. monocytogenes* and uncontaminated samples. The artificially contaminated samples were prepared by spiking them with suspensions containing a final level of 10^2 cfu/g *L. monocytogenes*. Samples were analysed in parallel with the reference method and *L. monocytogenes* capture ELISA. A total of 220 food samples were analysed, comprising 76 meat samples, 68 seafood samples and 76 dairy products (Table II).

Relative limit of detection

The relative limit of detection was determined using both the reference method and *L. monocytogenes* capture ELISA to analyse samples from each of the three food groups spiked with 5-10 cfu/g, 50-100 cfu/g and 100-1 000 cfu/g of *L. monocytogenes*, plus an uncontaminated sample from each food group as a negative control. Each sample was analysed six times with each method. The concentration of *L. monocytogenes* used to prepare the spiked samples was determined by plate titration and measurement of optical density.

Selectivity

Selectivity was determined by analysing samples spiked with target strains of *L. monocytogenes* and non-target bacterial strains (Table I). A total of 55 samples from the three food types were analysed, of which 35 were infected with a strain of *L. monocytogenes* and other, non-target strains, and the remaining 20 with non-target strains only.

Results

Monoclonal antibodies

A panel of 8 anti-*L. monocytogenes* MAbs was produced for use in the *L. monocytogenes* capture ELISA. Of these, MAb 9B8F7, isotype IgG_1, anti K was selected as the capture

Table II
Samples tested to assess the relative accuracy, sensitivity and specificity of the *Listeria monocytogenes* capture enzyme-linked immunosorbent assay

Type	Product	No. of positive samples NC	No. of positive samples AC	No. of negative samples
Meat	Sausage	–	2	–
	Salami	–	2	9
	Chicken flesh	–	13	34
	Processed chicken	2	13	–
	Raw ham	–	–	1
Seafood	Salmon	33	–	35
Dairy products	Gorgonzola	3	–	–
	Camembert	2	–	20
	Caciotta	32	–	19

NC naturally contaminated
AC artificially contaminated (spiked)

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antibody and 6F12C8, isotype IgG_3, anti K, conjugated with peroxidase as the secondary antibody. The other Mabs did not produce any signal that was significantly greater than that of the negative culture broth even at high bacterial concentrations.

Listeria monocytogenes capture enzyme-linked immunosorbent assay

L. monocytogenes capture ELISA revealed *L. monocytogenes*; cross-reactions were observed with *L. innocua* and *L. ivanovii*, while no cross-reactions were observed with the other bacterial suspensions used (Table III).

The monoclonal antibody 6F12C8-HRP was used at a working dilution of 1:20 000. The LOD for *L. monocytogenes* was calculated by extrapolating the mean absorbance value of the negative culture broth (N) plus three times its standard deviation from the standard curve. The LOD was found to be 6.6×10^3 cfu/ml and the cut-off value, expressed as S/N, was 1.55. Food samples producing an S/N greater than or equal to the cut-off value were considered positive for *L. monocytogenes*, while those with an S/N lower than the cut-off were considered to be negative. Among the food samples analysed using *L. monocytogenes* capture ELISA, 51 were found positive for *L. monocytogenes* and 22 negative. An analysis of the same samples with standard microbiological methods also revealed 51 samples positive for *L. monocytogenes* and 22 negative. When compared to the official method ISO 11290-1:1996 for the detection of *L. monocytogenes*, the sensitivity and specificity of our assay were found to be 100%, accuracy was 100% and the concordance index was significant, with K = 1.0 (Table IV).

The repeatability of *L. monocytogenes* capture ELISA, determined by analysing one sample positive and one negative for *L. monocytogenes*, was 11.9 CV% for the positive sample and 9.3 CV% for the negative sample, while reproducibility was 9.1 CV% for the positive sample and 10.8 CV% for the negative sample (Table V).

Table III
Bacterial strains and results of the cross-reactions test of the *Listeria monocytogenes* capture enzyme-linked immunosorbent assay

Bacterium (2×10^8 cfu/ml)	S/N	Bacterium (2×10^8 cfu/ml)	S/N
Bacillus cereus (ATCC 11778)	0.9	*L. monocytogenes*	6.5
Bacillus cereus	0.7	*Proteus vulgaris* (ATCC 49132)	0.9
Bacillus subtilis	1.2	*Pseudomonas aeruginosa*	0.9
Bordetella bronchiseptica	0.7	*Salmonella bredeney*	0.8
Citrobacter freundii	0.9	*Salmonella derby*	0.7
Enterobacter agglomerans	0.9	*Salmonella enteritidis* (ATCC 13076)	1.1
Enterobacter amnigenus	0.9	*Salmonella enteritidis*	1.0
Enterobacter cloacae	0.8	*Salmonella hadar*	0.8
Enterococcus faecium	0.3	*Salmonella muenchen*	0.7
Escherichia coli (ATCC 25922)	1.0	*Salmonella panama*	0.8
Escherichia coli O14 (BGVV)	1.1	*Salmonella saint-paul*	0.8
Escherichia coli O157:H7	0.9	*Salmonella typhimurium* (ATCC 14028)	0.9
Escherichia fergussoni	0.9	*Shigella flexneri* (ATCC 12022)	1.2
Klebsiella oxytoca (ATCC 49131)	0.9	*Staphylococcus aureus* (ATCC 25923)	0.6
Klebsiella pneumoniae	0.9	*Staphylococcus epidermidis*	1.1
Listeria innocua (ATCC 33090)	5.8	*Staphylococcus faecalis* (ATCC 29212)	0.6
Listeria ivanovii (ATCC 19119)	6.3	*Yersinia enterocolitica* (ATCC 23715)	0.8
L. monocytogenes (ATCC 7644)	6.3	*Yersinia enterocolitica*	0.8

S sample
N negative control

When the ratio S/N ≥1.55, the sample is considered positive
When ratio S/N <1.55, the sample did not show any cross-reaction

Table IV
Accuracy, sensitivity, specificity and concordance values of *Listeria monocytogenes* capture enzyme-linked immunosorbent assay compared to the ISO 11290-1:1996 results (confidence interval = 95%)

		ISO 11290-1:1996		
		Positive	Negative	Total
Listeria monocytogenes capture ELISA	Positive	51	0	51
	Negative	0	22	22
	Total	51	22	73
			LCL	UCL
Accuracy	100%		96.0%	100%
Sensitivity	100%		94.4%	100%
Specificity	100%		87.8%	100%
Kappa index = 1.0	$p<0.05$			

LCL lower confidence limit
UCL upper confidence limit

Table V
Repeatability and reproducibility values of *Listeria monocytogenes* capture enzyme-linked immunosorbent assay

Sample	Repeatability				Reproducibility			
	No.	S/N	SD	CV%	No.	S/N	SD	CV%
Positive	80	18.3	2.176	11.9	160	18.6	1.695	9.1
Negative	80	1.1	0.099	9.3	160	1.1	0.115	10.8

S sample
N negative control
SD standard deviation
CV coefficient of variation

Relative accuracy, sensitivity, specificity, limit of detection and selectivity

The results for relative accuracy, sensitivity and specificity with respect to analysis of samples from the three food groups using the reference method and *L. monocytogenes* capture ELISA are presented in Tables VI, VII and VIII. The relative limit of detection in meat, seafood and dairy products was 5-10 cfu/g (Table IX). With respect to selectivity, *L. monocytogenes* capture ELISA correctly identified as positive all samples spiked with *L. monocytogenes* and as negative all samples spiked with non-target strains (Table X).

Discussion

Human listeriosis is an infection with an apparent low incidence but with possible serious consequences. Regulation (CE) 2073/2005 of the European Commission (6) requires the use of ISO standards, or alternative methods, validated in accordance with ISO 16140:2003, for the detection of pathogens (safety criteria) and indicator organisms (hygiene criteria). Novel methods may be used, and are indeed recommended where they improve analytical response times, as long as they have been validated pursuant to ISO 17025:2005 (14). *L. monocytogenes* capture ELISA was therefore compared to the method ISO 11290-1:1996 (12) as specified by ISO 16140:2003 (13, 25). The relative accuracy, sensitivity and specificity found during validation in three types of food (Tables VI, VII and VIII) demonstrated the assay's high concordance with the reference method. The relative limit of detection (Table IX) is adequately correlated with the assay's LOD, given that the single food sample enrichment phase prior to analysis with *L. monocytogenes* capture ELISA considerably increases the number of cfu/ml, thus ensuring the detection

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Table VI
Relative accuracy, sensitivity, specificity and concordance of *Listeria monocytogenes* capture enzyme-linked immunosorbent assay in meat samples
(confidence interval = 95%)

		ISO 11290-1:1996		
		Positive	Negative	Total
Listeria monocytogenes capture ELISA	Positive	30	0	30
	Negative	2	44	46
	Total	32	44	76
			LCL	UCL
Relative accuracy	97.4%		90.9%	99.2%
Relative sensitivity	93.8%		79.8%	98.1%
Relative specificity	100.0%		93.6%	100.0%
Kappa index = 0.95	$p<0.05$			
McNemar χ^2 = 0.500	$p>0.05$			

LCL lower confidence limit
UCL upper confidence limit

Table VII
Relative accuracy, sensitivity, specificity and concordance of *Listeria monocytogenes* capture enzyme-linked immunosorbent assay in seafood samples
(confidence interval = 95%)

		ISO 11290-1:1996		
		Positive	Negative	Total
Listeria monocytogenes capture ELISA	Positive	31	5	36
	Negative	2	30	32
	Total	33	35	68
			LCL	UCL
Relative accuracy	89.7%		80.2%	94.9%
Relative sensitivity	93.9%		80.3%	99.3%
Relative specificity	85.7%		73.0%	85.7%
Kappa index = 0.79	$p<0.05$			
McNemar χ^2 = 0.570	$p>0.05$			

LCL lower confidence limit
UCL upper confidence limit

Table VIII
Relative accuracy, sensitivity, specificity and concordance of *Listeria monocytogenes* capture enzyme-linked immunosorbent assay in dairy product samples
(confidence interval = 95%)

		ISO 11290-1:1996		
		Positive	Negative	Total
Listeria monocytogenes capture ELISA	Positive	35	0	35
	Negative	2	39	41
	Total	37	39	76
			LCL	UCL
Relative accuracy	97.4%		90.7%	99.2%
Relative sensitivity	94.6%		82.3%	100.0%
Relative specificity	100.0%		92.8%	99.2%
Kappa index = 0.95	$p<0.05$			
McNemar χ^2 = 0.550	$p>0.05$			

LCL lower confidence limit
UCL upper confidence limit

Table IX
Relative limit of detection of the *Listeria monocytogenes* capture enzyme-linked immunosorbent assay in spiked samples

Samples	Contamination level (cfu/g)	Percent positivity	
		ISO 11290-1:1996	*Listeria monocytogenes* capture ELISA
Meat	0	0%	0%
Meat	5	40%	0%
Meat	10	100%	100%
Meat	100	100%	100%
Meat	1 000	100%	100%
Seafood	0	0%	0%
Seafood	5	40%	0%
Seafood	10	100%	100%
Seafood	100	100%	100%
Seafood	1 000	100%	100%
Dairy product	0	0%	0%
Dairy product	5	40%	0%
Dairy product	10	100%	100%
Dairy product	100	100%	100%
Dairy product	1 000	100%	100%

ELISA enzyme-linked immunosorbent assay

Table X
Results of the selectivity tests on meat, seafood and dairy products
(confidence interval = 95%)

		ISO 11290-1:1996		
		Present	Absent	Total
Listeria monocytogenes capture ELISA	Positive	35	0	35
	Negative	0	20	20
	Total	35	20	55

	Selectivity	LCL	UCL
Meat	100%	90.3%	100%
Seafood	100%	90.3%	100%
Dairy products	100%	90.3%	100%

ELISA enzyme-linked immunosorbent assay
LCL lower confidence limit
UCL upper confidence limit

of *L. monocytogenes* at the minimum contamination level determined. The selectivity study (Table X) revealed that the assay is specific for *L. monocytogenes* even in the presence of non-target bacteria, given that there were no cross-reactions, a frequent cause of false-positives in similar assays.

The standard microbiological techniques for the isolation and identification of *L. monocytogenes* are simple and effective, but relatively time-consuming, as they require two enrichment phases, taking a total of about 72 h, conducted in series on two different culture media (Demi-Fraser broth and Fraser broth) followed by a 24-h plate growth phase before biochemical identification of the suspect colonies. Of the alternatives, those using ELISA are the easiest to perform and allow simultaneous analysis of multiple samples detecting only the pathogens able to grow in the culture media.

The results of the validation against ISO 11290-1:1996 demonstrate that *L. monocytogenes* capture ELISA can be used in food screening,

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thereby reducing the assay time through the use of a single enrichment step in culture broth and avoiding the need to process all samples that give negative results with the official method. Moreover, the development of a ready-to-use kit would provide a valid diagnostic tool that could be used alongside official procedures for confirmation of *L. monocytogenes* in foods.

Grant support

The study was funded with a contribution from the Italian Ministry of Health, the 2000 Targeted Research Project 'Simultaneous determination of pathogens in foods with immunometric luminescent methods'.

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