Enzyme-Linked Immunosorbent Assay for General and Specific Detection of *Listeria* spp. and *monocytogenes* in Dairy Products

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Abstract: Bacteria of the genus *Listeria* are widely distributed in the environment and they frequently contaminate food. Among all species in the genus *Listeria*, only *L. monocytogenes* has been implicated in serious human illness. The other *Listeria* spp. are considered to be avirulent to man but they may cause a variety of disease symptoms or even death in animal. These bacteria are well equipped to survive food processing technologies. For example, they tolerate high concentrations of salt and relatively low pHs, and worst of all, they are able to multiply at refrigeration temperatures. This makes *Listeria* microorganisms a serious threat to food safety and ranks them among the microorganisms that most concern the food industry. The foods most frequently implicated are raw milk, soft cheeses (particularly those made from unpasteurized milk), ice cream, raw vegetables, fermented raw-meat sausages, raw and cooked poultry and raw and smoked fish. Food producers and distributors as well as public health authorities have great interest in timely detection of *Listeria* contamination. We present here a rapid antibody-based screening assay for the detection of both *Listeria* spp. and *Listeria monocytogenes* in dairy samples (milk, cheeses, ice-cream…). A detection system consists of an initial selective enrichment step, where both artificially contaminated samples and real dairy samples were cultivated, followed by direct sandwich format of ELISA using two different polyclonal antibodies; one specific for *Listeria* spp., and the other specific for *L. monocytogenes*.

Keywords: enzyme immunoassay; *Listeria monocytogenes*; milk; ice cream

INTRODUCTION

The genus *Listeria* contains only one species, *L. monocytogenes* that is a confirmed pathogen of man, causing the syndrome generally known as listeriosis (for a review, see [1]). The other *Listeria* species can be regarded as non-pathogenic, except in occasional, rare, circumstances. Further, members of the genus are very similar phenotypically [2] and can only be distinguished by biochemical profiling; even then, strain variation is seen. Thus, rapid diagnostic tests to discriminate the pathogenic *L. monocytogenes* from non-pathogenic *Listeria* are of great interest.

L. monocytogenes is known to be food-borne and is found in dairy products, meat (including poultry and fish) and, to a lesser extent, vegetables; refrigerated ready-to-eat meals are a special concern because the organism can grow at low temperatures. It is a normal resident of the intestinal tract in humans and animals, and is endogenous to the farm environment [1]. Control measures principally revolve around quality assurance systems, in particular hazard analysis critical control point (HACCP) methods. Central to control of *L. monocytogenes* in a HACCP system is extensive environmental and in-line sampling [1]. Here, rapid diagnostic tests as a replacement for, or as an adjunct to, the lengthy traditional culture methods could have a major impact in *L. monocytogenes* control by simplifying test procedures and thus allowing increased frequency of sampling. To be useful, of course, such tests must be specific for

L. monocytogenes and should not react with non-pathogenic *Listeria* species.

L. monocytogenes has a complex pathogenicity mechanism, with several virulence factors that allow infection to spread by direct cell-to-cell transmission. Although some *L. monocytogenes* virulence proteins have homologs in many other *Listeria* species, the internalin protein family (which mediates entry to host cells; is found only in *L. monocytogenes* and the animal pathogen *L. ivanovii*. Thus, these proteins offer a potential target for *L. monocytogenes*-specific diagnostic methods.

In this paper, we describe a detection of *L. monocytogenes* in milk and ice cream model samples that uses polyclonal antibodies raised to both, dead cells of *L. monocytogenes* and to internalin B, protein purified from *L. monocytogenes.*

EXPERIMENTAL

Extraction of internalin B protein. The method was adapted from that of Muller *et al*. [3] using an over-producing *L. monocytogenes* clone (EGD pERL3 50-1, kindly donated by Dr. T. Chakraborty (Giessen, Germany) [4]). Briefly, the organism was cultivated in 2 L Brain-Heart Infusion Broth (BHI; Oxoid Ltd, Basingstoke, UK) containing erythromycin (5 μg/ml) overnight at 37°C, with shaking. The culture was centrifuged and the supernatant discarded. The pellet was washed with phosphate-buffered saline (PBS; 0.14M NaCl, 0.0015M KH_2PO_4, 0.008M Na_2HPO_4, 0.0027M KCl; pH 7.4) and re-suspended in 50 ml Tris-HCl buffer (1M, pH 7.5). After incubating for 1h on ice, the sample was centrifuged and the supernatant ('InlB extract') filter-sterilised, dialysed at +2°C against 3 changes of aqueous $(NH_4)_2CO_3$ solution (25mM) and lyophilised.

Production of antibodies. Male and female white New Zealand rabbits (weighing 2.5 kg) were immunised with the InlB extract according to the scheme in published elsewhere [5]. Animals were kept singly in experimental cages 53 × 40 × 35 cm and fed a standard pellet diet, KKK/L (Ondra, Kralovany, Czech Republic), 120–150 g per day and tap water *ad libitum*; room temperature was 20–24°C, relative humidity 30–80%, and automatic ventillation 8–12 exchanges of air per hour**.** Blood was centrifuged (1000 g, 10 min, laboratory temperature), the serum separated and aminocapronic acid (0.3 g/l) and azide (1 g/l) were added. Sera were stored at 5°C.

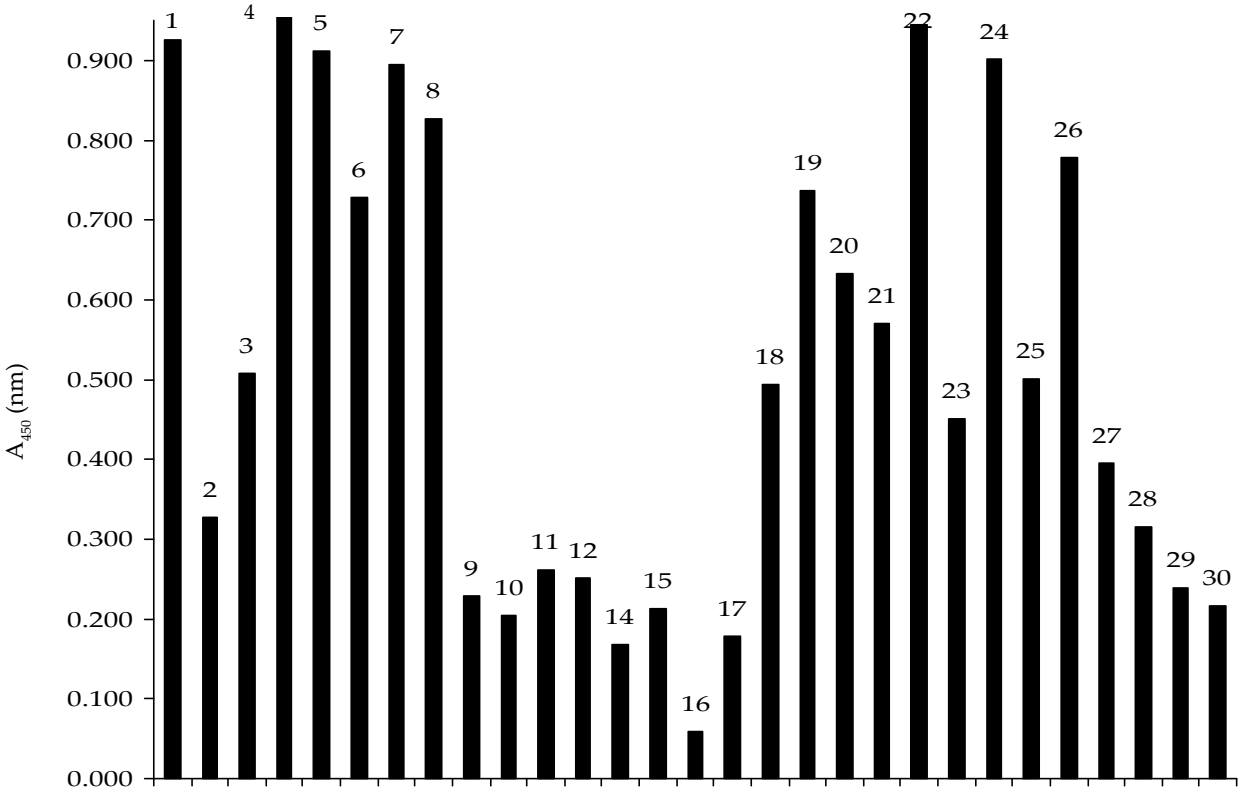
Purification of antibodies. IgG was purified from the serum with an affinity column of protein A immobilised on a glass carrier (Prosep A; Bioprocessing Ltd., Durham, UK), using binding buffer (0.01M phosphate buffer, containing NaCl 8 g/l, pH 7.4), elution buffer (0.1M citrate buffer, pH 5.8) and trapping buffer (1M Tris-HCl buffer, pH 7.4). All solutions and antisera were sterilised by membrane filtration. IgG fractions were lyophilised. The IgG fraction of the serum raised against internalin B extract is subsequently referred to in this paper as "InlB antibody".

Titration of antibodies against Listeria cells. *Listeria* strains were grown in trypticase soy broth (TSB; Oxoid Ltd., Basingstoke, UK) for 18 h at 37°C. Microplates (MaxiSorp; Nunc, Denmark) were coated (200 μl per well) for 2 h at 37°C with washed *Listeria* cells (~10^7 cells per ml in 0.05M carbonate buffer, pH 9.6). Coated microplates were washed 5 times with 250 μl per well PBST. Aliquots (200 μl) of a ten-fold dilution series of lyophilised InlB antibody, dissolved in PBST containing 50 g/l dried non-fat milk (PBSTM), were added to the wells and incubated for 2 h at 37°C. After further washing, goat anti-rabbit horseradish peroxidase conjugate (Sigma A6154; diluted 1:1000 v/v in PBSTM) was added (200 μl per well) and plates incubated for 1 h at 37°C. After final washing, the assay was developed with tetramethyl benzidine (TMB)-based substrate (200 μl per well; Vetoquinol, Bicester, UK), stopped with 2 M H_2SO_4 (50 μl per well), and absorbance measured at 450 nm.

RESULTS

Two new direct sandwich enzyme immunoassay formats have been developed. The former using antibody against internalin B (Ab/InlB) and the latter by means of antibody against dead *L. monocytogenes* (AbLm) cells were used as capture antibody. The same antibodies were used for preparation of conjugates with horseradish peroxidase (Ab/InlB-HRP) and served as a detector molecule. The detection limit for immunoassay with Ab/InlB was 4.5×10^7 CFU/ml for *L. m.* 88/049.

It was proved that both immunoassay formats give satisfactorily specific assay of *Listeria monocytogenes* cells with respect to other microorganisms tested. Both antibodies were found specific to *L. monocytogenes* and did not bind possible food contaminating microflora, e.g. *Enterococcus faecinum,*



Sample No.			Sample No.							
1	*L. monocytogenes*	88/049	16	*Enterococcus*						
2	*L. monocytogenes*	86/012	17	*Lactobacillus*						
3	*L. monocytogenes*	86/010	18	*L. monocytogenes*	88/049	+	*L. innocua*	88/025		
4	*L. monocytogenes*	89/009	19	*L. monocytogenes*	88/049	+	*L. ivanovii*	88/039		
5	*L. monocytogenes*	88/001	20	*L. monocytogenes*	88/049	+	*L. grayi*	88/044		
6	*L. monocytogenes*	87/017	21	*L. monocytogenes*	88/049	+	*Bacillus*			
7	*L. monocytogenes*	89/003	22	*L. monocytogenes*	88/049	+	*Enterobacter*			
8	*L. monocytogenes*	89/022	23	*L. monocytogenes*	88/049	+	*Enterococcus*			
9	*L. innocua*	88/025	24	*L. monocytogenes*	88/049	+	*Lactobacillus*			
10	*L. ivanovii*	88/039	25	*L. monocytogenes*	88/049	+	*L. innocua*	88/025	+	*Bacillus*
11	*L. welshimeri*	88/043	26	*L. monocytogenes*	88/049	+	*L. ivanovii*	88/039	+	*Enterobacter*
12	*L. seeligeri*	89/017	27	*L. monocytogenes*	88/049	+	other listeria			
13	*L. grayi*	88/044	28	*L. monocytogenes*	88/049	+	other listeria			
14	*Bacillus*		29	*L. monocytogenes*	88/049	+	all listeria			
15	*Enterobacter*		30	Blank						

Figure l. The detection of *Listeria monocyrogenes* in samples of chocolate ice cream contaminated by different microorganisms

Bacillus cereus, Lactobacillus plantarum, Enterobacter aerogenes. Both immunoassays have been tested for confirmation of *Listeria* collection. While assay with Ab/InlB interacts only with *Listeria monocytogenes* strains and thus was found specific only for *Listeria monocytogenes*, the assay with Ab/Lm bind all other *Listeria* species and was recognised as listeria genus specific.

Apply of immunoassay technique to milk and ice cream model samples contaminated by *Listeria monocytogenes*

Milk and chocolate ice cream were contaminated by different strains and species, as well as by other microorganisms and some combined samples. Totally 29 samples were prepared. All samples have been analysed by immunoassays with both antibodies. While immunoassay with Ab/InlB antibody recognised only those samples where *L. monocytogenes* was present, the assay with Ab/Lm possessed positive results in all samples where *Listeria* species were inoculated. No difference was seen when *L. monocytogenes* was assayed in the presence of high numbers of five other *Listeria* species (*innocua*, *seeligeri*, *grayi*, *ivanovii*, *welshimeri*) these other *Listeria* species, including *L. innocua*, showed no reactivity in the assay, even at high cell concentrations, nor did other microorganisms likely to be present in food samples. The results are presented only for the detection of samples by means Ab/InlB antibody and summarised in Figure 1.

CONCLUSIONS

We report here the development of a *L. monocytogenes*-specific immunoassay using specific antibody against internalin B (Ab/InlB). The assay, which clearly distinguishes the pathogenic *L. monocytogenes* species from the closely – related non-pathogenic species *L. innocua*, and from the other *Listeria* species, was developed by targeting antibody production to internalin B, a protein associated with the virulence of *L. monocytogenes*. The influence of milk and ice cream matrices is negligible. All 21 samples containing *L.monocytogenes* were recognised as positive. Only samples number 2, 28 and 29 was not possible statistically distinguished from control sample number 30.

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