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Rapid Detection of *Listeria monocytogenes*

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RAPID DETECTION OF *LISTERIA MONOCYTOGENES*

by

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ABSTRACT

Rapid Detection of *Listeria monocytogenes*

by

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Listeria monocytogenes is a foodborne pathogen that can cause severe illness and even death. It is found in dairy and meat products. The focus is on rapid detection since conventional methods are time consuming (4-5 days). Pre-enrichment steps, as part of those methods, are time consuming. Our objective was to develop a detection system without a pre-enrichment step, giving a final result within 2 to 4 h.

In the concept of "the need for speed," a detection system with an antibody-based capture technique, followed by polymerase chain reaction (PCR), was developed. Glass beads coated with a *Listeria* polyclonal antibody were added to the food sample. After a static incubation/capturing step, beads-cell complexes were separated from the food, and boiled to lyse the cells and release the DNA. In a final PCR/electrophoresis step the DNA samples were analyzed.

The use of a flow-based capturing system (ImmunoFlow) was also investigated. Using a bead-antibody complex in this ImmunoFlow setup has several advantages, including the possibility of concentrating the microorganisms out of large food samples

(with flow through setup), the exclusion of a pre-enrichment step, and the potential for automation.

Besides buffer solution (Tris), different kinds of milk, e.g., pasteurized, Ultra High Temperature (UHT), and raw milk, were also investigated. The detection limit in buffer solution was 1×10^6 CFU/ml no matter if the ImmunoFlow system or the static incubation was used. For the different pasteurized milk samples, the detection limit varied between 1×10^7 and 1×10^8 cells/ml in the static procedure. For UHT and raw milk, however, capturing of *Listeria monocytogenes* cells was not possible in the static or the ImmunoFlow setup.

In conclusion, we developed a rapid and specific detection system for *Listeria monocytogenes* at high concentration in pasteurized milk using a static capturing procedure. The total test time for this detection system is less than 4 h, which is much faster than the present detection systems (which are using an enrichment step prior to testing). Implementing a real-time PCR system after capture would further reduce this detection time.

(124 pages)

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Wim Lippens

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LIST OF SYMBOLS AND NOTATIONS

Ab = antibody

Ag = antigen

AOAC = Association of Official Analytical Chemists

APTES = γ-aminopropyl triethoxy silane

a_w = water activity

B = antibody B65420R

BSA = bovine albumine solution

cAMP = cyclic adenine monophosphate

CDC = Center of Disease Control

ddH_2O = double distillated water

DEX = dextran

DNA = dihydroxy ribonucleotide acid

dsDNA = double stranded DNA

EDTA = ethylenediaminetetra acetic acid

ELISA = enzyme linked immuno sorbent assay

EthBr = ethidium bromide

FDA = Food and Drug Administration

FSIS = Food Safety and Inspection Services

G = antibody G5-V99

GMP = good manufacturing practice

H = flagellar antigen

HACCP = hazard analysis critical control point

HTST = high temperature short time

IMS = immuno-magnetic separation

Ino2 = primer sequence name

K_A = affinity constant = [Ab – Ag] / [Ab] x [Ag]

KAc = potassium acetate

kGy = kilo grays

K_{off} = molar concentration of the unoccupied antibody binding sites multiplied with the molar concentration of the unoccupied antigen binding sites = [Ab] x [Ag]

K_{on} = molar concentration of the Ab-Ag complex = [Ab - Ag]

Lis1B = primer sequence name

MAP = modified atmosphere packaging

MonoA = primer sequence

Mpa = mega pascal

NASBA = Nucleic Acid Sequence-Based Amplification

O = somatic

PBS = phosphate buffer saline solution

PCR = polymerase chain reaction

PEG = polyethylene glycol

PNPP = *p*-nitrophenyl phosphate

R = antibody R4-V99

RIC = rapid ImmunoCapture

RNA = hydroxy ribonucleotide acid

RPM = revolutions per minute

RT = room tempeature

RT-PCR = reversed transcriptase polymerase chain reaction

S/N = signal over noise

SDS = sodium dodecyl sulfate

ssDNA = single stranded DNA

TAE = tris-acetate and EDTA containing buffer (1 M, pH 8.0)

TRIS = tris (hydroxymethyl) aminomethane

USU = Utah State University

WHO = World Health Organization

Y = antibody YVS4201

YOPI = young old pregnant ImmunoDeficient

CHAPTER 1

INTRODUCTION

The bacterium *Listeria monocytogenes* is a foodborne pathogen discovered in the early 1900's. Research throughout the years lead to a more detailed understanding of this organism. In the second half of the 20th century the pathogenic properties of this organism were clearly understood. Since then, increasing attention was given to *L. monocytogenes* as it impacts human health. The microorganism is not on top of the list of foodborne incidences (*Campylobacter*, *Salmonella*, and *Shigella* are above it), however, it is the leading cause of fatalities from food sources.

It causes invasive illnesses such as gastroenteritis and meningitis. The elderly, children, immuno-compromised and pregnant women are among the high-risk population commonly infected by this organism. Because of the high fatality rate, a lot of attention is given to *L. monocytogenes* by the food industry. Since 1989, a zero tolerance policy was introduced by Food Safety and Inspection Service (FSIS) for ready-to-eat products such as hot dogs. FSIS as well as Food and Drug Administration (FDA) together with the Center of Disease Control (CDC) are trying to increase the public awareness towards *L. monocytogenes* and to educate in order to prevent outbreaks (1, 2).

Testing food samples for *L. monocytogenes* is, however, time consuming. Very recently, new and faster procedures were introduced to aid this process. It takes 5-6 days to obtain a definite result. Even the new, faster methods, which have their focus on shortening the detection time, take on average up to 2 days, since an enrichment step is always included.

The focus of this research was to eliminate the enrichment step, build on the information found in literature about *L. monocytogenes* detection and use a system previously developed for the detection of *Escherichia coli* in our lab as the basis for this research project. The approach taken is an antibody based capture combined with a polymerase chain reaction (PCR) based detection in order to detect this microorganism in a fast, easy and accurate way. To reach this goal, glass beads were covalently coated with antibodies, specific for *Listeria*. The specificity of the test was obtained by combining the capture of the cells with a PCR step that allowed the multiplication of a specific DNA region of the *L. monocytogenes* genome.

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CHAPTER 2

LITERATURE REVIEW

Growth Properties and Danger

Listeria is a small (0.5 μm in diameter, 1-2 μm in length), non-spore-forming, Gram-positive rod. Cells are found singly or in short chains that can be isolated from a wide range of foods and environments (71, 88, 94). *Listeria monocytogenes* is non-motile and produces little or no detectable flagella (89). It is commonly found in soil, water, and plant material (24). However, the number of the organisms present in those habitats is very low (26, 93). In the food processing industry, *L. monocytogenes* is particularly difficult to control because it can adhere to food-contact surfaces and form a biofilm or coating that impedes the effectiveness of sanitation procedures (36). All *Listeria* species are phenotypically very similar, but can be distinguished on the basis of hemolysis and acid production from D-xylose, L-rhamnose, α-methyl-D-mannoside, and mannitol (Figure 2-1). *L. monocytogenes* is however the only pathogen for humans, where as *L. ivanovii* can cause severe illness among animals too (115).

Recent analysis of the 16s and 23s rRNA of *L. monocytogenes* has further clarified the phylogenetic position of *Listeria* in relation to other genera of gram-positive bacteria. However, the exact phylogenetic position of this genus remains controversial (89). Using those highly conserved genes, it is possible to detect and differentiate *Listeria* from other organisms in a genetic testing scheme. Differences between

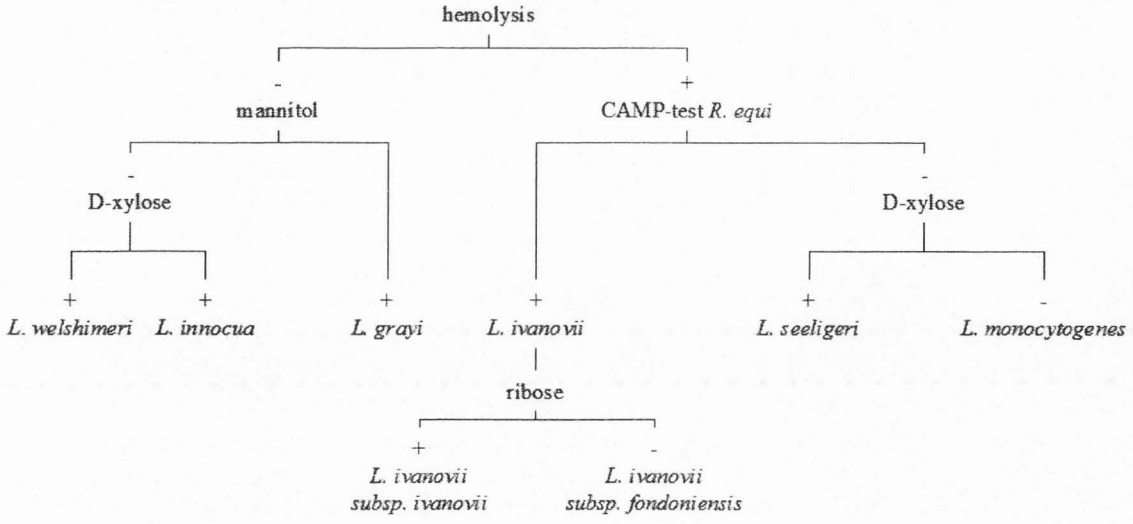

Figure 2-1. Identification of *Listeria* species (adapted from 89).

L. monocytogenes strains are identified by serological typing (serotyping), which is based on somatic (O) and flagellar (H) antigens. Other, less frequently used, conventional subtyping methods are bacteriophage typing, bacteriocin typing and antimicrobial susceptibility testing (37). The vast majority of human listeriosis (~90%) is associated with three serotypes (4b, 1/2a, and 1/2b) (28, 53, 100, 104). Currently, molecular-based methods are gaining more interest since they are more specific and easier to perform than growth or Ab-based tests (37).

With the combined inhibitory effect of a low pH and the activity of lactic starters, the growth of *Listeria* in cheese can be retarded, but it is difficult and had limited success (77). The survival and growth depends on the conditions during manufacture, ripening, and storage of the cheese products. In an outbreak in France, the microorganism was found throughout the entire period of ripening and storage in soft lactic cheeses made from raw goat milk and led to several cases of listeriosis (22, 34, 77). *L. monocytogenes*

is also found in other dairy products, eggs, chicken, meat products, vegetables, and seafood (2, 17, 29, 35, 41, 74, 94). A survey of 204 small abattoirs in Switzerland revealed that almost 11% of all meat samples were positive for *L. monocytogenes* (25). The microorganism has a wide growth temperature range and can withstand low pH and high salt concentrations (reviewed in 65). Sado et al. (94) reported the survival of *L. monocytogenes* in unpasteurized fruit juice at pH 3.75. Furthermore, this bacterium has the capability to grow at refrigerated temperatures (7°C and lower) in aerobic or anaerobic conditions (reviewed in 89). The so-called ready-to-eat products, which do not receive any heat treatment by the consumer, but are packaged under vacuum or modified atmosphere (MAP), are potential threats to consumer safety towards this bacterium (88, 96, 110). In an effort to understand and control *L. monocytogenes* growth and contamination, researchers have developed predictive growth models, quantitative modeling, and risk assessment formats (45).

Preservation Techniques

Methods used in food preservation involve physical, chemical, and biological factors. Physical preservation includes heating, cooling, freezing, irradiation, pulsed light, and high pressure. Chemical treatments include addition of anti-microbial, acidifying, and curing agents. Preservation by biological means includes fermentation, which control spoilage and pathogenic microorganisms through gradually lowering the pH (65). Based on the properties of *L. monocytogenes*, proper preservation techniques can be applied to control this bacterium (65).

L. monocytogenes is psychotropic and therefore can grow in the range 0°C to 45°C (8). Temperatures below 0°C will slow down growth or moderately inactivate this pathogen. It is important to stress that the bacterium is not killed when frozen. It is true however, that although freezing will only cause a limited decrease in viability, such treatments can injure and thus sensitize the organism to other anti-microbial treatments. Conversely, temperatures higher than 50°C are lethal to *L. monocytogenes* (105). The CDC, FDA and the World Health Organization (WHO) concluded that High Temperature Short Time (HTST) treatment and milk pasteurization (71°C, 15 s) are safe processes that effectively reduce the number of *L. monocytogenes* to levels that do not pose an appreciable risk to human health in healthy populations (65, 106).

Water activity (a_w) is an important parameter in an attempt to control microbial growth. Like most bacteria, *L. monocytogenes* grows optimally at a_w ~0.97. However, it can multiply at a_w values as low as 0.90 and can survive for extended periods at a_w-values lower then 0.9 (65).

High pressure treatment (375 Mpa for 15 min) was sufficient to inactivate more than 1 x 10^5 CFU/ml in a phosphate buffer (65). Applications of this process in food products are limited, but look very promising especially in beverages.

Listeria spp. are more resistant to gamma irradiation compared to other non-spore-forming food borne pathogens (1.7-4.0 kGy for reduction of 7 orders of magnitude). Ultraviolet radiation and high-intensity pulsed light are only useful to inactivate microorganisms on the surface of foods since they only have limited penetration power (65).

L. monocytogenes is acid tolerant. The minimum pH that this species, and most of the other *Listeria* spp. can grow, is between 4.5 -5.0. This, however, is only possible if the organism is incubated near optimum temperatures (37°C) and sufficient time is given to overcome an extended lag phase (at least 4 hours) (reviewed in 63, 65).

Bacteriocins have had limited applications in food as bio-preservatives. Three examples of bio-preservation are bacteriocins including pediocin, nisin, and lactisin. The first one is produced by certain strains of *Pedicoccus acidilactici*, the last two are produced by some strains of *Lactococcus lactis* ssp. *lactis* (14). All three proteins inhibit *L. monocytogenes* in food, but their activity is strongly influenced by various environmental conditions such as pH and temperature (65).

Infection and Risk Populations

Infection with *L. monocytogenes* causes influenza-like symptoms that progress to gastroenteritis, convulsions, and even spontaneous abortions if untreated (46). From the mouth, the pathogen travels to the stomach, across the intestine, and spreads through the bloodstream (reviewed in 89, 98). Bacterial binding to the intestinal cells prior to invasion is established by the interaction of the bacterial protein internalin with an intestinal receptor called E-cadherin (27, 89). Listeriosis is commonly associated with infections in the uterus, sepsis and the central nervous system. However, it can also cause disease without invading the host cell; therefore, it is classified as facultative intracellular pathogen (57).

The entry of *L. monocytogenes* into non-phagocytic cells occurs without any detectable perturbation of host cell morphology. The invasion is mediated by the membrane protein internalin, encoded by the genes *inlA*, *inlB*, and *inlC* (27, 91, 98). Protein p60, encoded by the gene *iap* (invasion associated protein), has also been associated with the entry into host cells (87). The cells will first appear in a vacuole that has a single membrane which is subsequently lysed, allowing *L. monocytogenes* cells to escape to the macrophage cytoplasm (28, 58). One of the proteins responsible for this lysis is listeriolysin O (LLO). It is a hemolysin, encoded by the *hly* gene (87, 98). After lysing the membrane by the protein, the bacterial cell is released in the cytoplasm. At this stage, the microorganism can multiply rapidly, but can also move into adjacent cells, eventually becoming wide spread in the host organs (28, 38) (Figure 2-2). To move within and between host cells, the *L. monocytogenes* cells use host actin filaments by disrupting the host cytoskeleton. This is called actin-based motility (87). The actin filaments are organized into long polar tails. The process of asymmetric actin nucleation by *L. monocytogenes* requires only one surface protein called ActA (98). This tail produces the propulsive force that moves the *Listeria* through the cytoplasm of the host cell at ~1.5 μm/s. Continuous *de novo* actin polymerization is required for this movement (28, 58). The bacterium enters neighboring cells by producing special cell structures (protrusions) ending up in a double membrane vacuole. Escaping into the cytoplasm of the new host involves *prfA*, *plcA*, and *plcB* genes (98).

In order to eliminate the pathogen, macrophages will ingest the cells very rapidly, and intracellular killing starts shortly after phagocytosis and leads to destruction of most of the ingested bacteria through acid production (58).

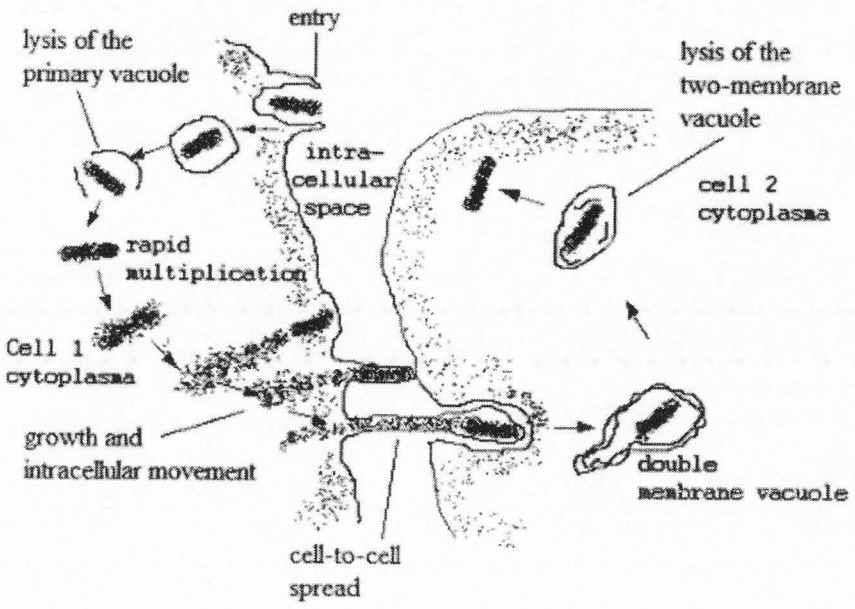

Figure 2-2. The intracellular life cycle of *L. monocytogenes* during host infection (adapted from 28).

The minimum infectious dose for humans ranges between 10^3-10^4 CFU/g of food (107). Ingestion of such a dose creates a significant health threat for people, usually in certain well-defined high-risk groups such as newborns, children, immuno-compromised and elderly people grouped under the acronym YOPIs (Young, Old, Pregnant, and ImmunoCompromised) (58, 101). In these people, the mortality rate from listeriosis can be as high as 30%, which is very high compared to for example *E. coli* O157:H7 with a fatality rate around 4% (110). *L. monocytogenes* is one of the few infectious microbes that can cross the placenta and infect the fetus. Although the pregnant women might not experience any symptoms, the fetus can be infected (98). People not related to one of the groups above however, can also suffer severely after ingestion of high numbers of cells (49).

Table 2-1. Clinical syndromes associated with infection with *L. monocytogenes* (63).

Population	Clinical presentation	Diagnosis	Predisposing conditions or circumstances
Pregnant women	Fever, myalgias, diarrhea Preterm delivery Abortion Stillbirth	Blood culture Amniotic fluid culture	
Newborns			
<7 days old	Sepsis, pneumonia, meningitis, sepsis	Blood culture Cerebrospinal fluid culture	Premature
=7 days old			
Non pregnant adults	Sepsis, meningitis, focal infections	Culture of blood, cerebrospinal fluid, or other normally sterile site	Immuno suppression advanced age
Healthy adults	Diarrhea and fever	Stool culture in selective enrichment broth	Possibly large inoculum

Less frequently reported susceptible populations are diabetics, cirrhotics, and asthmatics (Table 2-1). In a recent study, the frequency of meningitis caused by *L. monocytogenes* was 7.5%, with a death rate of 40%, which was the highest among all the organisms that were investigated (50).

Conventional Detection Systems

In 1926, Murray and coworkers stated (78), "The isolation of the infecting organism *L. monocytogenes* is not easy and we found this to remain true even after we had established the cause of the disease." It is interesting to notice that this statement is still true today. Extensive work has been done to develop pre-enrichment media for the isolation of *L. monocytogenes* from foods (23). However, this leaves a more rapid detection unaddressed and places a burden on processors in the current zero tolerance practice, requiring many to hold their products for 7-10 days while waiting for the microbiology results.

Plating methods

In 1948, M. L. Gray introduced a cold enrichment procedure to isolate *L. monocytogenes*. The incubation time, however, which is 5 to 13 weeks at 4°C, was a big disadvantage. Because of the need of shorter incubation times, selective enrichment and plating media were developed that further narrowed the selective nature of the medium and decreased the growth time (reviewed in 23). In 1960, the first widely used plating medium was introduced, being the McBride *Listeria* Agar (MLA). A short chronological overview is given below (Table 2-2). Results with traditional plating methods are typically obtained after 4 or 5 working days, making conventional plating methods time consuming and too slow for practical use in today's processing environment. Although these media are selective for *L. monocytogenes*, pre-enrichment with a specific broth is preferable in almost every case. Enrichment broths commonly used include tryptose broth, University of Vermont Medium (UVM), and Fraser Broth (23).

Table 2-2. The major selective media for isolation and enrichment of *Listeria* (Adapted from 23).

Year	Name
1959	Modified McBride *Listeria* Agar (MLA2)
1960	McBride *Listeria* Agar (MLA)
1986	Lithium chloride Phenylethanol Moxalactam Agar (LPM Agar)
1987	FDA-Modified McBride *Listeria* Agar (FDA-MMLA)
1988	RAPAMY and ALPAMY
1989	Polymixin B, Lithium chloride, acriflavine and ceftazidime (PALCAM Agar)
1989	Oxford Agar (OXA)
1990	Modified Oxford Agar (MOX)

Biochemical methods

Plating is in most cases followed by biochemical tests (49). These tests, used to identify the different *Listeria* species, include β-hemolysis, Gram reaction, motility, carbohydrate utilization profile and cAMP reaction (Figure 2-1). Total test time is typically 8-10 days (67). Various commercial miniaturized multitest assays are used (89), including 20S API-ZYM (Anal. Prod., Plainview, NY), API *Listeria* Vitek Automicrobic Sys. (BioMérieux, Hazelwood; MO), and MICRO-ID kit (Organon Teknika, Durham, NC).

Rapid Detection Systems

The need for speed

The total testing time for a food sample with conventional detection methods takes easily 48 to 96 h. These techniques, in use since the 1980's, are not sufficiently rapid to assure the safety of perishable food products before consumption (7). If the manufacturer wants to be sure about the microbial safety of the finished product however, two major disadvantages arise. First, expensive and big storage facilities are needed to store the product while it is held waiting for the bacterial results. Second, the storage time shortens the shelf life of the product. These factors often force companies to ship their products before testing is completed, with the risk of recalls.

In most cases, this risk is acceptable especially when Good Manufacturing Practice (GMP) is followed. However, positive results of the samples after shipment will force the manufacture to launch a recall for the product (69). Besides the risk of

fatalities, economic damage, reputation and salability are affected, and have lead to bankruptcy in some cases. Contaminated finished meat products at Bil Mar Foods (Illinois, December 1998/January 1999) resulted in a major recall. Contaminated sausages produced by this large company were distributed nationwide and caused hundreds of illnesses and several consumers died. The company decided to recall all of its sausages which amounted to 13,000 metric tons from its distribution chain. In the end, 21 consumers died and a class action lawsuit was filed against the company (61). And very recently, in October 2002, 27.4 million pounds of fresh and frozen ready-to-eat turkey and chicken products were voluntarily recalled by Pilgrim's Pride Corporation (Wampler Foods, Inc.) because of *L. monocytogenes* contamination (30). Other recent recalls include 150 pounds of pork dumplings from Goldon Coin Food Industries, Hawaii (Oct. 2002) and 200,000 pounds of fresh and frozen poultry products from J. L. Foods Company Inc., NJ (Nov. 2002) (31, 32).

Because of the reasons mentioned earlier (shortening shelf life, risk of outbreaks and/or recalls, etc.), a fast, sensitive and accurate detection system is needed in order to trace *L. monocytogenes* before shipment and yet provides a confident level of consumer safety. Angèles d'Auriac et al. (3) stated this as follows: "To be useful, such methods [detection systems] ought to produce results within one working day; be quantitative, sensitive, and specific; require less work than the current standard methods; have a high throughput; and be nondestructive to the target organisms so as to allow confirmation work."

In the context of "the need for speed," several investigators mention the direct need of a rapid, sensitive, and specific method for the early detection of *L.*

monocytogenes (56, 60, 85). Rapid detection systems can roughly be divided into three groups (with some overlap): ImmunoAssay based methods, ImmunoLatex agglutination based methods, and DNA/RNA based methods.

Immunoassay-based methods

Antibody-based detection systems are very popular and are widely used commercially. This is not surprising considering the fact that antibody detection systems have been applied in clinical testing for over 20 years and are versatile in their applications. The use of antibody systems, mainly as ELISAs, for analyzing food samples, is fast after a pre-enrichment step (10).

Three criteria are very important to judge the usefulness of an antibody in a rapid detection system: specificity, nonspecific binding, and affinity (12). Specificity indicates how well an antibody distinguishes between different antigen structures (epitopes) on a variety of different bacteria. This is determined by the interactions between the variable chains and the antigen. When a non-target organism has a similar surface epitope as the target organism, a cross reaction occurs, thereby producing a result that may be considered as a false positive. For example, an antibody against *Lactobacillus* that also reacts with *E. coli*, will lead to a false positive. Cross-reaction is mediated through the antigen-binding site (idiotype), not through non-specific molecular interactions. Non-specific interactions are often a limiting factor in ELISA testing because it leads to high background values. This causes problems with the detection limit and may also be related to false positive results or ambiguous readings. A great deal of effort is made in the selection of the antibody used in the test to maximize specificity and minimize

nonspecific binding. Affinity, the ratio of K_{on} to K_{off}, measures the binding strength between the antibody and the epitope and is expressed by the affinity constant (K_A). It is another important factor to evaluate an antibody (12).

In a standard ELISA format, cells are directly absorbed or immuno-captured onto wells of a microtiter plate (e.g. 96 well plate) using mono- or polyclonal Abs. The captured cells are then detected (indirect or direct) using an antibody that carries either a hapten or an enzyme reporter (20). Positive samples however, must be confirmed using standard microbiological culture methods (7). Some commercially available ELISAs are *Listeria*-Tek (Organon Teknika, Durham, NC), Reveal for *Listeria* (Neogen, Lansing, MI), TECRA *Listeria* Visual Immuno Assay (Int. BioProducts, Redmond, WA), and Transia Plate *Listeria monocytogenes* (Diffchamb AB, Västra Frölunda, Sweden). All these techniques require at least 48 h to complete (enrichment step not included) and identify at the genus level. A method, similar to ELISA is the enzyme linked fluorescent immunoassay (ELFA). In 2000, BioMérieux (Durham, NC) developed the Vidas Lis and Vidas Lmo tests, which are based on ELFA for detection of *Listeria* and *L. monocytogenes*. The Official Methods Board of AOAC International adopted the Vidas Lis method. Results are obtained after 48 h (enrichment not included) (33). Clearview (Oxoid, Ogdensburg, NY) is another rapid, antibody based detection system for *L. monocytogenes*. Antibodies for capturing the microorganisms are immobilized on a porous membrane strip and the detection is based on a colorimetric blue signal. Total test time is 44 h but detection is limited to genus level (90).

A technique that is very interesting uses magnetic beads. Particles coated with antibodies capture and concentrate microorganisms and are separated from the food or air particles with a magnet (54, 68). The technique is called ImmunoMagnetic separation (IMS). This system has been made commercially available by Dynal (Oslo, Norway) but other products such as Captiva O157 (International Diagnostic Group, Bury, UK) and ListerTest (Vicam, Watertown, MA) are now also on the market (86). Dynabeads are monosized polystyrene spheres with super paramagnetic properties. The Dynabead anti-*Listeria* beads are pre-coated with high-affinity antibodies against surface markers of live *Listeria* (80).

The paramagnetic beads are removed from the food sample with a magnet. Although this system can be a useful tool to separate the food compounds from a sample and to selectively concentrate targeted cells, some disadvantages were found. Some food components can interfere with this process. Considerable non-specific binding was observed and no increased ratio of *L. monocytogenes* to non-*Listeria* flora was found (109).

Since polyclonal antibodies (Abs) are used, a combination of the immuno capture device with PCR is required to give a definite result about the presence of *L. monocytogenes*. Furthermore, intact dead cells can also bind to the immuno magnetic beads (84). However, incorporation of two washing steps seems to remove dead cells (49, 109). This means that the affinity for the Ab-dead cells complex is lower than for the complex with living cells. Collecting Dynabeads out of a food sample suspension can be impaired, and as a result, only a partial recovery of the beads was possible (15). This problem cold be circumvented by diluting the suspension before or after adding the

Dynabeads. The drawback was that only at the highest inoculum level (40 CFU/g cheese) *L. monocytogenes* cells could be detected (109). O'Conner et al. (84) concluded that food components such as fats and food debris can interfere with the antibody-organism interaction. Uyttendaele et al. (109) formulated two remarkable conclusions about IMS: 1) IMS is not appropriate for separation of *L. monocytogenes* from a concentrated cheese homogenate and 2) IMS with Dynabeads anti-*Listeria* did not allow a selective enrichment or concentration of *L. monocytogenes*. The overall conclusion was that there is no justification to include IMS using Dynabeads anti-*Listeria* in an isolation procedure for *L. monocytogenes*. Hudson et al. (49) found that after three washes, the recovery of *L. monocytogenes* cells was only 1% and a poor detection limit was observed for skim milk at 2×10^5 CFU/ml (80). Contrary to those findings, IMS was very successfully used in the detection and concentration of *E. coli* in samples of raw vegetables and raw meat products (16, 95), the capturing of *Cryptosporidium* oocysts in water (48), and the detection of *Pneumocystis carinii* DNA (68). The same positive results were obtained for *Salmonella* in food and stool samples (102). The fact that an article from the University of Oslo in cooperation with Dynal itself mentions detection of *L. monocytogenes* cells only at a cell concentration of 10^7 and 10^8 CFU/ml, is remarkable (92). It highlights the difficulty of creating a sensitive system. EiaFoss *Listeria* (Foss North America, Eden Prairie, MN) is a commercially available system that combines IMS with ELISA. In a similar system, Blake and Weimer (11) combined an IMS technique for capturing with a sandwich ELISA for the detection of *Bacillus stearothermophilus* with a lower detection limit of 8×10^3 CFU/ml.

Another way to concentrate foodborne bacteria is by adding metal hydroxides. Using this method, researchers found a 50-fold sample concentration and the recovery of bacteria was between 65 and 96% (64). Fiber optics is another way to rapid detect *L. monocytogenes*. The system is based on a sandwich immunoassay using cyanine 5 dye-labeled polyclonal antibodies. Biotin-avidin interactions are used to attach the antibodies onto the fiber probe. A sensitivity of 3-30 CFU/ml (ground beef samples) was obtained. Two major drawbacks of the system are the inability to give information about bacterial viability and the small, 100 µl, sample size (21).

Immunolatex agglutination-based methods

Latex agglutination tests are mainly used in the health care sector to detect the presence of antibodies or antigens in bodily fluids. If a sample contains the corresponding antigen or antibodies, the latex beads will agglutinate when mixed with the sample. This method can provide a result in 15 min. Examples of the immuno-latex agglutination based method are Micro-ID *Listeria* (Remel, Lexena, KS) and *Listeria* Rapid Test (Oxoid, Ogdensburg, NY).

DNA/RNA-based methods

DNA and RNA based methods have seen an immense increase in popularity over the last two decades. Their specificity and sensitivity are two main advantages that play an important role.

Nucleic acid hybridization techniques. The main purpose of nucleic acid hybridization, first introduced in the 70's, was to study specific genes (97). Several improvements over the last decade increased the sensitivity and reproducibility

drastically. One of the major changes was the replacement of nitrocellulose membranes by nylon membranes. Due to these improvements, nucleic acid hybridization techniques are currently used for the detection of pathogens. GenProbe (San Diego, CA) introduced AccuProbe, which is specific for the detection of *L. monocytogenes*. Detection is based on a single strand DNA probe with a chemiluminescent label that hybridizes to the rRNA of the target organism. The stable DNA:RNA hybrids are measured in a luminometer. This system is, however, only tested on pure cultures after enrichment. Two assays have been developed by Gene-Trak (Hopkinton, MA); one to detect all species of *Listeria* and another specific for *L. monocytogenes*. For both assays, a dipstick is placed in the cell lysate solution with a probe present, which was added in a previous step. The 16S rRNA gene is targeted and the read out is colorimetric (7). The company claims a sensitivity of 1-5 CFU/25 g of sample in 2 h after 48 h enrichment.

Polymerase Chain Reaction (PCR). Genetic tests are focused on PCR methods for the detection of pathogens. Initially, these methods extracted DNA directly from food with limited success for all food types. As such, commercial methods were developed based on a pre-enrichment step prior to the PCR detection as a strategy to simplify the extraction process, and as a way to increase low-density pathogen populations. More recently, PCR-based methods have been developed to extract DNA from some foods and environmental samples. Other DNA detection systems have been used to track strain relatedness, but they only provide historical information useful in epidemiological studies.

Detection techniques based on PCR are very promising because simplicity is combined with specificity and sensitivity for detection of the pathogen, if sufficient

quality DNA can be extracted from the sample (13, 39). In vitro amplification of specific DNA sequences by PCR allows direct detection and identification of the pathogen. Chen et al. (18) claimed a sensitivity of 10 fg DNA, which theoretically corresponds to approximately two *L. monocytogenes* organisms per ml (18). Sensitivity is sometimes approached by researchers based on the volume present in the PCR tube (which generally contains only 25 to 50 µl). However, others refer to the initial present cell number before an enrichment step for cell number calculations. A detection limit lower than 10^3 CFU/ml without previous enrichment is seldom reported in literature. Shifting toward food samples reduces this sensitivity drastically.

A prerequisite for PCR is obtaining DNA (or RNA) for amplification, thereby relying on a robust lysis procedure. Different methods for lysis are present such as physical, chemical and enzymatic. The different methods can be compared to each other using absorbance and fluorescence techniques (4, 5, 6). Among physical methods, a boiling step for 5-10 min is most commonly used (75). This crude DNA can be "cleaned up" by implementing an extra purification/centrifugation step to remove cell debris (39, 40). The use of a bead beater is also used to lyse cells (111). This mechanical lysis method is based on shear forces between the cells and beads added to the cell suspension. Alkaline lysis is a chemical method and uses a solution of NaOH and sodium dodecyl sulfate (SDS) often in combination with a heating step (44, 60, 75). It became clear, however, that the detergent SDS inhibits the polymerase activity in PCR. The easiness of foaming (even at concentrations of 0.1%) and the toxicity of SDS make its use unfavorable in food product analysis (97). Triton X-100 is another chemical used for cell lysis (1, 19, 52, 97, 112). The two most commonly used enzymes are lysozyme and

proteinase K. Lysozyme degrades the bacterial cell wall and is therefore often used in extraction protocols. It is applied alone or in combination with other enzymes (e.g. proteinase K, pronase E) and/or chemicals such as SDS (49, 62, 103). Incubation time with lysozyme varies from 5 min to 30 min at 37°C. Proteinase K, as the name reveals, degrades proteins in the cell wall. O'Connor et al. (84) used this enzyme alone to degrade the cell wall; Subsequently, heating at 95°C for 10 min inactivates the enzyme prior to subjecting to PCR. A combination of lysozyme (15 min) with proteinase K (60 min) at respectively 37°C and 60°C, produced the highest amount of DNA release in *L. monocytogenes* cells (84).

PCR involves the enzymatic amplification of a targeted nucleic acid sequence using a thermo stable DNA polymerase and a primer that uniquely defines the target. Theoretically a single copy of target DNA can be amplified to 10^6 copies in only 30-40 cycles, generally completed within 1-3 h (81). The choice of the sequence targeted is often based on the genes encoding for the virulence factor. For *L. monocytogenes,* this is most commonly the gene encoding for listeriolysin O, *hlyA*, used for host cell entry. Other target genes include *iap* (invasion associated protein p60), *inl*A (internaline), *prf*A, or *ima*A (44, 51, 56, 79, 81, 83).

Although PCR is a very promising technique, the poor sensitivity in food analysis due partly to reaction inhibitors and the potential for false-positives reactions, have limited the routine application of PCR-based screening assays (81). The industry tries to accommodate this by offering DNA/RNA clean up kits including InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA), Lyse-N-Go PCR Reagent (Pierce, Rockford, IL), Dynal beads DNA Direct (Dynal, Oslo, Norway), and the High Pure PCR Template preparation

kit (Roche, Indianapolis, IN). The primary functions of these kits are to concentrate the template and to separate the inhibitors before the PCR analysis. The latter acts generally at one or more of three essential points in the PCR reaction: interfering with cell lysis, interfering with nucleic acid degradation, and inhibiting the polymerase activity for amplification of the target DNA (116). PCR applications commercially available for detection of *L. monocytogenes* include BAX system (Qualicon, Wilmington, DE), Probelia (Bio-Rad laboratories, Hercules, CA), and DNA-Detect (Vita-Tech, Markham, Ontario, Canada).

A PCR reaction cannot distinguish DNA from live or dead cells (81) and therefore might result in false positives. Klein and Juneja (56) amplified *L. monocytogenes* DNA 6 h after autoclaving, which shows the stability of DNA. Herman (43) concluded that *L. monocytogenes* DNA could be detected by PCR more than 30 days after cell viability was lost due to various inactivation treatments. This is particularly important in foods and the environment, where nonviable, inactivated pathogens may be present after treatments such as thermal or irradiated processed food (73). The chances of false positives with a PCR detection system are therefore high. Hence, the use of enrichment to overcome this problem by providing large amounts of DNA from an actively growing culture, is common. The insertion of an enrichment step however, has its own disadvantages. The most obvious disadvantage is the time needed to enrich (56, 81). The 2 h used by Klein and Juneja (56) is unlikely to be sufficient to allow growth since a growth curve after incubation in a specific, pure grow medium like BHI, shows a log phase of at least 2 h (this work). To isolate *L. monocytogenes*, Zhao and Doyle (117) concluded that a minimum of 24 h incubation at 37°C is required to obtain a 10^4 CFU/ml population.

High numbers of competing microorganisms (especially *L. innocua*) in food samples can overgrow and mask the presence of *L. monocytogenes* (the cells might not multiply to detectable numbers), leading to false negative results (99). Their presence will reduce the sensitivity of the PCR assay (16). Stressed cells may not multiply at all, but they may maintain metabolic activity.

The enrichment procedures are labor-intensive and don't allow high throughput or automation. All commercially available DNA testing kits use an enrichment step to increase the microbe concentration and to ensure that enough DNA (or RNA) is present (60). Zhao and Doyle (117) concluded that a short enrichment step of 6 h for heat-injured *L. monocytogenes* is not enough, and most kits use at least 8 to 24 h enrichments. Microorganisms can be sub-lethally stressed by the food environment and so require a period of recovery preceding enrichment (79, 117).

The use of rRNA, a much less stable molecule, in reverse transcriptase PCR (RT-PCR) to form cDNA prior to PCR is another solution to the problem of detecting false positives due to the presents of dead cells (44, 56, 73, 76). Taq polymerase does not amplify RNA, therefore it may be possible to selectively assess the active cells in the sample, even after autoclaving (56). Messenger RNA (mRNA) is also a promising target for detection assays because it is produced only by viable cells and is rapidly degraded after cell death. It can also be used as an internal signal amplification system because cells produce multiple copies of a single mRNA transcript during gene expression making it theoretically possible to increase the assay sensitivity (81, 82).

In addition to the several RT-PCR methods, an alternative approach was taken with the Nucleic Acid Sequence-Based Amplification system (NASBA) (108). This

method does not need the use of a thermocycler (isothermal reaction) and eliminates the separate RT-step for RNA amplification. Although RNA detection might have multiple advantages over DNA detection, the instability might lead to a false negative if the detection system is not fast or is contaminated with degradation enzymes, which are very common. Novak and Juneja (83) demonstrated that heat-injured *L. monocytogenes* cells were occasionally undetectable by RT-PCR. They concluded that RT-PCR was ineffective in detecting low levels of heat-injured cells until mRNA synthesis was reestablished.

A broader viewpoint in favor of DNA detection is GMP and HACCP conditions. If, in a sample of the end product DNA of *L. monocytogenes* is detected (from dead or alive cells), then improper handling techniques were used somewhere in the production chain. This brings us back to the introduction of this proposal were Graham et al. (35) stated that *L. monocytogenes* should be considered as an indicator organism. Presence of high numbers (10^3 CFU/ml or higher) of this microorganism in any of the ground material supplies, or in any food-processing step, must be traced and eliminated. Relying just on a heating step at the end of the processing chain may not be sufficient. The main problem with *L. monocytogenes* is usually growth in food during storage, which means that the problem of detecting dead cells is of minor concern anyway (80). In short, detecting a nucleic acid band on a gel after PCR amplification should raise concern irrespectively if this band is a result of DNA from dead or alive cells (assuming that bands are a result of 10^3 CFU/ml or higher).

An aspect independent of whether RNA or DNA is used, is the issue of false positive results due to cross contamination in PCR. Proper handling techniques are

required during PCR to avoid cross-contamination since it is considered as the main reason for false positive results (116). The development of single tube PCR products, like the Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ) or the BAX PCR amplification kit (DuPont Qualicon, Wilmington, DE), reduces the risk of cross contamination. An advantage of the BAX system is that all the reagents necessary for DNA amplification are present in a pellet form. The total analysis time is 52 h and detection is specific for *L. monocytogenes*. After PCR, gel electrophoresis is performed. Sensitivity was however poor and high numbers of false negatives were obtained (90). Other researchers however concluded that after including an enrichment step, the BAX™ system was very convenient with a detection limit of 1 CFU/25 g of meat sample (9, 81). By reducing the handling (adding and transferring components) cross-contamination of the PCR reaction can be reduced. The use of the enzyme uracil-n-glycosylase (UNG) or uracil DNA glycosylase (UDG) can also reduce this risk. After incorporating uracil into amplicons during PCR, specific degradation of fragments containing uracil will occur when treated with UNG or UDG. Thus products carried over from previous assays can no longer serve as templates (81).

Real-time PCR. Once the thermocylcer has amplified the DNA fragment, an agarose gel is loaded with the sample in the research laboratory. This gel electrophoresis step takes about 1 hour and in the concept of 'the need for speed', researchers are trying to avoid this step. To speed up PCR and obtain quick results, real-time PCR is used. This system is based on the detection and quantification of a fluorescent reporter as the amplicon is produced, so a real-time result is visible via photomultiplier tubes and no gel electrophoresis is needed. Molecular beacons, SYBR Green, and Taqman probes are

commonly used to create a fluorescence signal. Real-time thermocyclers such as the ABI PRISM 7700 (Perkin-Elmer, Foster City, CA), the Smart Cycler (Cepheid, Sunnyvale, CA), GeneAmp 5700 (PE Biosystems, Foster City, CA), Lightcycler (Roche, Indianapolis, IN) and Opticon (MJ Research Waltham, MA) are now commercially available. The chief disadvantage of real time PCR is cost. The machines are expensive to purchase and the maintenance and running costs are considerable (97). By avoiding post-PCR processing, the risk of cross-contamination is again reduced (42), but sample handling is still a primary concern. Quantification is also possible and a higher sensitivity compared to standard PCR is claimed (55, 80). The implementation of a real-time PCR step for *L. monocytogenes* detection has become more common (42, 55, 66, 80, 81).

ImmunoFlow system

The ImmunoFlow system developed and investigated in this research project uses the same basic idea as the IMS system in which cell capturing relies on antibodies covalently bound to beads. However the ImmunoFlow system uses an upstream flow-through, creating a fluidized bead bed. Furthermore, 3 mm in diameter glass beads are used which are very different from the 2.8 μm used for IMS beads. The ImmunoFlow system consists of a cartridge filled with glass beads that are coated with antibodies (Ab) and which will capture *L. monocytogenes* cells flushed through the system. By using the fluidized bed principle (flow of food samples is in upward direction through the bead bed), the food sample resides in the chamber and stays in contact with the beads longer than predicted by the linear flow rate, thus capturing and detecting *L. monocytogenes* is

theoretically, more efficient. Furthermore, the continuous movement of the beads will delay clogging of the capture device during food testing. This system was already successfully applied by Weimer et al. (113, 114) for the detection of *Bacillus globigii* spores and *Escherichia coli O157:H7*. A detection limit of 1 spore/cell, independent of the sample, size was reported (113). The detection procedure is based on an indirect sandwich ELISA. After running the sample over the beads, a specific, secondary Ab solution is run through the cartridge to bind the microorganisms captured by the Abs on the beads in the previous step. The following Ab solution, a tertiary Ab labeled with an enzyme, will attach to the secondary Ab. By adding a substrate in a next step, a color change will occur which can be quantitatively analyzed. If a presumptive positive result is obtained, a PCR/gel electrophoresis step can be performed on the DNA extracted from the captured cells. The linking of a real-time PCR system to the ImmunoFlow system should significantly reduce the analysis time (49, 72).

Although the ImmunoFlow system might have similar capturing efficiency problems as found in the IMS system (both systems use Ab), several disadvantages are overcome. First, the glass beads in ImmunoFlow are not separated from the fluid – eliminating the loss of beads as was previously experienced in IMS (11, 15, 109). By automating the system, the labor intensity and the analysis time will also be reduced significantly. Furthermore, the system uses large sample volumes (up to 5 L) that can be pumped through the bead bed at a flow rate to fluidize the beads, leading to a turbulent flow. The issue of small sample volumes for IMS was also recognized by Chandler et al. (15) and solved it by developing an injection flow capture system with a detection limit of 10^3 cells/ml. Concerning the flow dynamics, the same researchers came to the

important conclusion that only by inducing enough turbulence within the flow cell (fast trap-release) could efficient cell capture be obtained [i.e. the fluidized concept reported by Weimer et al. (113)].

Improving Detection Sensitivity

The focus on PCR methods is based on the preparation of DNA from the sample (either separation of the bacteria from the food or DNA extraction), template concentration techniques, and primer selection to amplify the target organism in a sea of other potential template DNA (60). All these elements present a challenge to research. The reason that those factors are important, lies in the sensitivity of PCR. First, as mentioned above, a minimum amount of DNA has to be present before amplification can occur. Second, polymerases, the core elements of the PCR reaction, are very sensitive to inhibition by many elements present in food products. Third, simultaneous detection by PCR of different microorganisms is difficult when one of the target organisms is present in lower numbers compared to the other ones (47). With complex matrices such as food, steps must be taken to limit the effect of any potentially inhibitory compounds present that may limit PCR amplification and therefore reduce the sensitivity (59, 70). At the same time, effort in concentrating the microorganisms (i.e. the DNA/RNA) to increase the sensitivity of the detection method must be done. In order to fully exploit the sensitivity and specificity of PCR, certain devices must be incorporated before the actual DNA amplification step in the thermocycler.

This study merged a capture/concentration method with PCR to eliminate enrichment in an effort to produce an assay that was fast (< 4 h), specific, and sensitive.

The cell separation/concentration step based on immuno-technology was combined with a DNA amplification step to obtain a final result. This approach eliminated several problems that came across in the past as mentioned throughout this literature review. By using an ImmunoFlow system with sampling station (i.e. circulation of the food sample over the beads), the ultimate goal was to eliminate any kind of enrichment step, which would increase the analysis time. A sample run in the ImmunoFlow system was completed within 30 min. A presumptive positive result will be confirmed with PCR after lysing the cells and extracting the DNA. This confirmation step might take another hour. This means that a positive sample confirmation will be obtained within 2 h.

Hypothesis

Listeria monocytogenes can be captured from milk using a fluidized-bed solid-phase capture system with covalently attached antibody-beads, allowing elimination of the enrichment step, and followed by PCR for specific detection.

Objectives

1. Optimize Rapid Immuno Capture (RIC) in buffer solution to capture *L. monocytogenes* after finding the optimum antibody combination and concentration.
2. Determine a method to lyse the cells after capture on to beads.
3. Combine Immuno-capture and PCR to detect *Listeria* and identify *L. monocytogenes* in pure culture and food samples.

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CHAPTER 3

RAPID DETECTION OF *LISTERIA MONOCYTOGENES* IN MILK VIA SOLID PHASE CAPTURE AND PCR[1]

Abstract

Listeria monocytogenes is a food borne pathogen that causes severe illness and death. Illness is frequently associated with dairy products and processed, ready-to-eat meat products. Detection of *Listeria* with antibody-based methods is limited to a genus only assay for AOAC approved methods. Other assays using PCR without additional treatments, such as pre-enrichment, have not been reported in food products. In this study, *L. monocytogenes* ATCC 43215 was captured with glass beads covalently coated with anti-*Listeria* polyclonal antibodies followed by detection with an ELISA or PCR. Capture was achieved with a 20-min shaking incubation or a 5 min fluidized bed step. Subsequently, the beads were washed, boiled, and used for detection with PCR. No detection was observed with PCR without solid phase capture from any kind of milk inoculated with as much as 10^8 CFU/ml added. Detection with solid phase capture decreased significantly ($p<0.05$) in milk samples compared to buffer. Further investigation of this observation demonstrated that heat treatment and increasing fat content of milk samples significantly decreased ($p<0.05$) capture in flow, but not in shaking capture setup. Both solid-phase capture conditions consistently bound cells in Tris buffer with a detection limit of 10^6 CFU/ml. The detection limit with PCR for the invasion associate protein gene (*iap*) in pure culture suspended in Tris buffer (without

[1] Coauthored by Wim Lippens, Marie Walsh, and Bart Weimer.

using glass beads) was $1.0x10^1$ CFU/ml. These data lead us to conclude that solid phase capture before PCR was required to detect *Listeria monocytogenes* in pasteurized milk. This approach provided a result in 4 h or less by eliminating the enrichment step.

Introduction

Despite the progress seen in recent times in medical care and food technology, food- and waterborne diseases are of increasing concern for human health worldwide (11). Listeria infections are of particular concern because the mortality is high and it persists to cause chronic diseases like gastroenteritis. *L. monocytogenes* is a gram-positive, psychotropic, foodborne pathogen found in milk and ready to eat food products (38). Due to its wide spread distribution in soil and raw foods, people frequently encounter this bacterium through various sources, like fresh cheeses, meat, poultry and eggs, fish and other seafood products (14, 41). This emerging pathogen causes listeriosis with influenza-like symptoms that progress to gastroenteritis, convulsions, and even spontaneous abortions among pregnant women if untreated (39). According to CDC, an estimated 2,500 people become seriously ill with listeriosis each year. Of those, about 500 people die each year (4, 36, 47). *L. monocytogenes* is the only species of concern for disease within the genus *Listeria*. The other five species, *Listeria ivanovii, Listeria innocua, Listeria welshimeri, Listeria seeligeri, and Listeria grayi* are rarely pathogenic for humans. Identification of the microorganism to the species level is therefore important to avoid false positive results in food safety analysis. This discrimination is more commonly done using DNA-based methods such as polymerase chain reaction

(PCR) or microarray-based assays (48).

Currently, detection requires 4-8 days before a definitive identification is obtained with plating and immunoassays. This a substantial limitation in detecting this organism (8). As a result, the rapid pathogen detection industry is a growing market with an expected grow of the testing market to $192 million and 34 million tests by 2005 (2). The latest developments in this field use bio- and microchip technology with promising results towards sensitivity and selectivity (17, 34).

An enrichment step is generally used to facilitate recovery of injured or stressed organisms, but also increases the time. Culture methods used by the FDA and the USDA-FSIS, have emerged as the most commonly used protocols in the United States. Both methods use selective (Fraser broth, FB; Oxford medium, OXA) and non-selective enrichment broths (Buffered *Listeria* enrichment broth, BLEB; Brain Heart Infusion, BHI; University of Vermont broth, UVM) as enrichment media (3, 22). There is a risk, however, that during enrichment other non-pathogenic *Listeria* spp., especially *L. innocua*, will overgrow *L. monocytogenes* (36). Direct PCR from the broth or the sample may overcome this problem.

Aznar and Alarcon (5) found that PCR methods were more sensitive since they were able to detect more positive samples. As a result, immuno- and DNA-based detection methods are of interest for rapid detection of *L. monocytogenes*. A major problem however, with DNA-based techniques such as PCR, is the inhibitory components in the food sample. Therefore, a separation from the food and the microorganism of interest is necessary in many food types. Several techniques to separate the bacterial cells or the genetic material from the sample before analysis have

been reported. These include surface adhesion immunofluorescent techniques (SAIF), probe membrane-based systems, and DNA absorbing magnetic beads (32, 33, 35 ,40, 43). Herman et al. (20) used a chemical extraction method combined with several centrifugation steps prior to analysis with nested PCR. Physical collection or separation of the cells by centrifugation, filtration and immunomagnetic separation (IMS) before analysis are also used (36). The latter method combined with bacteriophage assays, a chemiluminescence fiber-optic biosensor, slide agglutination or DNA probes besides PCR-based techniques, have also been reported (13, 25, 30, 45). However, after evaluating the efficiency and specificity of the IMS technique in different food samples, it was concluded that IMS was not appropriate for separation of *L. monocytogenes* from a concentrated cheese homogenate. Presumably, the Ab specificity and avidity were insufficient (23, 27, 35, 46). This work highlights the need for Ab that bind tightly to obtain a test that is sufficiently sensitive.

Weimer et al. (50, 51) developed an alternative Ab capture method for physical removal of bacteria before analysis. This approach uses a fluidized bed devise that allows turbid samples to flow into a cartridge containing 3 mm (in diameter) glass beads at 100 to 2,500 ml/min flow rates. Using the fluidized bed allows the cells to be captured within 5 to 20 min, thereby eliminating the pre-enrichment step. The sensitivity of this sample scheme varied with Ab, sample size, and bead volume. In each of these cases, Ab capture was coupled to ELISA detection. Additionally, Fluit et al. (15), used monoclonal Ab-based capture combined with PCR to provide a species specific result for *Listeria*. However, the latter detection method included an enrichment step, which brought the total detection time to 55 h.

Several *L. monocytogenes* PCR detection systems are commercially available. Pre-enrichment and sample preparation steps are part of all commercial methods, resulting in a minimum detection time of ~48 h (36). The hypothesis of this study was that *Listeria* can be captured from milk using a fluidized-bed solid-phase capture system with covalently attached antibody-beads, eliminating the enrichment step, and that this can be couple to a PCR reaction for specific *L. monocytogenes* detection. This hypothesis was confirmed in pure culture and in milk (however, only at cell concentrations simular to those after enrichment), whereby the enrichment step was eliminated using a 5 to 15 min capture step before detection. Milk fat reduced solid phase binding in flow, but had no effect on shaking capture in milk.

Materials and Methods

Bacterial strains and storage

The strains used in this project were *L. monocytogenes* ATCC 43251 and *Listeria innocua* ATCC 33090 (Rockville, MD). Stock cultures of each bacterium were prepared from single colony isolates after growth as described by the ATCC instruction sheet. Each culture was inoculated (1%) into 10% non-fat dry milk powder containing 33% sterile glycerol and stored in liquid nitrogen for subsequent use.

Inoculated sample preparation

Before each use, a new vial of the stock culture was thawed, inoculated (1%) into BHI broth, and grown for 12-14 h at 37°C on a shaking incubator (Lab-Line, Melrose Park, Il) at 150 rpm to a density of ~1 x 10^9 CFU/ml. The cells were washed twice with

an equal volume of 50 mM Tris (pH 7.2), collected by centrifugation (3,000 x *g* for 5 min at 4°C), and resuspended in the same volume of sterile 50 mM Tris (pH 7.2). This suspension was used to inoculate milk samples at 1:10 dilution of the cell preparation with various milk types and Tris buffer 10 min prior to using them for Immunocapture.

Ultra high temperature (UHT) milk products were purchased from Gossner Foods (Logan, UT). Pasteurized milk was obtained from a local grocery store. Raw milk was obtained from the Gary H. Richardson dairy processing plant in the Nutrition and Food Sciences Department at Utah State University (Logan, UT). Samples with different fat content (0%, 2%, and 3.5%) were tested for each type of milk, except raw milk, which only had one fat level (3.5%). For pasteurized milk, a sample with 1% fat was also included. Milk without *Listeria* spp. added was used as a negative control for each sample.

Antibody selection

Each Ab was titered using a procedure described by Harlow and Lane (19). To obtain the working concentrations and acceptable combinations, every Ab was tested against each other in an indirect ELISA format at various concentrations. This was done by diluting each Ab over a range of 1:100 to 1:50,000 in PBS (pH 7.2) in a matrix format (i.e. dilution series of two different Abs were tested at the same time on the 96-well plate). The amount of Ab in each dilution was calculated to the molecule number per ml using equation 1, with the assumption of 150,000 kD as the molecular weight of the Ab:

$$Ab\ molecules/ml = \frac{protein\ \ concentration}{molecular\ \ weight} \times \frac{Avogadro's\ \ number}{1000} \qquad \text{Eq. 1}$$

Four polyclonal Abs were used to determine acceptable combinations of primary and secondary Ab: R4-V99 and G5-V99 (OEM Concepts, Toms River, NJ), YVS4201 (Accurate Chemicals, Westbury, NY), and B65420R (Biodesign International, Saco, ME). The G5-V99 Ab was produced in goat, the other three Ab were made in rabbits (Appendix A). The tertiary Ab was an alkaline phosphatase conjugate of goat anti-rabbit IgG (Sigma, St. Louis, MO).

An ELISA was done in order to find the optimum antibody combination/ concentration. For each dilution of the primary Ab (1:100 to 1:50000), a volume of 75 µl was dispensed into the wells of a 96-well plate (clear polysterene, Fisher Scientific, Pittsburgh, PA) followed by overnight incubation at 4°C to passively coat the well with the Ab. Residual Ab was removed with four washings of 250 µl phosphate buffered saline (PBS) (pH 7.2). The well surface was blocked with 150 µl of a 2% bovine serum albumin (BSA, fraction V, Sigma, St. Louis, MO), with an incubation of 1 h on a platform shaker (C1 New Brunswick Scientific, Edison, NJ) at 100 rpm and 37°C. Subsequently, 75 µl of a ~10^9 CFU/ml suspension (see above) of *Listeria* was added to each well. The 96-well plates were incubated at 37°C for 2 h for binding.

Each secondary Ab was added (75 µl) in a dilution format perpendicular to those of the primary Ab coating so that each Ab was used in all possible combinations/concentration. Plates were incubated at 37°C for 1 h, and washed four times with 250 µl of PBS. The tertiary Ab was added (75 µl of 1:5,000 dilution, ~9.6 x 10^{10} molecules) to all the wells, incubated at room temperature ~25°C (RT) for 30 min and washed 4 times with 250 µl of PBS. *p*-Nitrophenyl phosphate (pNPP, Sigma,) at 1

mg/ml in 0.1 M glycine buffer (pH 10.4) containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$ was added to the wells (75 µl) before incubating the assays for 20 min on a shaker in the dark at RT.

Color development in each well was measured using a BioAssay 7000 plate reader (Perkin Elmer, Norwalk, CT) at a wavelength of 405 nm. Each experiment contained controls of 1) ELISA without cells (blank), 2) the primary Ab combined with the tertiary Ab, 3) the secondary Ab combined with the tertiary Ab, and 4) the tertiary Ab alone (Appendix A). Each assay was done in duplicate for each replicate. A matrix of the signal to noise (S/N) ratios was prepared to define the appropriate combination for each Ab that was used as primary and secondary Ab. The S/N ratio was calculated by dividing the absorbance value of the treatment by the appropriate absorbance for the blank of each Ab combination. An acceptable result was considered to be a S/N >1.2.

DNA amplification protocols

Primers specific for the *iap* gene of *L. monocytogenes* were used as described by Bubert et al. (7), except the PCR conditions were modified as follows: the forward primer (MonoA) of 5'-CAAACTGCTAACACAGCTACT-3' with a reverse primer (Lis1B) of 5'-TTATACGCGACCGAAGCCAAC-3' was used to amplify a 660 base pair product. Another primer set specific for the *iap* gene of *L. innocua,* was used to amplify a 870 base pair product with the forward primer (Ino2) 5'-ACTAGCACTCCAGTTGTT AAAC-3' and a reverse primer (Lis1B) of 5'-TTATACGCGACCGAAGCCAAC-3') (7). Primers sets were made by and purchased from Qiagon Operon (Alameda, CA) (Appendix B).

To amplify the selected DNA sequences puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ) were used as described by the manufacturers instructions. The PCR reaction mixture was made to a final volume of 25 µl and contained 1 Ready-To-Go bead, 1.5 µl of a 20 nM solution of each primer, 5 µl of the lysis buffer, and 17 µl of sterile ddH_2O. PCR was performed using a PTC-200 Peltier Thermo cycler (MJ Research, Reno, NV). The PCR procedure of Bubert et al. (7) was used for *L. monocytogenes*, except the program was modified as follows: denaturation for 1 min at 95°C, 40 cycles (15 s) at 95°C, annealing for 30 s at 58°C, and 45 s of extension at 72°C. An extra extension step of 72°C for 4 min was added at the end of the thermocycling program. The PCR conditions for *L. innocua* were 1 min denaturation at 95°C, 30 thermocycles at 95°C for 15 s, annealing at 58°C for 30 s, and extension at 72°C for 50 s. After completion of PCR, the samples were held at 4°C until proceeded to the electrophoresis step.

The entire volume (25 µl) of the PCR mixture was combined with 1 µl gel-loading dye (BlueJuice, Gibco BRL, CA), mixed, and loaded into 1.3% agarose gels (FMC Bioproducts, Rockland, ME). Electrophoresis was performed for 90 min at 4°C using a voltage of 80 V/cm in 1x TAE buffer (40 mM Tris base, 40 mM acetic acid, and 1 mM EDTA). The gel was stained with ethidium bromide (1 µg/l) for 20 min and the bands were detected on a transeluminator (UVP, Upland, CA). Photographs of the gel were obtained using a Polaroid Land Camera MP4. A Hi-Lo DNA ladder (MBI Fermentas, Hanover, MD) was included in each gel. PCR fragments of approximately

660 and 870 bp were indicative of the presence of *L. monocytogenes* and *L. innocua*, respectively.

Cell lysis

L. monocytogenes was grown overnight in BHI for 12-14h at 37°C in an Environ-Shaker (Lab-Line, Melrose Park, Ill) at 150 rpm to a population density of ~10^9 CFU/ml. Before adding cells to a sample, the pure culture was washed twice (1 ml cell pellet resuspended twice in 1ml of 50 mM Tris buffer pH 7.2). Cells were harvested by centrifugation (model CR3i-V1, Jouan S.A., Saint-Herblain, France) at 3,000 x *g* for 5 min and the cell pellet suspended into each lysis solution to a concentration of 1 x 10^8 CFU/ml. These preparations were used to compare lysis procedures for DNA release from the captured cells (Appendix C). The treatments included lysis by physical disruption (boiling 5 to 10 min, shear by vortexing for 5 min), chemical extraction (1% Triton X-100, alkaline lysis with 0.05 M NaOH and 0.02% SDS), and enzymatic digestion (1.5 mg/ml lysozyme, 30 min, 37°C, 500 μg/ml proteinase K, 30 min, 60°C) procedures. A combination of 6 min boiling of a 1% Triton X-100 solution followed by a 30-min incubation with lysozyme (1.5 mg/ml) at 37°C) was also investigated.

Initially, PicoGreen (Molecular Probes, Eugene, OR) was used to determine in a quantitative way the amount of dsDNA released by each lysis method (Appendix C). After each treatment, 500 μl extracted DNA solution was put into a new 2 ml Eppendorf tube (Brinkman, Westbury, NY) together with 500 μl of a 1:200 diluted PicoGreen solution, vortexed and incubated in the dark for 5 min. Each sample (200 μl) was dispensed into a well of a 96-well plate (Fisher Transparent Sterile Plate) and a

fluorescence reading was taken on a plate reader (HTSoft 7000 BioAssay (Perkin Elmer, Norwalk, CT) in duplicate, using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. A five-point standard curve was created with DNA amounts ranging from 1 ηg/ml to 1 μg/ml (Appendix C) to determine the concentration of the release DNA using equation 2.

$$Y = 4.3643X + 2349 \quad (R^2=0.99) \qquad \text{Eq. 2}$$

The blank readings for each treatment were subtracted from the sample readings; therefore, the simplified zero-adjusted equation Y = 4.3643X was used to calculate the net DNA release. Each lysis procedure was done in duplicate and replicate. The efficiency of the different lysis procedures in pure cultures were rank based on the total amount of DNA released (Appendix C). Based on this ranking, a lysis method was selected for further use.

Bead preparation and activity testing

The polyclonal Ab anti-*Listeria* G5-V99 (lot # 500-39737) was covalently linked to glass beads (3 mm) using a dextran (Sigma) spacer as described by Weimer et al. (52) (Appendix D). Briefly, 100 g of 3 mm glass beads were derivatized with APTES (Sigma A3648), modified with dextran, air dried, and stored at room temperature until the primary Ab was covalently bound. The antigen-affinity purified polyclonal IgG was purchased in a buffered salt solution (10 mM phosphate, pH 7.4 containing 150 mM NaCl and 0.1% NaN_3) from OEM Concepts (Toms River, NJ). Five hundred micrograms of the affinity purified (>95% by SDS-PAGE) primary Ab was desalted over a D-Salt™ Dextran plastic desalting size exclusion column (Pierce, Rockford, IL) before it was

covalently attached onto 100 g of the spacer-coated beads. The beads were blocked using 50 ml of a 2% BSA in 50 mM Tris (pH 7.2) containing 0.02% sodium azide, for 2 h at RT. The beads were stored at 4°C in this solution until further use.

After the primary Ab coating, an activity test was done with 10 beads from each batch using the rapid immunocapture procedure (RIC) (Figure 3-1). Briefly, 10 glass beads were put into a 2 ml Eppendorf tube (Brinkman, Westbury, NY) using sterile tweezers. An overnight cell culture was washed twice with 50 mM Tris (pH 7.2), and 10^8 CFU (1ml) were added to the beads. The tubes were incubated on a platform shaker (New Brunswick Scientific, Edison, NJ) at 150 rpm for 20 min at RT (~25°C). On a ceramic filter, each tube with beads was washed four times with 25 ml aliquots 50 mM Tris buffer (pH 7.2), and transferred to a new 2 ml tube. The anti-*Listeria* species IgG B65420R (lot # 11K33401), was added (1 ml or 3.61×10^{13} molecules) and the tubes were incubated on the shaker for 20 min at 150 rpm. After repeating the wash step and transferring the beads to a new tube, 1 ml of the alkaline phosphatase-labeled tertiary antibody anti-rabbit IgG (Sigma, St. Louis, NJ) was added at a molecule concentration of 1.3×10^{12} molecules/ml. After incubation (150 rpm for 20 min at room temperature), the beads were washed (4 x 25 ml 50 mM Tris buffer pH 7.2) and then transferred into a 48-well plate (each set of 10 beads in a separate well). The pNPP substrate (Sigma) was dissolved in a 0.1 M glycine buffer (pH 10.4) containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$ to a working solution of 1 mg/ml, and added (600 µl) into each well. The plate was wrapped in aluminum foil, incubated for 20 min at 150 rpm before 200 µl of the solution was transferred into a new well, and the absorbance at 405 nm was measured on a BioAssay 7000 plate reader (Perkin Elmer). Negative controls for each test were

included and underwent the same treatment as the samples, except that the cell suspension was replaced by 50 mM Tris buffer (pH 7.2). The RIC procedure was used to determine the detection limit in 50 mM Tris (pH 7.2) buffer before using the method in flow capture.

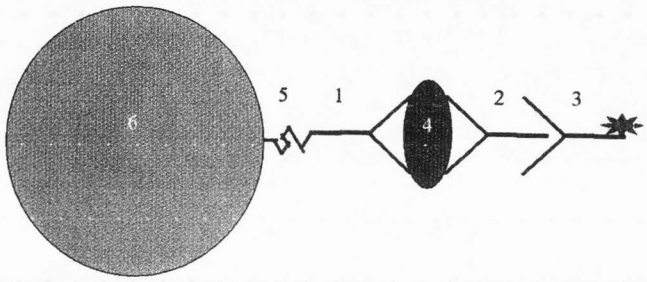

Figure 3-1. Diagram of a bead surface used for the activity test for each batch of glass beads and for the indirect ELISA used in this study: 1) primary Ab; 2) secondary Ab; 3) phosphatase-labeled tertiary Ab; 4) *L. monocytogenes* cell; 5) dextran spacer molecule; 6) 3 mm glass bead.

Fluidized bed capture

A 10-fold dilution series, ranging from 1 x 10^8 CFU/ml to 1 x 10^5 CFU/ml, was prepared in a 50 ml volume of 50 mM Tris buffer or milk samples. The inoculated samples were incubated on a rocking table Roto-Shake Genie™ (Scientific Industries Inc., Bohemia, NY) for 10 min prior to running them through the capturing device. *L. monocytogenes* cells were captured in a single use fluidized bed module that contained 60 glass beads (~ 2 gram) with 12 ml of dead volume connected to 180 PVC plastic tubing (1/8 ID x 3/16 OD, 1/32 wall) (Nalgene, Rochester, NY) on the inlet and outlet. The inlet tubing (18 cm) was put into the sample and the outlet (98 cm) was attached to a peristaltic pump (Masterflex, Cole-Parmer, Chicago, IL) before leading back to the 50 ml

sample (Figure 3-2), such that sample recycling through the capture module was allowed. The sample was pumped at a flow rate of 100 ml/min through this closed system in an upward flow direction. After capture for 5 min (i.e. ~42 column volumes), the glass beads were washed inside the chamber four times with 25 ml aliquots of 50 mM Tris buffer (pH 7.2).

The final wash solution was completely decanted before transferring the beads to a sterile 15 ml centrifuge tube (Becton Dickinson, Franklin Lakes, NJ). Double distilled H_20 was added (1 ml) to cover the washed beads before the beads were boiled for 6 min. The ddH_2O containing the extracted DNA from the captured cells was collected and used for PCR detection.

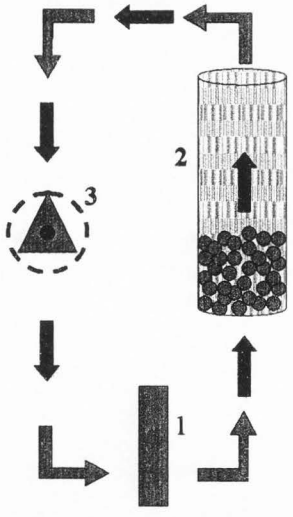

Figure 3-2. Diagram of the fluidized bed capture module and closed loop pump system used in this study: 1) food sample; 2) cartridge with glass beads; 3) peristaltic pump.

Shaking capture

The same 10-fold dilution series used in the fluidized bed experiments was used with the shaking capture as a control condition. After inoculation and thoroughly mixing the 50 ml samples as described above, 60 Ab-coated beads were added and the samples incubated for 20 min on a rocking table at RT. After incubation, each sample was washed 4 times with 25-ml aliquots of 50 mM Tris buffer (pH 7.2) on a ceramic filter-funnel before transferring the bead-cell complex to a sterile 15 ml centrifuge tube. Double distilled H_20 was added (1 ml) to cover the beads and boiled for 6 min. The resulting DNA-containing solution from the captured cells was collected and used for PCR detection.

Each experiment was done in replicate. Statistical analysis was done using JMP 3.1.5 (SAS Institute Inc., Cary, NC) as a completely randomized ANOVA with as the sources of variation were capture method, fat percentage and heat treatment (Appendix E). Means were compared using the Fisher's pair wise comparison. The threshold for statistical significance was set to $\alpha = 0.05$.

Results

Antibody selection

Screening was done to match the primary and secondary Ab (Appendix A) since it was suspected that the Ab avidity (i.e. the overall stability between Ab and antigen (Ag) complex) for this large Ag would be weak. The goat anti-*Listeria* G5-V99 was selected as the primary Ab and it was paired with the rabbit anti-*Listeria* B65420R as the

secondary Ab (Figure 3 -3). This combination provided a S/N > 1.5 and the signal was insensitive to minor concentration changes in the primary Ab concentration. Despite selecting the Ab combination for minimal influence due to concentration, the signal was dependent on the concentration of the secondary Ab. Therefore, a concentration of 3.6 x 10^{12} molecules/ml (1:500 dilution of the stock solution) (Figure 3-3) for the secondary Ab was confirmed within a narrower dilution range. Subsequently, the optimal (i.e. cost vs. signal) tertiary Ab concentration was determined as well with the optimal secondary Ab concentration of 3.6 x 10^{12} molecules/ml (Figure 3-4).

Combinations of the secondary and tertiary Ab were examined to determine the optimum concentration (i.e. concentration vs. cost vs. signal) to maximize the detection signal with minimal background (Figure 3-4). S/N ratios > 2 were obtained for

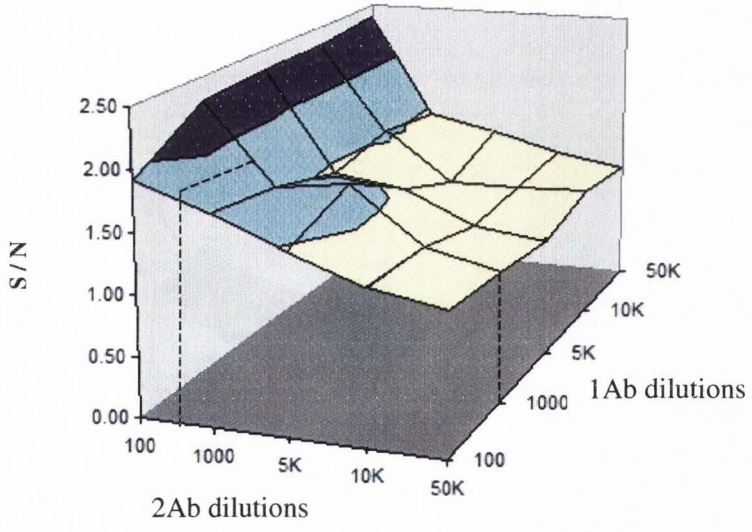

Figure 3 -3. Determination of the primary and secondary Ab concentration. Tertiary Ab was an anti-rabbit alkaline phosphatase fixed at 12.8 x 10^9 molecules/µl; 1Ab = primary Ab G5-V99; 2Ab = secondary Ab B65420R, ----- indicates selected concentrations for 1Ab and 2Ab.

combinations of the secondary Ab diluted 1:100 and 1:500 with the tertiary Ab diluted 1/1000 or 1/10,000. A dilution of the tertiary Ab of 1:1,000 gave the highest S/N ratios, however, a higher dilution was selected (1:5,000) for further experiments to reduce the amount of background with this Ab (Figure 3-4).

In all further experiments, the primary Ab (G5-V99) was used to the coupling reaction at a concentration of 6.3 x 10^9 molecules/mm^2 (dilution 1:25,000). The secondary Ab (B65420R) concentration of 3.6 x 10^{12} molecules/ml (dilution 1:500) with a tertiary Ab (Sigma, A3687) dilution of 1:5,000 (molecule number not known) of the stock was used in the ELISA assays. The average S/N ratio for all bead batches with these conditions was 2.1 ± 0.4. Batches with a S/N below 1.2 were not used.

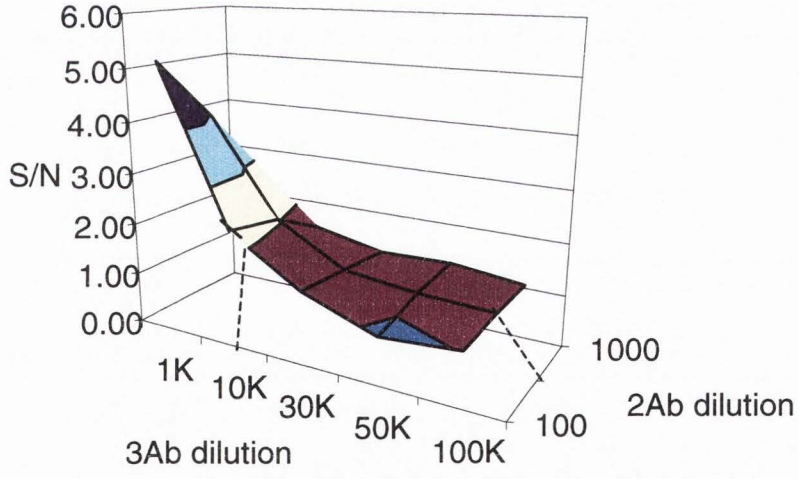

Figure 3-4. Influence of the secondary Ab B65420R and tertiary Ab A3687 concentration on the S/N. The primary Ab was fixed at 6.3 x 10^{12} molecules/mm^2. S/N = signal to noise.

Lysis treatments and primer specificity testing

The DNA extraction comparisons are summarized in Table 3-1. Six minutes of boiling significantly (p-value < 0.05) increased DNA release (1615 ± 80 ng) compared to a non-boiling step (355 ± 17 ng) of DNA. An alkaline lysis method, with or without a 3 M KAc solution, together with the control (ddH_2O), resulted in a significantly lower ($p < 0.05$) amount of DNA release compared to the other treatments (475 ng vs. 96 ng, respectively). Addition of a 6.7×10^4 units/ml of lysozyme with a 30-min incubation at 37°C significant increased the release of DNA (1393 ng DNA). The same was true with a 6-min boiling step of a 1% Triton X-100 solution (1466 ng DNA). The combination of 1% Triton X-100 with lysozyme however did not lead to an increased DNA release compared to the two individual treatments. Boiling was selected for further use.

The PCR protocol was modified to increase the sensitivity by adding a 1-min denaturation step at the beginning of the thermocycler program, increasing the cycles to 40, and inserting a 4-min extension step. In pure culture this resulted in a detection limit of 10^1 CFU/ml. To achieve this detection limit, a cell pellet from 1-ml cell culture

Table 3-1. DNA release for various lysis treatments. The different letters indicate significantly different ($p<0.05$) groups using Fisher's pairwise comparisons.

Treatment	DNA released (ng)
Boiling for 6 min	1615[a]
Lysozyme	1477[a]
Boiling for 6 min with 1% Triton X-100	1466[a]
lysozyme with 1% Triton X-100	1393[a]
Alkaline with 3 M KAc	475[b]
DdH_2O, no boiling	355[b]
Alkaline treatment	96[b]

(10^9 CFU/ml) was resuspended in 100 µl of a lysis buffer containing 500 µg/ml proteinase K solution (13.8 units/mg) (Appendix C). Although proteinase K gave a very low detection limit in pure culture, it was abandoned considering the poor detection results in a solid phase capture environment, as described below.

Since *L. monocytogenes* is the only pathogen within the genus *Listeria*, it is appropriate to make the PCR detection very specific to this species. The primer set for *L. monocytogenes* was specific for this organism only, as indicated by an amplicon of 660 bp for the *iap* gene in *L. monocytogenes*, but absent in *L. innocua*. This observation was verified with an additional primer set specific for *L. innocua* (Figure 3-5).

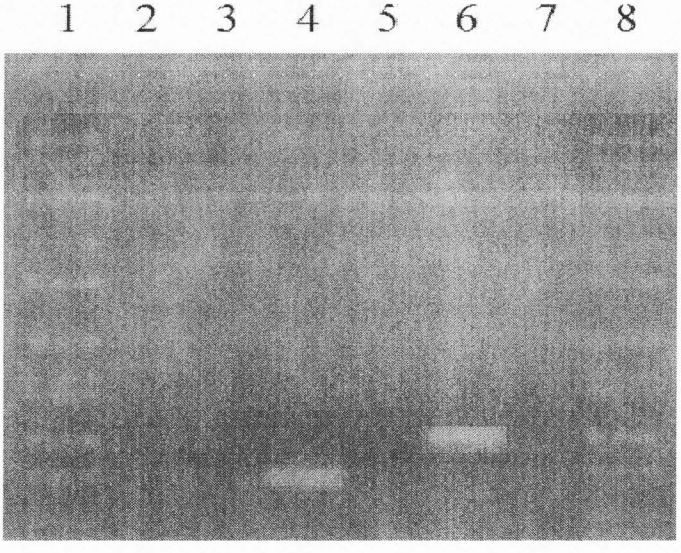

Figure 3-5. Primer specificity for *L. monocytogenes* and *L. innocua*. The lanes are: 1&8) DNA size markers (from top to bottom: 10,000, 8,000, 6,000, 4,000, 3,000, 2,000, 1,550, 1,400, 1,000, 750, 500 bp; lane 2-4) PCR results from primer set of *L. monocytogenes iap* primers *MonoA–Lis1B* with no DNA (2); *L. innocua* DNA (3); *L. monocytogenes* DNA (4); lane 5-7) primer set of *L. innocua Ino2-Lis1B* with no DNA (5); *L. innocua* DNA (6); *L. monocytogenes* DNA (7).

Solid-phase Capture of *L. monocytogenes*

Freshly prepared beads were made with a dextran spacer (Appendix D) and tested for activity before each use, using 10 beads using RIC in Tris buffer. If the S/N was ≤1.2, the beads were not used. The detection limit of this ELISA procedure was between 1 x 10^4 CFU/ml and 1 x 10^6 CFU/ml in buffer (Figure 3-6).

With the lysis treatment and the PCR protocol determined, the capture flow rate for the fluidized-bed technique (ImmunoFlow) (50) was determined. This experiment was done and compared to a control of shaking capture for 20-min at low speed (100 rpm) followed by an ELISA detection. The flow through of the samples during ImmunoFlow continued for 5 minutes in all settings and varied from 75 to 150 ml/min The maximum capture was observed at 100 ml/min (Figure 3-7). At this flow rate and time, 41.6 column volumes flowed past the beads.

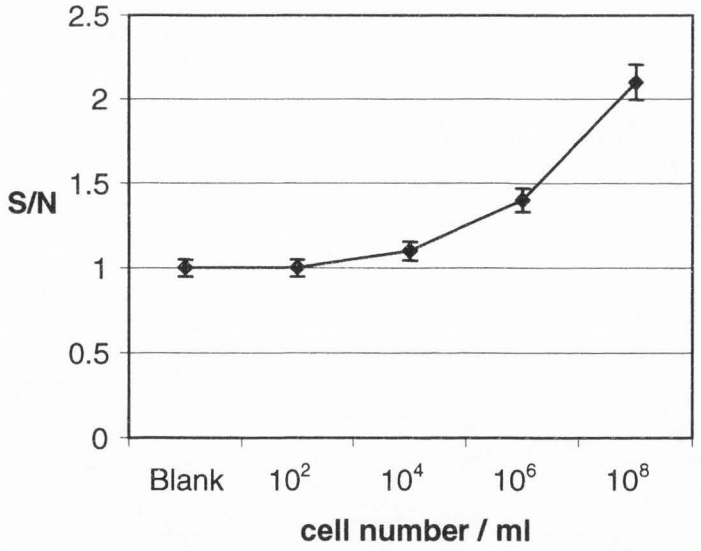

Figure 3-6. Determination of the detection limit in 50 mM Tris buffer for *L.. monocytogenes* cells using RIC. The blank contained only ddH_2O.

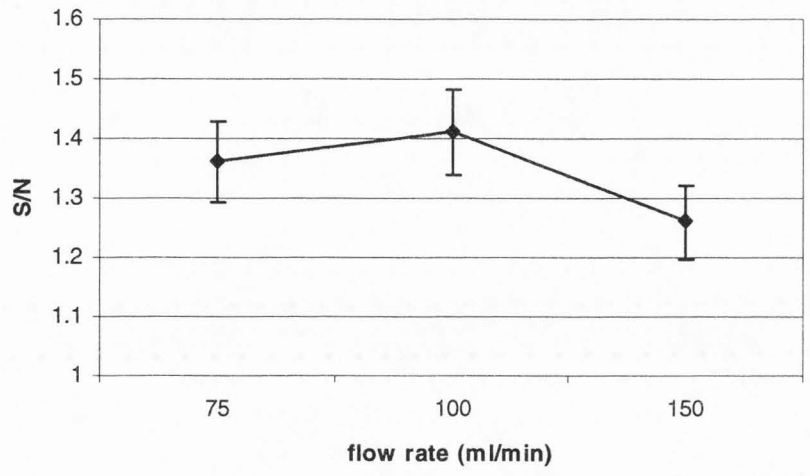

Figure 3-7. Determination of the flow rate for capture in ImmunoFlow with RIC. The sample was recirculated for 5 min with 10^8 CFU added to a total volume of 50 ml of 0.05 M Tris buffer (pH 7.2).

After establishing the flow rate, the detection limit was determined in Tris buffer using 50 ml samples with PCR detection and electrophoresis analysis. Once captured, the cells were lysed by boiling for 6 min and gave a detection limit of 1 x 10^6 CFU/ml in 50 mM Tris (pH 7.2) using a 50 ml sample.

After addition of the cells to milk, capture was done directly without any pre-enrichment. Capture was sporadic in flow with milk samples. With the shaking capture technique, consistent results were observed for pasteurized milk samples, but varied with milk treatment. Therefore, further work with milk samples was done using the shaking capture format. In addition, no signal was detected in milk samples inoculated with the highest level of *L. monocytogenes* (10^8 CFU/ml) directly subjected to PCR (i.e. no capture step) (Figure 3-8, lanes 10-12).

In pasteurized milk, detection was consistently observed at the highest inoculation level (1 x 10^8 CFU/ml) at all fat levels tested. The amplicon for the 2% fat pasteurized milk sample was less pronounced than the ones observed with skim and 1% fat pasteurized milk samples, Tris buffer, or the positive control bands (Figure 3-8), indicating that fat content in this solid-phase capture technique remains a factor that leads to reduced detection. However, capture was possible at all fat percentages, but capture decreased as the fat content increased ($p > 0.05$).

In UHT and raw milk samples, only sporadic capture was observed even at the highest inoculation level (1 x 10^8 CFU/ml). Significantly lower ($p < 0.05$) capture was observed with UHT and raw milk samples than with pasteurized milk samples.

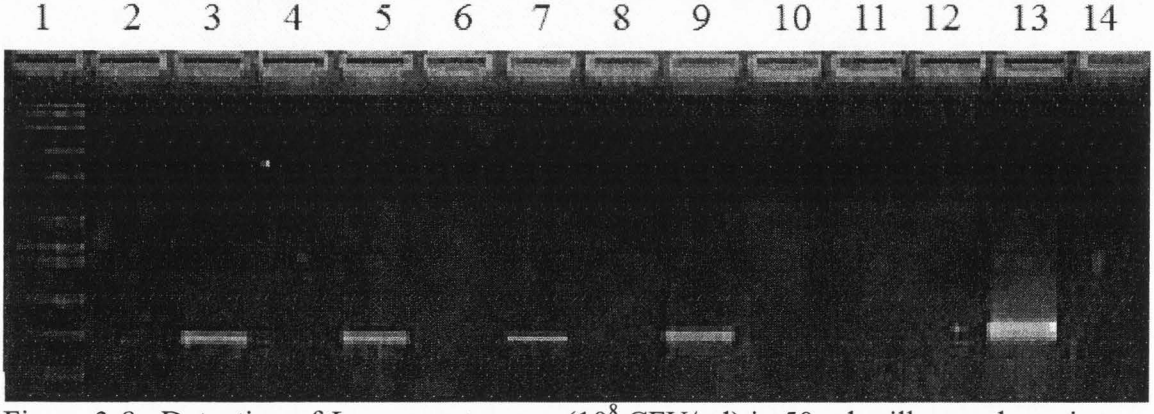

Figure 3-8. Detection of *L. monocytogenes* (10^8 CFU/ml) in 50 ml milk samples using the static capture technique. Lanes: 1) molecular size DNA ladder same as figure 3; 2,4,6,8) negative controls for skim milk, 1%, 2%, pasteurized milk and TRIS buffer respectively i.e. milk samples without cells added; 3,5,7,9) same samples inoculated with *L. monocytogenes* cells; 10,11,12) direct PCR on pasteurized milk samples (skim, 1%, and 2%) without capture step; 13) positive control being 10^8 CFU/ml *L. monocytogenes* cells boiled in water 6 min; 14) negative control, dH_2O.

Discussion

The trend in pathogen testing emphasizes the need for biosensors for the food industry that are fast and specific, especially since conventional methods are laborious and time consuming thereby adding cost to the food production (2). Combining DNA amplification with techniques that concentrate microorganism from food particles is likely to provide specific results faster. Separation of the microbial cells from the food is required to avoid interference by natural PCR inhibitors (15, 26, 28, 37). Immuno-based capture techniques that use paramagnetic beads is common, but are limited to small sample sizes and still rely in most cases on pre-enrichment before capture (6, 10, 15, 29, 46, 49). The hypothesis of this study was that the enrichment step can be completely eliminated to provide a rapid detection system. This hypothesis was demonstrated in pure culture, but the detection limit was not sufficiently low in milk to make the assay a practical replacement.

Selection and optimization of the Abs used in this solid-phase capture technique is a crucial step (27). Since the assay was assembled as an indirect ELISA, 3 Abs were selected and an optimal working concentration determined. By testing different dilutions and combinations of Abs, a selection was made based on the obtained 3D graphic surface areas for each Ab combination (Figure 3-3). A high S/N was set as a key factor in determining a meaningful end point above the background. The primary Ab selected G5-V99 (OEM Concepts) was covalently bound onto the glass beads at a concentration of 6.3×10^{9} molecules/mm^2, the secondary Ab, B65420R (Biodesign), was added at a concentration of 3.6×10^{13} molecules/ml and the alkaline phosphatase IgG (Sigma) was

selected as the tertiary Ab at a fixed concentration of 1.3×10^{12} molecules/ml. A S/N of 2.3 ± 0.1 was obtained for this Ab combination.

After capturing the cells onto the glass beads, a lysis procedure was selected in order to release the DNA for PCR analysis directly from the glass beads. The PicoGreen assay (Molecular Probes) was used to analyze different lysis method divided into three groups: physical, chemical or enzymatic. Combinations between different groups were also tested. Using only a heating step, the 6 min boiling was significantly more effective ($p < 0.05$) in releasing DNA from the cells for PCR. Among the chemical treatments, the use of a 1% Triton X-100 solution on a pure culture, combined with a 6-min boiling step, which resulted in the highest amount of DNA release out of a 1×10^8 CFU/ml *L. monocytogenes* cell suspension (1466 ng DNA). Those findings were consistent with the observation of Abolmaaty et al. (1) who used Triton X-100 in combination with NaN_3 at pH 8.0. The alkaline lysis procedure resulted in significant lower amount of DNA released ($p < 0.05$) compared to boiling. This can be explained by the fact that this lysis procedure is developed in the first place for Gram-negative bacteria, not for Gram-positive bacteria, such as *L. monocytogenes*. Lysozyme in a concentrated solution (1.5 mg/ml) as the lysis treatment was also evaluated with PicoGreen (Molecular Probes) and resulted in the release of high amounts of dsDNA (1393 ng) out of a 1-ml cell suspension (1×10^8 CFU/ml). Several researchers combined lysozyme and proteinase K in an effort to lyse the *L. monocytogenes* cells efficiently. The detection limits varied from 1×10^2 to 1×10^5 CFU/ml in food depending on the specific procedure used (9, 18, 24, 42, 44). Considering time and effort besides the amount of DNA extracted, the proteinase K treatment was selected first to apply on the bead-cell complexes. It was believed that the

use of a proteinase would help the release of DNA in a protein-rich environment (Ab, BSA, and cellular proteins) into the extracting medium and therefore increases sensitivity. However, using a proteinase K solution to lyse the cells in the bead-cell complexes, brought the detection limit to 1 x 10^6 CFU/ml in 50 mM Tris buffer, which is less sensitive compared to the 1 x 10^1 CFU/ml in pure culture solution. Furthermore, non-specific bands showed up on the gel after electrophoresis. It is not known why exactly such a difference in detection rates was observed but DNA binding to the beads might be a possible contributing factor. Since the same detection limit in 50 mM Tris buffer (pH 7.2) could be obtained by simply boiling the bead-cell complexes in ddH_20 for 6 min instead of a time-consuming enzyme procedure, the latter was used.

DNA amplification was done using a primer set specific for the *iap* gene of *L. monocytogenes*. Modifying the thermocycling protocol of Bubert et al. (7) by increasing the cycling time from 30 to 40 and adding an extra elongation step of 4 min at 72°C, the sensitivity in DNA detection could be increased by 1 order of magnitude. Although the selected primer combinations was not the same as the one preferred by Aznar and Alarcon (5), similar specificity was obtained. No false positives were observed, even when the primers were exposed to DNA from the most closely related species, *L. innocua*. Aznar and Alarcon (5) also tested these primers, against several other species in the genus and found the same results.

Shaking and fluidized-bed capture were investigated to collect cells from the sample. The turbulent flow of sample solution through the fluidized-bed cartridge should increase the contact time between the Ag and the Ab coated onto the glass beads by creating a fluidized bed. Therefore, a better detection was hypothesized with an

increased sample size of (50 ml) and no pre-enrichment. No significant differences were observed ($p > 0.05$) between the different capturing methods in 50 mM Tris buffer with *L. monocytogenes* cells added. A detection limit of 1 x 10^6 CFU/ml was observed in both methods. Capturing cells from inoculated milk was not consistent basis using the fluidized-bed technique even after adjusting the flow rate to maximize capture (Figure 3-7). In the shaking procedure, a consistent positive result was detected for pasteurized milk samples, inoculated at the highest level (1 x 10^8 CFU/ml) in all fat conditions. Except in UHT and in raw milk, where no detection was achieved at any of the cell concentrations tested. Although the detection limit in pasteurized milk samples was limited to 1 x 10^8 CFU/ml, direct PCR from milk samples for the detection of the microorganism was not observed. This is in accordance with the findings of Wernars et al. (53), who described a strong inhibition of the PCR reaction when *Listeria* DNA was directly extracted from soft cheese.

It is not known why the low sensitivity during capturing was observed, but the binding strength of the Ab (avidity) could be an underlying mechanism to explain these observations. Previous studies found that the protocols to coat the Abs on the beads are well established and work efficient for other microorganism such as *E. coli* O157 (27, 51). Therefore, any lack of sensitivity should be linked to the specific antibodies used. The inability to develop a sensitive and specific Immunocapture technique for *L. monocytogenes*, is reported by several other researchers (12, 27, 43).

The shaking capturing technique generates less shear force on the Ab/Ag complex, compared to the more intense flow-through dynamics in the fluidized-bed method, which might explain the poor capture results for the latter. Low Ab avidity was

also noticed by Jung et al. (27). Four different polyclonal anti-*Listeria* Ab were evaluated in a direct and indirect assays to capture *L. monocytogenes* using IMS separation techniques. The researchers concluded that none of the tested Ab exhibited sufficient specificity or avidity to allow sufficient separation and detection of *L. monocytogenes* for a useful test. However, the use of the ImmunoFlow method has reportedly been successful with *E. coli* and *Salmonella* (10, 50), suggesting again that the anti-*Listeria* Ab used in this study have low avidity as shown by Jung et al. (27). The possibility of interfering proteins in the Ab stock solution as a reason for the low sensitivity is not very plausible since according to OEM Concepts, no proteins other than the Ab should be present in the Ab stock solution. Very small amounts of albumin could however be present, but not enough to show up on SDS-PAGE.

The different heat treatments or homogenizations among the milk samples explain the differences in the obtained results. Changes in protein structures in the milk seem to make the pasteurized milk the most suitable for this solid-phase capture technique. It is believed that the protein structures in UHT and raw milk are shaped in such a way that they prevent strong Ab-Ag binding. This change of protein structure seems to be more important than the fat amount for interference in binding capacity.

Most rapid detection system compromise on both aspects of detection time and detection limit by adding a small enrichment step before solid-phase capture (6, 10, 25, 29). At this time, without any enrichment, the solid-phase capture techniques for *L. monocytogenes* do not reach the desired sensitivity for the food industry (1-100 CFU/ml) (23, 27, 35, 46). The same conclusions were made in this research project.

However, rapid detection of *L. monocytogenes* cells at high cell concentration in pasteurized milk can be done with the static immunocapture method used in this work to reduce the time. Using real-time PCR technology might reduce the detection time (less than 4 hours) even further and make quantification possible (16, 21, 31). Future work needs to be done to isolate Ab with higher affinity for *L. monocytogenes* cells for use with milk and fluidized bed capture.

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CHAPTER 4

SUMMARY AND CONCLUSIONS

Rapid capture and/or detection of food borne pathogenic microorganisms are pressing needs in the food industry. Although the knowledge and the awareness have increased with respect to the dangers of an increasing number of pathogens, there is still a lack of a reliable, accurate, and fast bacterial detection method for use with food. A better surveillance system, more control points, and concerned consumers are part of this trend, the lack of a robust detection system for routine use in processing facilities remains a significant problem.

Several rapid detection methods are commercially available. However, all of these assays require a enrichment step to increase the cell population to 10^8 CFU/ml prior to detection that is at least 8 h and as long as 48 h for a final result is available. DNA amplification techniques are among the most promising tools for rapid and specific detection. However, the quality of the DNA sample to be amplified is crucial and many foods contain PCR inhibitors. The combination of cell separation/DNA clean-up techniques from the food samples with PCR techniques is therefore of great interest. The use of immuno-based techniques used for cell separation, has the extra advantage of being selective for the microorganisms of interest.

Hypothesis

Listeria monocytogenes can be captured from milk using a fluidized-bed solid-phase capture system with covalently attached antibody-beads, allowing elimination of the enrichment step, and followed by PCR for specific detection.

Objectives

1. Optimize Rapid Immuno Capture (RIC) in buffer solution to capture *L. monocytogenes* after finding the optimum antibody combination and concentration
2. Determine a method to lyse the cells after capture on to beads
3. Combine Immuno-capture and PCR to detect *Listeria* and identify *L. monocytogenes* in pure culture and food samples.

Ab selection for the development of an immunocapture device was crucial and seemed to be a key factor in the poor sensitivity of the capture step. The success of ELISA systems rely on the quality of the Ab for capture efficiency and the properties of the Ab. Therefore objective 1 was done to select commercially available Ab pairs that were appropriate for capture and detection. From the multiple combinations and dilution series of different antibodies tested, the indirect ELISA combination with the highest cell binding capacity was selected (Figure 3-3).

In Chapter 2, a protocol for selecting the optimum Ab combination and titer was described. Using an indirect ELISA format, different Ab combination and concentrations were compared. Ultimately, a polyclonal Ab (G5-V99) was selected as the primary Ab

that was covalently linked to the glass beads through a dextran spacer molecule. The secondary Ab was produced in a rabbit (B65420R) and an IgG (A3687), with alkaline phosphatase bound, was used as tertiary Ab. After optimizing the concentrations using an ELISA format, a similar procedure was used to further optimize the Ab concentration using RIC (Appendix A). The optimal concentration of Abs were the following: the primary Ab was covalently bound onto the glass beads at a concentration of 6.3×10^9 molecules/mm^2, the secondary Ab was added at a concentration of 3.6×10^{13} molecules/ml and the alkaline phosphatase IgG was fixed at a concentration of 1.3×10^{12} molecules/ml as the tertiary Ab.

The most appropriate lysis procedure for *L. monocytogenes* after solid phase capture was determined to be a 6-min boiling step. The supposition that protein and cell wall degradative enzymes, in particular proteinase K, could help to release DNA out of the protein complexes surrounding the bead surface into the ddH_20 and therefore increasing the detection limit was not observed.

Primers specific for *L. monocytogenes* cells were selected for PCR analysis (Figure 3-5). The detection limit, when using the solid-phase capture technique, was 1×10^6 CFU/ml with the 6-min boiling step in 50 mM Tris buffer. The same procedure was used as the lysis step for the detection experiments with the different milk samples.

Objective 3 was completed using the solid-phase capture system in two formats (ImmunoFlow and static or shaking capture) on different milk samples and on TRIS buffer. Detection was done using PCR. The influence of the fat percentage of the milk (skim, 1%, 2%, 3.5%) as well as on the heat treatment of the milk (raw, pasteurized, UHT) was determined. The fluidized-bed system was consistently positive in Tris buffer

to a cell concentration of 1 x 10^6 CFU/ml. No consistency in capture was observed in milk using flow, while the shaking capture assay was consistent. In this format, *L. monocytogenes* was detected consistently in pasteurized milk at a concentration of 1 x 10^8 cells/ml at all fat concentrations tested (Figure 3-8). In UHT and raw milk this was however not possible. Presumably, the heat treatment influenced the composition of the different milk components in such a way that they interfere with the Ab in the case of raw and UHT milk.

It was shown that performing a PCR step on a milk sample without the implementation of a capture step was not possible (Figure 3-8), making a solid phase capture step required for elimination of pre-enrichment. Milk components must therefore inhibit the PCR reaction. Although the selection of Abs was done carefully, we are convinced that the properties of the available *L. monocytogenes* Abs towards binding capacity were not optimal.

In conclusion, direct detection of *L. monocytogenes* out of milk with PCR was not possible, therefore a capture or removal step before PCR analysis, was required with milk. In pure culture, a lysis and PCR procedure was developed with a detection limit of 10^1 CFU/ml. During solid-phase capture using ImmunoFlow or static capture, the detection limit was found to be 10^6 CFU/ml in Tris buffer. The detection limit in pasteurized milk was 1 x 10^8 CFU/ml and was similar to that reported for an IMS system developed by Dynal. Elimination of the enrichment step for *L. monocytogenes* detection was achieved using a solid phase capture strategy. The static capture procedure was completed in about 4 h without enrichment and at the same detection limit as commercial assays.

APPENDICES

Appendix A.
Antibody Selection and Titers

Searches for anti-*Listeria* Abs was done online with Abcam software (http://www.abcam.com) from Abcam Ltd. (Cambridge, UK). Four different anti-*Listeria* Abs were selected and tested against each other to use as primary or secondary Ab, in an indirect ELISA format (Table A-1). For the tertiary Ab in the indirect ELISA setup two different Ab were tested. Both Abs had a phosphatase enzyme linked to it (Table A-2).

Table A-1. Details of the different antibodies (primary and/or secondary)

Name Ab	**Produced in**	**Conc (mg/ml) Lot number**	**Company**
G5-V99	Goat	5.74 mg/ml 500-39737	OEM Concepts Toms River, NJ
R4-V99	Rabbit	4-5 mg/ml 101-26091	OEM Concepts Toms River, NJ
B65420R	Rabbit	4-5 mg/ml 11K33401	Biodesign Int. Saco, ME
YVS4201	Rabbit	4-5 mg/ml H3898	Accurate Chem. Westbury, NY

Table A-2. Details of the different tertiary antibodies

Name Ab	**Produced in**	**Conc (mg/ml) Lot number**	**Company**
Alkaline Phosphatase Conj. Goat IgG	Rabbit	N/A A3687	Sigma Saint Louis, MO
Phosphatase labeled affinity Ab	Goat	0.1mg/ml 05-90-90	K&P Lab Inc. Gaithersburg, MD

Sixteen different combinations were tested in a matrix, indirect ELISA format (Table A-3). One restriction in the possible combinations was the inability to combine an anti-rabbit tertiary Ab with an Ab produced in goat and vice versa. At this point the concentration of the tertiary Ab was kept constant at the titer given by the manufacture (1:40,000 for A3687 and 1:100 for 05-90-90).

All the different Ab concentrations were based on dilutions. For each Ab, 5 different dilutions were made: 1:100; 1:1,000; 1:5,000; 1:10,000 and 1:50,000 and every dilution combination was done in duplicate (Figure A-1).

Table A-3. Sixteen different ELISA: G = G5-V99, R = R4-V99, B = B65420R, Y = YSV4201, anti-R = anti-Rabit, anti-G = anti-Goat

Primary Ab (1Ab)	**Secondary Ab (2Ab)**	**Tertiary Ab (3Ab)**
G	R	Anti-R
G	B	Anti-R
G	Y	Anti-R
G	G	Anti-G
R	R	Anti-R
R	B	Anti-R
R	Y	Anti-R
R	G	Anti-G
B	R	Anti-R
B	B	Anti-R
B	Y	Anti-R
B	G	Anti-G
Y	R	Anti-R
Y	B	Anti-R
Y	Y	Anti-R
Y	G	Anti-G

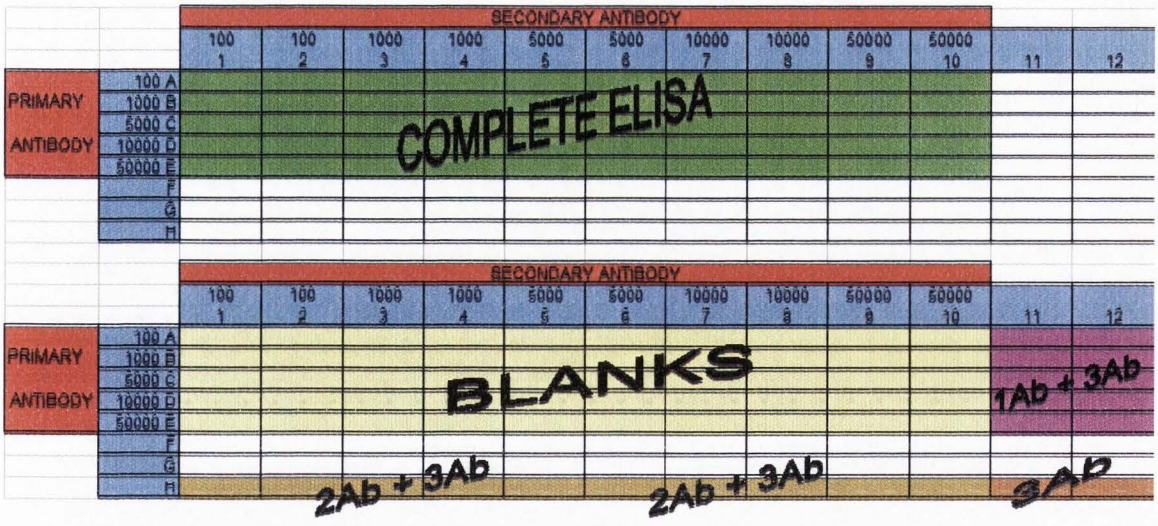


Figure A-1. Format for the 16 ELISA titer experiments.

In addition to the complete indirect ELISA, 4 different controls were added (Figure A-1)

- Same indirect ELISA without the microorganism i.e. blank readings
- The primary Ab (dilutions) combined with the tertiary Ab
- The secondary Ab (dilutions) combined with the tertiary Ab
- The tertiary Ab on its own

A 3D surface area chart was created by plotting in Microsoft Excel 2000 one Ab dilution on the X-axis, one on the Z-axis and the signal to noise on the vertical Y-axis (Figure A-2,Figure A-3,Figure A-4,Figure A-5). G5-V99 was selected as the primary Ab. The secondary Ab was B65420R and as the tertiary Ab alkaline phosphatase A3687 was chosen (Figure A-2, graphic B). The experiments was done twice: once in March 2001 and a second time in May 2002. Each time, every Ab concentration/ combination was done in duplicate.

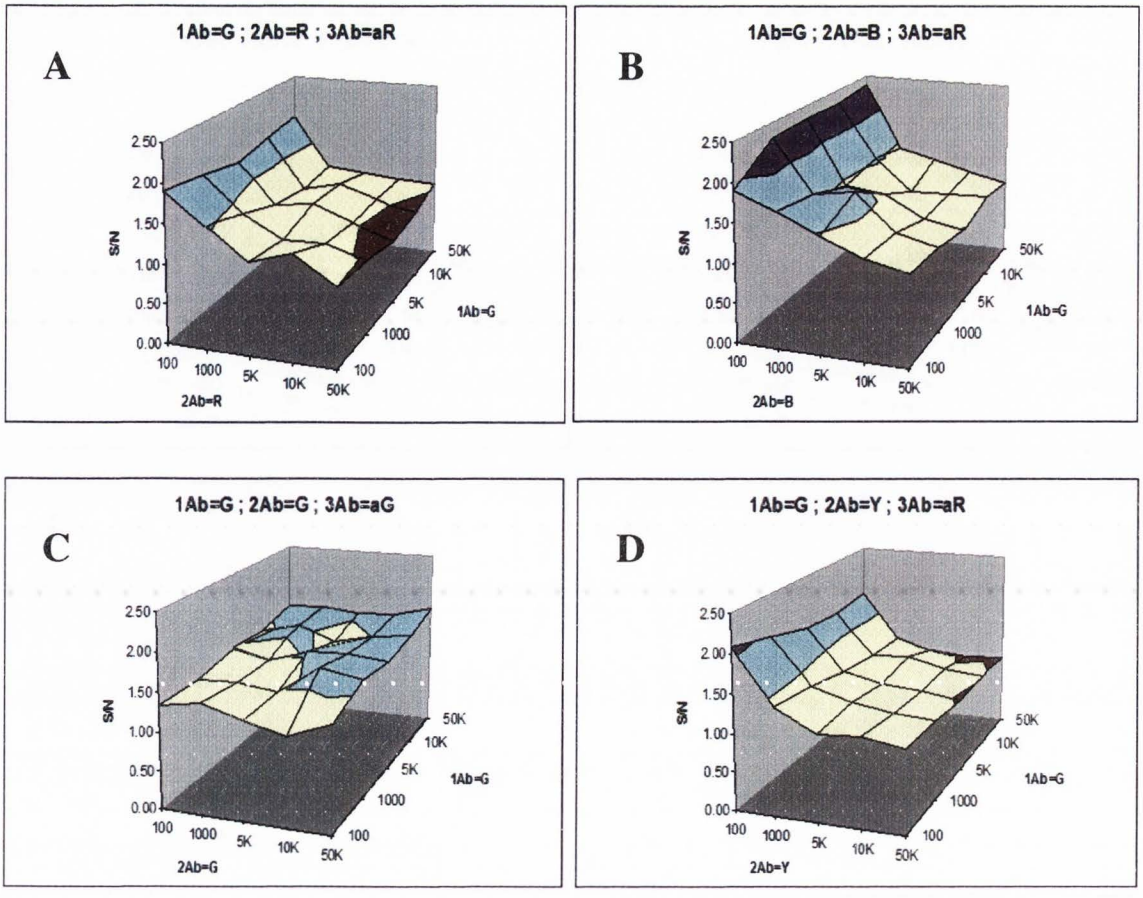


Figure A-2. Four different 3D-graphic presentations of the signal from different Ab combinations and concentrations with G5-V99 as primary Ab (1Ab); 2Ab= secondary Ab; X- and Z-axis are dilution series; Y-axis represents S/N = Signal/Noise. R = Ab R4-V99, G = Ab G5-V99, B = Ab B65420R, Y = Ab YVS4201.

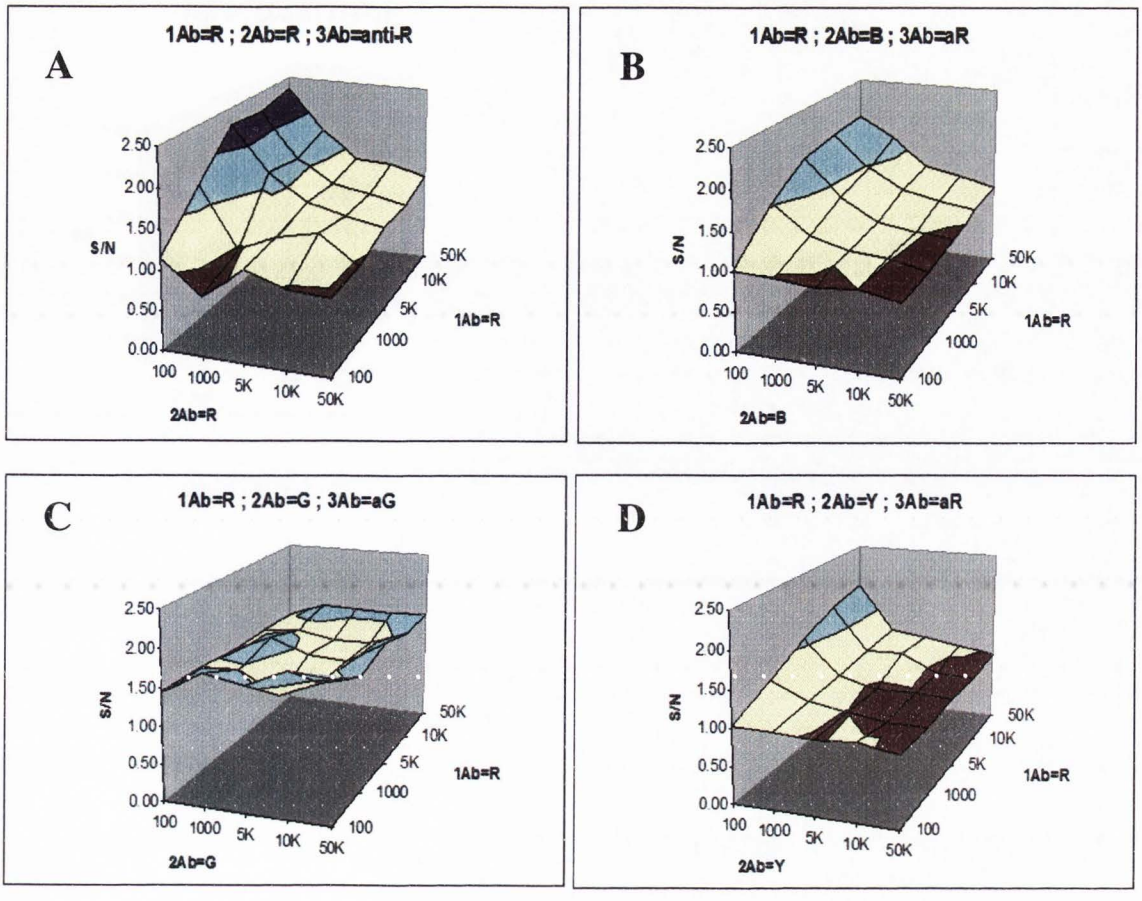


Figure A-3. Four different 3D-graphic presentations of the signal from different Ab combinations and concentrations with R4-V99 as primary Ab (1Ab); 2Ab= secondary Ab; X- and Z-axis are dilution series; Y-axis represents S/N = Signal/Noise. R = Ab R4-V99, G = Ab G5-V99, B = Ab B65420R, Y = Ab YVS4201.

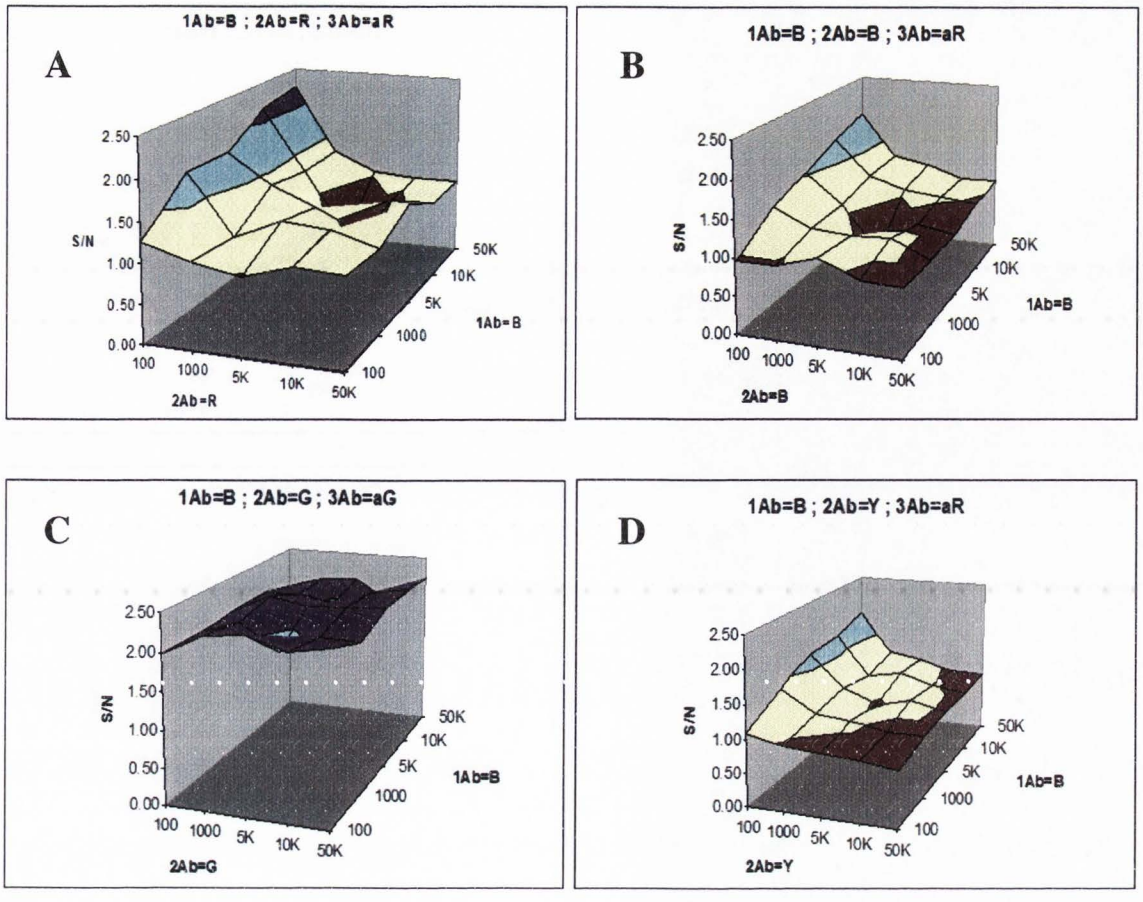


Figure A-4. Four different 3D-graphic presentations of the signal from different Ab combinations and concentrations with B65420R as primary Ab (1Ab); 2Ab= secondary Ab; X- and Z-axis are dilution series; Y-axis represents S/N = Signal/Noise. R = Ab R4-V99, G = Ab G5-V99, B = Ab B65420R, Y = Ab YVS4201.

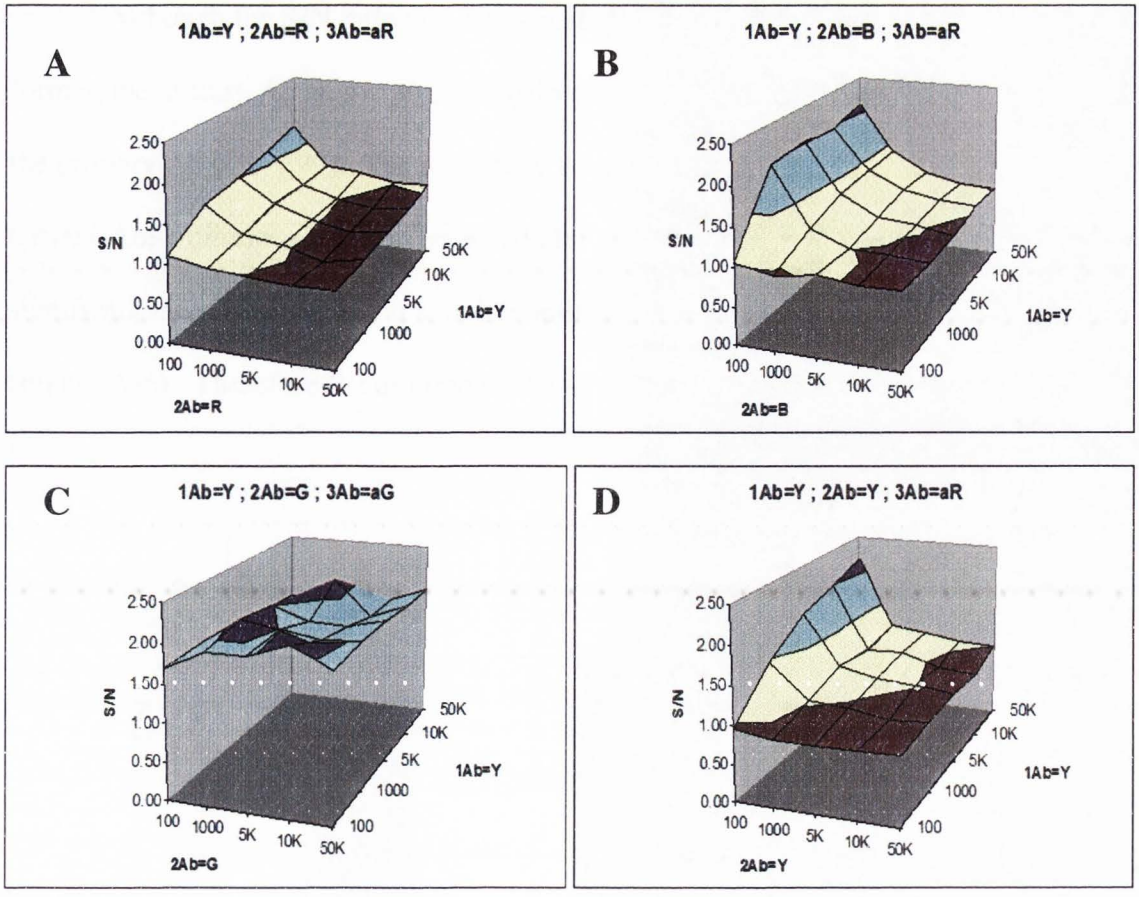


Figure A-5. Four different 3D-graphic presentations of the signal from different Ab combinations and concentrations with YVS4201 as primary Ab (1Ab); 2Ab= secondary Ab; X- and Z-axis are dilution series; Y-axis represents S/N = Signal/Noise. R = Ab R4-V99, G = Ab G5-V99, B = Ab B65420R, Y = Ab YVS4201.

Evaluation of the 3Ab concentration in the bead format

Although the S/N ratio was investigated and optimized in the indirect ELISA format, the tertiary Ab was evaluated again using 3mm glass beads already coated with the primary Ab (G5-V99). The secondary Ab concentration was kept at 1:500. For the tertiary Ab, 3 dilutions were investigated (1:1,000; 1:2,500; 1:5,000) (Figure A-6). No significant increase in S/N at at a higher antibody concentration 1:5,000 was observed (Figure A-6). Therefore the dilution of the tertiary Ab was set to 1:5,000.

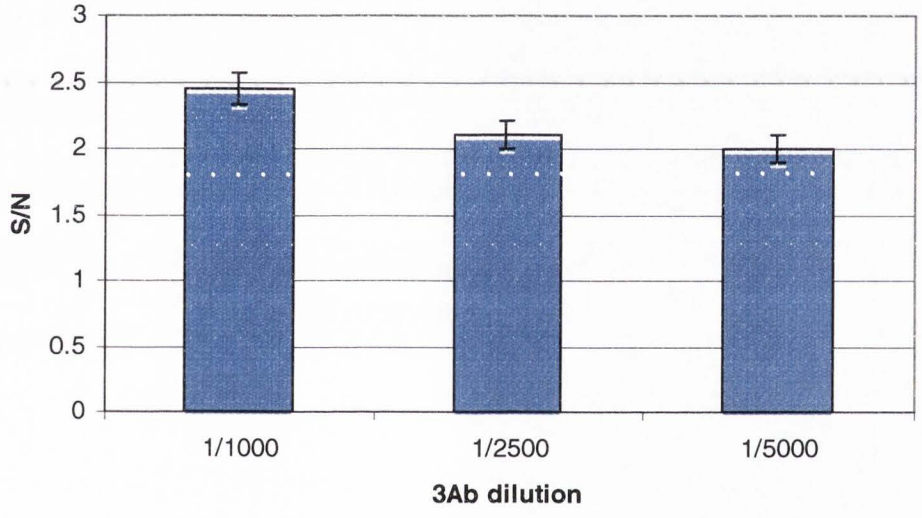


Figure A-6. Determining tertiary Ab concentration in RIC format: S/N = Signal/Noise; 3Ab = Sigma A3687.

Appendix B.

Optimization of PCR and PCR as the Analyzing Tool after Capturing

Optimization of the PCR protocol and selection of primer sets

Although PicoGreen can give quantitative results towards the amount of DNA released, it does not tell if the DNA is from *L. monocytogenes*. In a next step, a PCR approach was taken to identify the identity of the lysed cells. PCR has become very popular as a detection and confirmation system because of its speed, specificity and accuracy.

After a literature search, different primer set were selected (based on selectivity and specificity) and order at Qiagen Operon (Alameda,CA). Table B-1 shows a list of all the different primers selected. In order to be consistent, all stock primer solutions were aliquot to 20 nmol in 100 µl. The diluting agent was ddH_2O and stored at –20°C.

All sample preparations were done following a standard procedure protocol. Seventeen microliter ddH_2O was added to the puReTaq™Ready-To-Go™PCR Beads (Amersham Biosciences, Piscataway, NJ). Next 1.5 µl of each primer (forward and reverse) was added to the side of the tube in order to make sure that the total volume was dispensed. Finally 5 µl of the sample DNA solution was added (also to the side of the tube). After a short centrifuge step of 5 sec at 6000 x g (Eppendorf Centrifuge 5415 C),

Table B-1. Selected primers used in this study.

Seq name	Reference	Seq 5' to 3'	Stock (nmol)
WIM1	1	ACTAGCACTCCAGTTGTTAAAC	43.96
WIM2	1	TTATACGCGACCGAAGCCAAC	37.85
WIM3	3	CCTAAGACGCCAATCGAAAAGAAA	42.76
WIM4	3	TAGTTCTACATCACCTGAGACAGA	39.66
WIM5	2	CCTAAGACGCCAATCGAA	40.27
WIM6	2	AAGCGCTTGCAACTGCTC	42.99
WIM7	1	CAAACTGCTAACACAGCTACT	81.00

the different tubes were vortexed for 2-3 sec and another centrifuge step (5 sec at 6000 x g) was performed. Carefully the tubes were transferred to the PTC-200 Peltier Thermo Cycler (MJ Research, Reno, NV) after a final check that the total 25 µl volume was located on the bottom of each tube. All the four different primer sets were tested with the appropriate cycling parameters. The primer set WIM 5-6 and WIM 7-2 gave very clear bands after the amplified samples were subjected to gel electrophoresis.

In a first approach primer set WIM 5-6 was chosen for the multiplication of *L. monocytogenes* DNA in the PCR reaction. A ten-fold dilution series ranging from 1 x 10^8 to 1 x 10^1 CFU/ml was made, boiled 6min and subjected to the thermocycler protocol. The cycling parameter were as follows: 1min denaturation at 95°C followed by 30 cycles of 95°C for 15 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s. An extra extension step of 72°C for 6 min was added at the end. After the procedure was completed, the samples were held at 4°C until preceded to the electrophoresis step (90 min, 80 V, 4°C. A band was only obtained on the 1 x 10^8 CFU/ml lane. The sensitivity was improved by 1 log unit by increasing the cycle number from 30 to 45.

Unsatisfied with the obtained results, primer set WIM7-2 was used. The protocol of the latter primer set was immediately modified from the original 30 cycles to 40 cycles and an extension step of 72°C for 4 min was added. The full cycling parameters were as follows: 95°C for 1 min followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 45 s. After that the extension step of 72°C for 4 min was added. This time, bands showed up on the agarose gel on the lanes of 1 x 10^8, 1 x 10^7, and 1 x 10^6 CFU/ml (Figure B-1) i.e. the sensitivity was improved by 1 log.

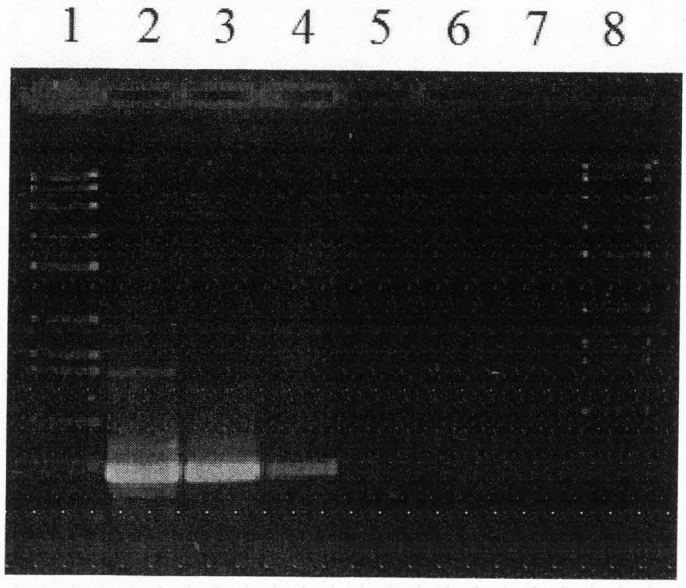


Figure B-1. Agarose gel image for primer set WIM 7-2. Lanes: 1&8 DNA size markers (from top to bottom: 10000, 8000, 6000, 4000, 3000, 2000, 1550, 1400, 1000, 750, 500 bp); 2 to 7 tenfold dilution serie $1x10^8$ to $1x10^3$ CFU/ml.

Reference

1. Bubert, A., I. Hein, M. Rauch, A. Lehner, B. Yoon, W. Goebel and M. Wagner. 1999. Detection and differentiation of *Listeria* spp. by a single reaction based on multiplex PCR. *Appl.Environ. Microbiol.* 65:4688-4692.

2. Chen, W., D. Li, B. Paulus, I. Wilson, and V. Chadwick. 2000. Detection of *Listeria monocytogenes* by polymerase chain reaction in intestinal mucosal biopsies from patients with inflammatory bowel diseases and controls. *J. Gastroenterol. and Hepatol.* 15:1145-1150.

3. Norton, D. M. 2002. Polymerase chain reaction-based methods for detection of *Listeria monocytogenes*: toward real-time screening for food and environmental samples. *J. AOAC Int.* 85:505-515.

Appendix C.

Lysis Procedures using PicoGreen and PCR as the Analyzing Tools

Standard curve with PicoGreen

In order to link the numbers produced by the spectrofluorometer to amounts of dsDNA, a DNA standard curve was created following the company's instruction sheet. *Lamda* DNA (1 ml of 100 µl/ml in TE buffer), provided by the PicoGreen dsDNA kit, was used to create a 5-point standard curve at two levels: high range and low range (Figure C-1, C-2).

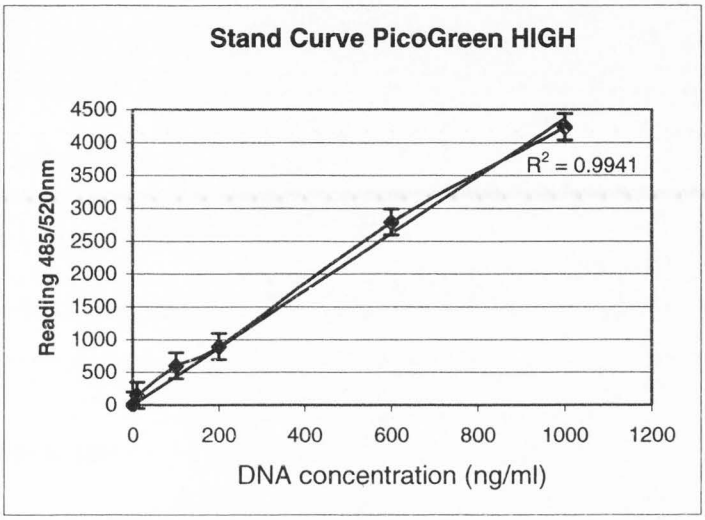


Figure C-1. High-range standard curve for PicoGreen.

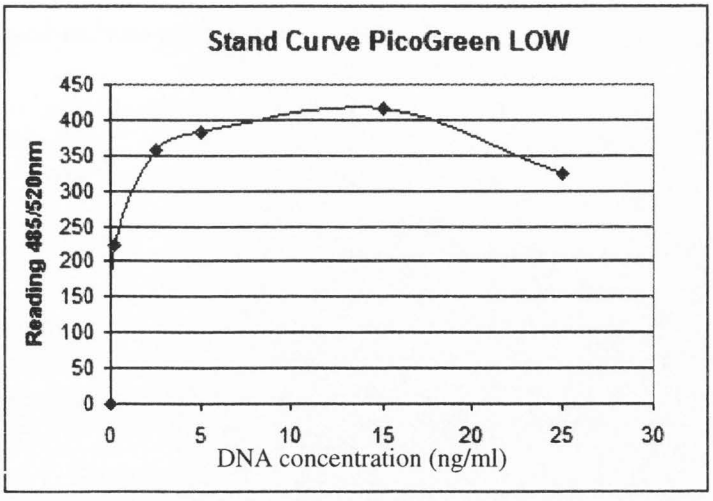


Figure C-2. Low-range standard curve for PicoGreen.

The high-range standard curve gave a very good fit with $r^2 = 0.99$. For the low-range standard curve however, no linear correlation was obtained. Self quenching of the PicoGreen dye is probably responsible for this observation. The lysis procedures tested, extracted all dsDNA amounts in the high range. Therefore no further attention was given to the results of the low-range standard curve.

Lysis with ddH_2O

The harvested cells were resuspended in ddH_2O in order to obtain a 10^9 CFU/ml. One sample was kept at room temperature (RT) for 5 min, the other one was put into a water bath at 96° C for 5 min. The latter was put on ice for 2 min immediately after the heating step. For both samples, a blank (ddH_2O) was included (Table C-1).

Lysis with Triton X-100

Triton X-100 (octylphenol ethylene oxide condensate, Sigma, St. Louis, MO) is a nonionic detergent, which is used in biochemical applications to solubilize proteins. It is therefore often used in lysis procedures, alone or in combination with other lysis products. For the lysis procedure, a 1% TritonX-100 solution was used. The same conditions as for ddH_2O, were used (Table C-1).

Lysis with lysozyme

One milliliter of 10^9 CFU/ml cell culture was pelleted, resuspended into 1 ml of a lysozyme solution (1.5 mg/ml), and incubated at RT for 30 min (Table C-1).

Lysis with Triton X-100 and lysozyme combined

A combination of Triton X-100 with lysozyme (1.5 mg/ml) was also investigated. After spinning down 1 ml of a washed cell suspension (10^9 cells/ml), the pellet was resuspended in 500 µl 1% Triton X-100 solution. After boiling the sample for 6 min, the tubes were put on ice for 2 min before adding 500 µl of a lysozyme solution (1.5 mg/ml). After a short vortex step, the samples were held at RT for 30 min (Table C-1).

Lysis using the Alkaline Lysis method

The alkaline lysis procedure used NaOH (alkaline solution) and sodium dodecyl sulfate (SDS). The first one "loosens" the cell wall and denatures plasmid and chromosomal DNA, the second compound denatures lipids and proteins and therefore pops holes in the cell membranes. The alkaline working solution contained 0.05 M NaOH and 0.2% SDS. One-milliliter cell suspensions (1 x 10^9 CFU/ml) were washed, pelleted and resuspended in 1 ml alkaline lysis solution. One sample was boiled, the other one was held on ice for 5 min. Again, for each treatment a blank was included.

The previous experiment was repeated but after lysing the cells, potassium acetate (KAc, 500 µl) was added. The working solution contained 3 M potassium and 5 M acetate. This chemical helps to get ride of 'garbage' in the sample solution. It precipitates ssDNA (since large ssDNA molecules are insoluble in high salt) and furthermore the potassium reacts with SDS to form KDS, which is insoluble (Table C-1).

Table C-1. Overview of different lysis treatments and the amounts of DNA released after a non-boiling or 6-min boiling step of 1 x 10^8 *L. monocytogenes* cells.

Lysis component	**Non Boiled DNA release (ng)**	**Boiled DNA release (ng)**
ddH_2O	355	1615
1% Triton X-100	596	1466
Lysozyme (1.5 mg/ml)	1393	N/A
1% Triton X-100 + Lysozyme	797	1393
Alkaline procedure	298	96
Alkaline + KAc	805	475

Lysis with lysozyme over time

To investigate the lysis efficiency of lysozyme and to investigate the time needed to obtain a certain amount of extracted DNA, a time-depending experiment with lysozyme was performed, ranging from 5 to 30 min (Figure C-7). The most commonly used incubation time with lysozyme is 30 min. The main purpose was to investigate if a shorter time period could lead to similar lysis efficiency.

After washing the *L. monocytogenes* cells twice, 500 µl of a 10^9 CFU/ml suspension was spinned down, the pellet resuspended in a 500 µl lysozyme solution (1.5 mg/ml), and incubated at RT for different time intervals (5, 10, 15, and 30 min). Within 5 min of incubation, lysozyme already performs with a high activity (Figure C-3). The longer the incubation time however, the more DNA is released.

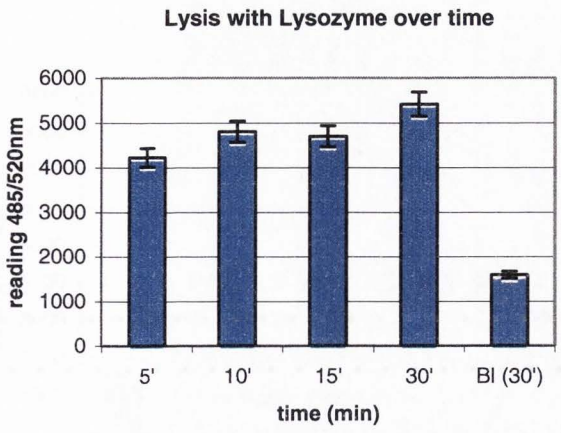


Figure C-3. Lysis using lysozyme over time; Blank = ddH_2O.

Lysis with lysozyme at RT and 37°C

To investigate the influence of temperature on the lysozyme activity during lysis, an assay with 2 different temperatures was setup. The main purpose was to find out if the incubation step could be performed at RT (25°C) or if a higher incubation temperature (37°C) was necessary. During this experiment the incubation time was set to 30 min. Same procedure was used as in the time-depending experiment. No significant differences were observed between RT and 37°C incubation temperatures. For 37°C, a net DNA release of 1477 ng was obtained. For RT this was slightly less, being 1353 ng (Figure C-4).

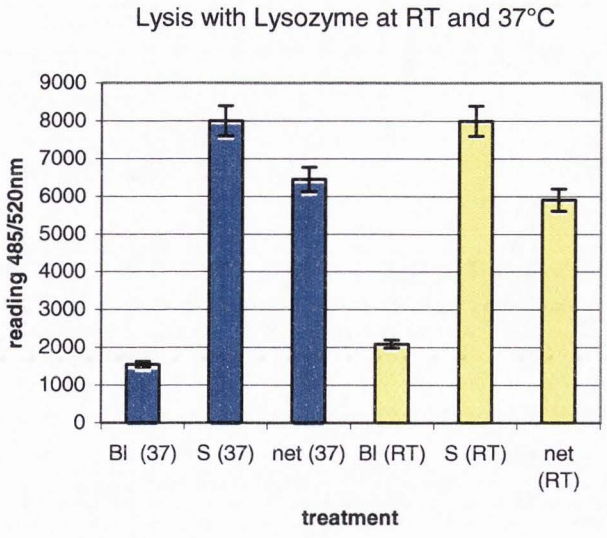


Figure C-4. Lysis using lysozyme at two different incubation temperature (RT and 37°C) for 30 min; Bl = lysozyme solution without cells; S = lysozyme solution with 10^8 CFU/ml.

Analyzing lysis solutions with PCR

After selecting the primer set and optimizing the cycling parameters for the thermocycler, the focus on increasing the detection limit and sensitivity on the agarose gel was now approached from a lysis standpoint.

Small experiments to improve lysis and DNA release included boiling time and boiling volume. A total boiling time of 6 min was chosen since after a 10-min boiling step non-specific bands were observed (data not shown). Having a total volume of 1 ml or only 100 µl didn't make a difference, as long as no precipitation step was included (data not shown). Besides boiling, the enzymes lysozyme and proteinase K were looked into with PCR as the analyzing tool. The latter enzyme was not investigated with PicoGreen earlier.

For the investigation of lysozyme (L-7001, Grade III, 45,000 units/mg solid, Sigma), an overnight culture was washed twice, diluted and resuspended in a 1.5 mg/ml lysozyme solution. The samples were put into a water bath for 15 min at 37°C followed by a 6 min boiling step. Surprisingly, no results (i.e. no bands on the agarose gel) were obtained using lysozyme as the lysis tool. Using Tris-EDTA (TE) buffer solution (10 mM Tris, 1 mM EDTA, pH 8.0) instead of ddH_2O to resuspend the cells did not solve this problem (data not shown). A clear explanation for this result could not be given. It is not know why inability to detect DNA with lysozyme was observed, but Dnase activity during the incubation step could be a possible reason.

Proteinase K (P-0390, 13,8 units/mg solid, Sigma) was added to the list of components for lysis. An overnight culture was diluted out (1×10^6 to 1×10^1 cells/ml) in 1 ml volumes, washed twice with 50mM Tris buffer (pH 7.2) and resuspended in 100 µl of a 500 µg/ml proteinase K solution. The different dilutions were incubated 30 min in a water bath at 60°C followed by a 6 min boiling step. Bands showed up on the 1.3% agarose gel for all the cell dilutions (1×10^6 to 1×10^1 cells/ml) (Figure C-5).

The experiment was repeated with the difference that the cells dilutions were first boiled for 4 min in 50 µl TE buffer. After cooling the samples on ice (5 min) 50 µl of a 1 mg/ml proteinase K solution was added, incubated for 30 min, and boiled for another 4 min. Although even more clear bands were expected for all the samples compared to the previous experiment, bands showed up only on the lines for 1×10^6 to 1×10^2 cells/ml.

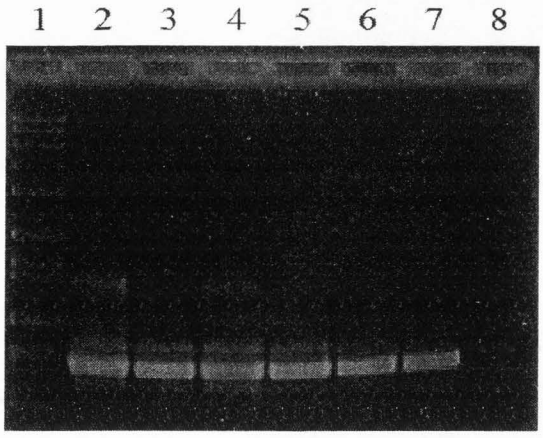


Figure C-5. Lane 1) DNA size marker (from top to bottom: 10,000; 8,000; 6,000; 4,000; 3,000; 2,000; 1,550; 1,400; 1,000; 750; 500 bp), lane 2 to 7) dilution series of pure culture with $1x10^6$ (2), $1x10^5$ (3), $1x10^4$ (4), $1x10^3$ (5), $1x10^2$ (6), $1x10^1$ (7) CFU/ml; lane 8) negative control with ddH_2O.

The combination of lysozyme (1 mg/ml, Sigma) and proteinase K (500 µg/ml, Sigma) was also investigated. One milliliter of each cell dilution was pelleted, resuspended in 100 µl lysozyme solution (1.5 mg/ml) and incubated for 15 min at 37°C. In a next step, 100 µl of the proteinase K solution (500µg/ml) was added and the samples were incubated at 60°C for 30 min. To inactivate the enzyme activities and to increase the DNA extraction, the cell solutions were boiled for 6 min before advancing to PCR. Somehow the lysozyme treatment must have influenced the results in a negative way since the detection limit was only 1 x 10^4 CFU/ml. It is not completely understand why this reduction in lysis efficiency was observed. Therefore it could be concluded that the use of one proteinase K treatment (500 µg/ml) for 30 min at 60°C followed by a boiling step of 6 min gave the best results.

Appendix D.
Spacer Comparison

Selecting the right spacer molecule

Before coating an Ab onto the glass beads, a spacer molecule had to be selected. Two molecules, polyethylene glycol (PEG, P-4463, Sigma, St. Louis, MO) and polydextran (DEX, D-4133, Sigma, St. Louis, MO) were tested and compared. The main purpose of a spacer molecule is to reduce the steric interference between the individual primary antibodies when covalently linked to the beads. The spacer is attached to the plain glass beads and binds on his turn the primary Ab. Two batches (100 grams) were prepared, each with a different spacer. To investigate the effect of the spacers, the rapid immuno capture (RIC) procedure was followed.

Figure D-1 (A) shows the direct readings of the different samples. Not only were the sample readings (signal, S) for DEX beads higher than those for PEG beads, but also the blank reading (noise, N) was lower in 3 of the 4 dilutions compared to the blanks for PEG. Converting those results to S/N ratios, it became clear that beads with polydextran as the spacer molecule lead to higher S/N values (Figure D-1, B). Up to a dilution of 1:10,000 of the secondary Ab, the S/N ratios increased for DEX coated beads (from 2.6 for 1:1,000 to 3.4 for 1:10,000). Only at 1:50,000 a higher signal for the PEG beads was observed (Figure D-1, B).

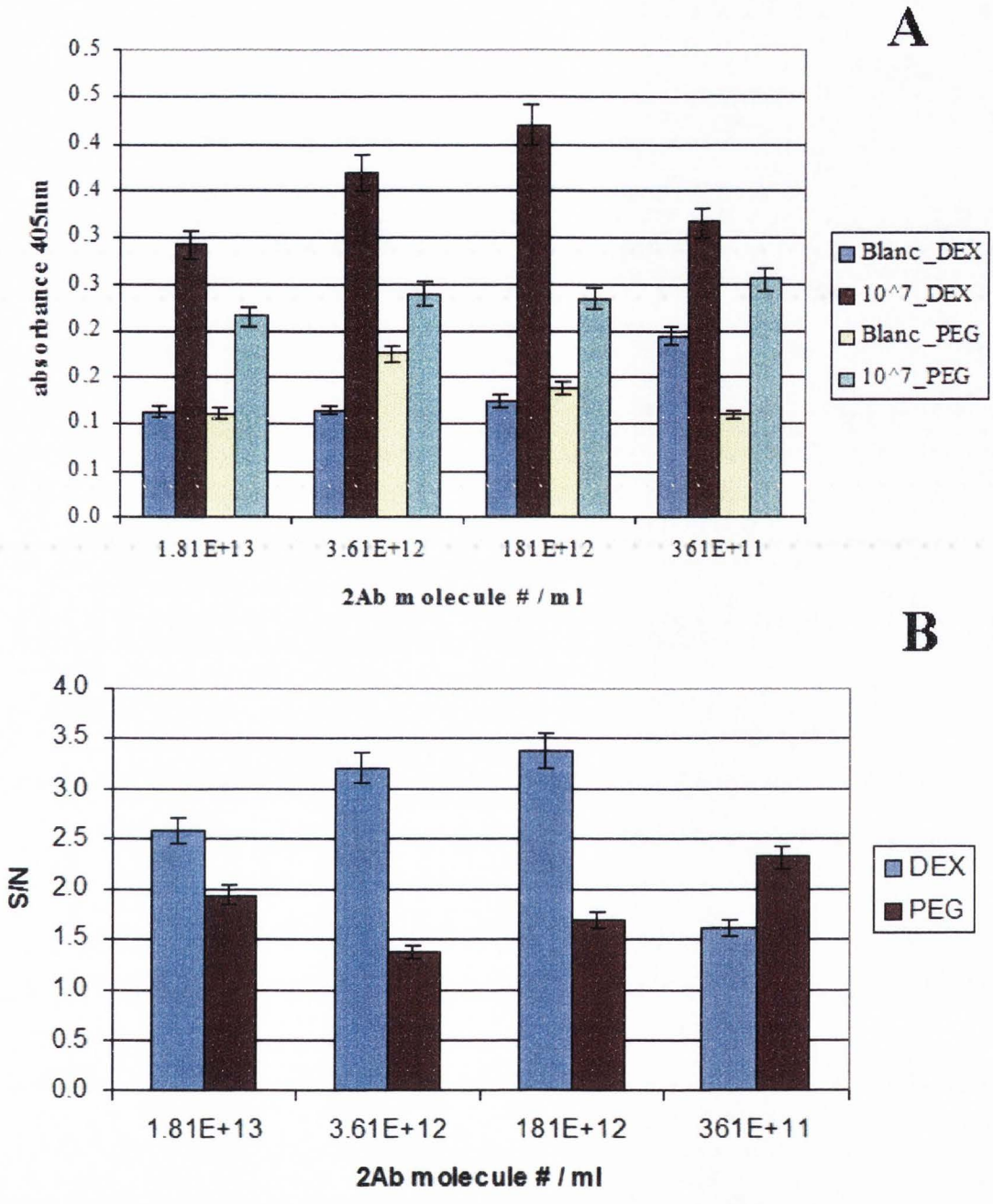


Figure D-1. Comparison between the spacers PEG and DEX on glass beads. A: absorbance readings; B: Signal/Noise.

Appendix E.

Statistical Analysis with JMP 3.1.5

SAS Institute Inc.

Statistical analysis of lysis treatments

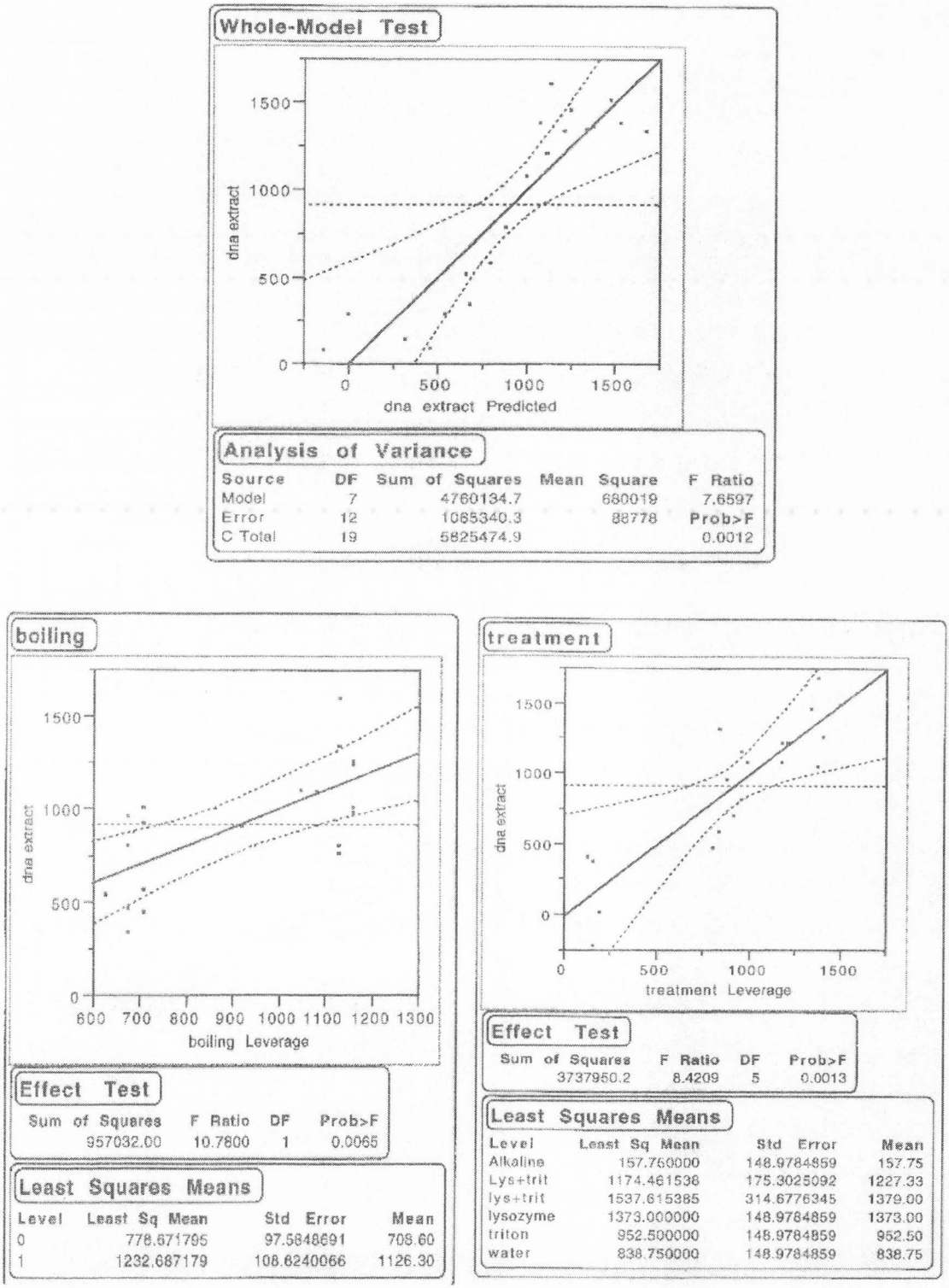


Figure E-1. Statistical analysis of lysis data: 0 = no boling, 1 = boiling.

Response: dna extract

Summary of Fit

RSquare	0.817124
RSquare Adj	0.710446
Root Mean Square Error	297.957
Mean of Response	917.45
Observations (or Sum Wgts)	20

Parameter Estimates

Effect Test

Source	Nparm	DF	Sum of Squares	F Ratio	Prob>F
reps	1	1	86868.0	0.9785	0.3421
treatment	5	5	3737950.2	8.4209	0.0013
boiling	1	1	957032.0	10.7800	0.0065

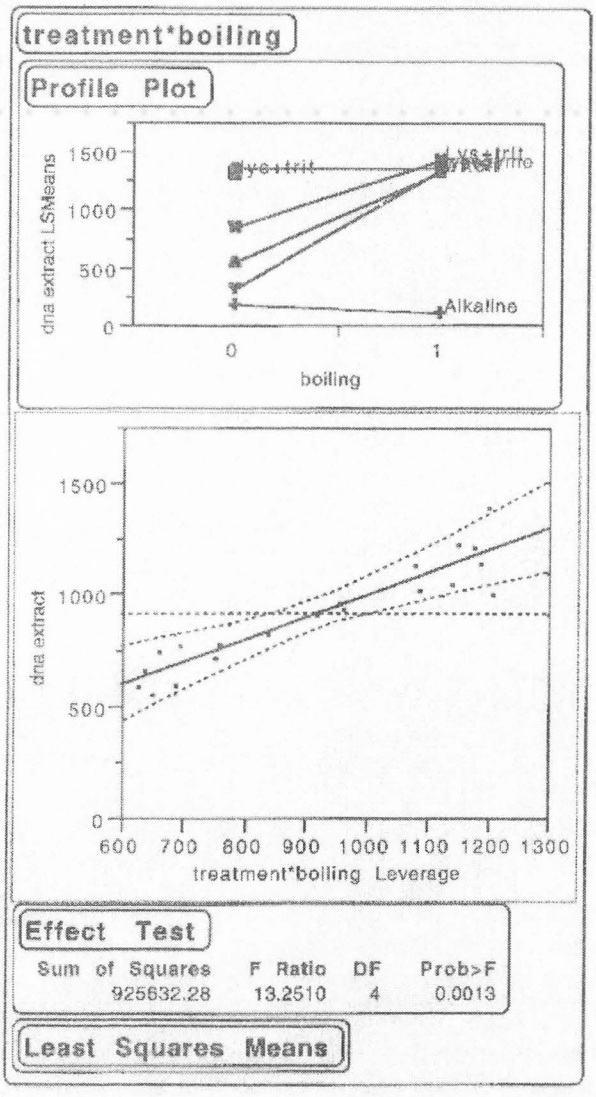


Figure E-2. Statistical analysis of lysis data: treatment vs. boiling.

Statistical analysis of capture results

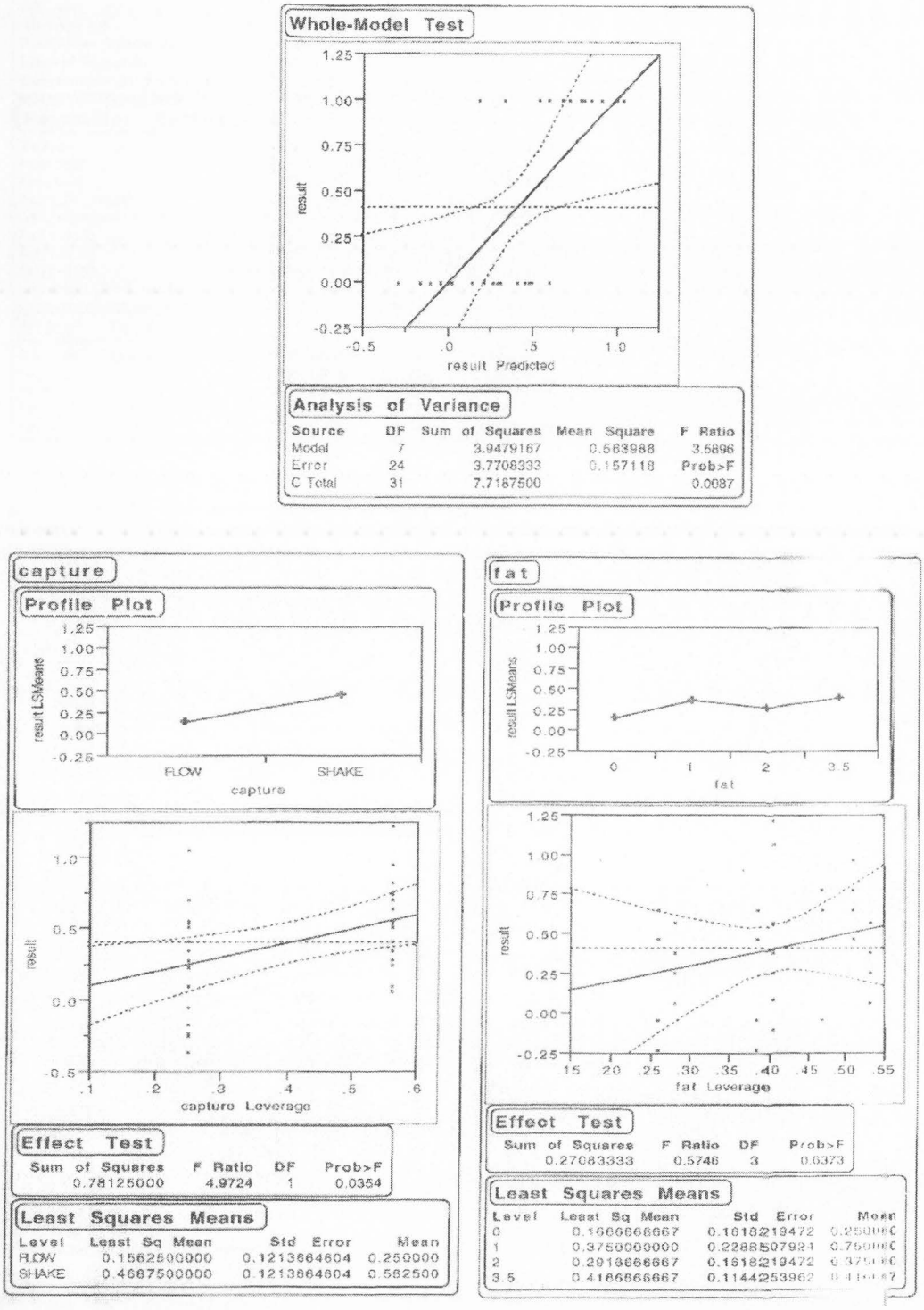


Figure E-3. Statistical analysis of cell capture data: Flow vs. shaking capture, Fat influence (skim, 1%, 2% and 3.5%).

Response: result

Summary of Fit

RSquare	0.511471
RSquare Adj	0.368983
Root Mean Square Error	0.396381
Mean of Response	0.40625
Observations (or Sum Wgts)	32

Parameter Estimates

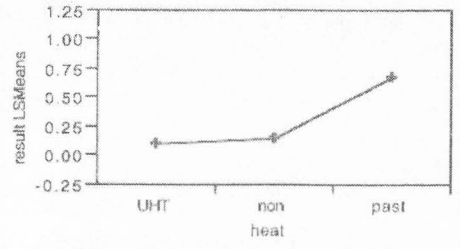
Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	0.3125	0.099095	3.15	0.0043
rep[1-2]	0.09375	0.070071	1.34	0.1935
heat[UHT-past]	-0.208333	0.114425	-1.82	0.0811
heat[non-past]	-0.166667	0.161822	-1.03	0.3133
fat[0-3.5]	-0.145833	0.127931	-1.14	0.2856
fat[1-3.5]	0.0625	0.171638	0.36	0.7189
fat[2-3.5]	-0.020833	0.127931	-0.16	0.8720
capture[FLOW-SHAKE]	-0.15625	0.070071	-2.23	0.0354

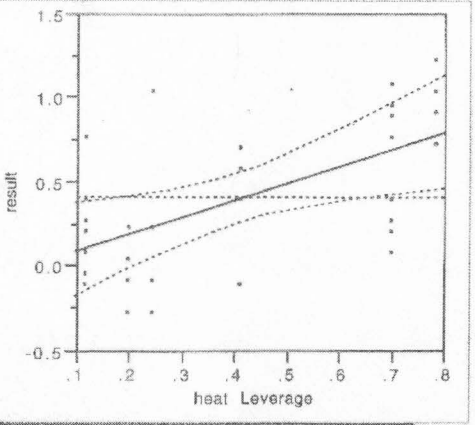
Effect Test

Source	Nparm	DF	Sum of Squares	F Ratio	Prob>F
rep	1	1	0.2812500	1.7901	0.1935
heat	2	2	2.2083333	7.0276	0.0040
fat	3	3	0.2708333	0.5746	0.6373
capture	1	1	0.7812500	4.9724	0.0354

heat

Profile Plot





Effect Test

Sum of Squares	F Ratio	DF	Prob>F
2.2083333	7.0276	2	0.0040

Least Squares Means

Level	Least Sq Mean	Std Error	Mean
UHT	0.1041666667	0.1279314821	0.083333
non	0.1458333333	0.2358939974	0.250000
past	0.6875000000	0.0990953000	0.687500

Figure E-4. Statistical analysis of cell capture data: heat treatment (UHT, non i.e. raw milk, pasteurized milk).