The logo of Purdue University’s Center for Food Safety Engineering (CFSE) represents the center in many more ways than one. Understanding the iconography of its images is one way to understand and appreciate CFSE.

The Gear—Engineering

One of the primary components of the CFSE logo is a gear, symbolizing the engineering component of the center. CFSE engages many of the different branches of engineering, and the gear calls to mind the basics of that discipline.

The teeth of the gear bear the insignia of the five Purdue schools that contribute to the center, organized alphabetically in a counterclockwise arc starting from the upper right.

- The first insignia represents the School of Agriculture;
- The second insignia represents the School of Consumer and Family Sciences;
- The third insignia represents the Schools of Engineering;
- The fourth insignia represents the School of Science; and
- The fifth insignia represents the School of Veterinary Medicine.

The Circuit Board—Progress

The circuit board in the center is actually part of the revolutionary biochip developed by Purdue researchers. In the CFSE logo, the biochip symbolizes the combination of engineering and food safety for technological progress.

The Petri Dish—Food Safety

The petri dish in the lower portion represents food safety, as epitomized by traditional microbial identification methods. One of the center’s goals is to develop more rapid methods of identifying microbes.

The Circle—Synthesis

The circle bounding the petri dish and the biochip inside the gear represents the synthesis of old and new technologies for improved food safety.

CFSE logo rendering courtesy of Kellen Maicher, Purdue University Department of Computer Graphics Technology
As the Center for Food Safety Engineering (CFSE) at Purdue University approaches its third year, our focus remains the same, but we continue to grow. Our main goal is to build multidisciplinary teams and develop better approaches for detection and control of foodborne hazards that affect the U.S. food supply. We plan to develop detection systems for *Listeria monocytogenes*, *E. coli O157:H7*, *Campylobacter* spp., *Salmonella* spp., and emerging pathogens, including those that can present a biosecurity risk to our food supply.

Our strength lies in our unique ability to combine the talents of Engineering scientists with researchers from Agriculture, Consumer and Family Sciences, Science, and Veterinary Sciences.

The following report highlights projects funded under our cooperative agreement with the United States Department of Agriculture-Agricultural Research Programs (USDA-ARS), as well as research projects developed through other funding opportunities. We have faculty teams working on methods to detect pathogenic bacteria, molds and seafood toxins, and teams working on bioluminescence techniques that use infrared sensors. Our combined efforts this year led to 28 refereed journal publications and 43 presentations at national/international meetings.

Our largest project to date, led by Drs. Ladisch, Bashir, and Bhunia, involves the development of a biosensor to detect *Listeria monocytogenes* in ready-to-eat processed meat systems. During the past year, they improved sample preparation procedures and optimized a detection-based platform — advances that bring us one step closer to commercial use of the biosensor. We hope this approach can be quickly adapted for other foodborne pathogens and in other food systems.

We are very excited about our collaboration with USDA-ARS scientists at the Eastern Regional Research Center (ERRC) in Wyndmoor, Pennsylvania. This year we jointly began development of multi-array sensors, optical sensors, and microbial detection using a cantilever approach.

CFSE also has initiated a graduate student internship program and an undergraduate research program. Most recently, Kristen Naschansky Gray gained invaluable experience during a graduate internship with researchers at USDA-ARS ERRC. More recently, the undergraduate research program linked undergraduates in their junior and senior years with CFSE faculty in independent research projects. As this program grows, we hope to offer internship opportunities for undergraduates.

These are certainly very exciting times for CFSE and Purdue University. I am proud of our combined efforts and look forward to our growth in the coming years. To learn more about our center, please visit our Web site at www.cfse.purdue.edu or feel free to contact me directly.
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What major problem or issue are you resolving, and how are you resolving it?

The public has become increasingly concerned about microbial contamination of meat and, more importantly, of fresh fruits and vegetables due to an increased emphasis on these foods in healthy diets and the recognition of new foodborne pathogens such as *Campylobacter jejuni*, *Escherichia coli* O157:H7, and *Listeria monocytogenes*. In response to this rising concern, the President announced a Food Safety Initiative in 1997. As part of this initiative, a joint agency guide on approaches to minimizing microbial contamination of fresh fruits and vegetables was recently released. While the recommended practices provide a framework for minimizing risk, we could better protect the public if we could more rapidly detect pathogens in the production, processing, and distribution systems.

Our long-term research goals are to improve detection of food-contaminating pathogens by

1. genetically constructing pathogen-specific bacteriophage capable of inducing bioluminescence from bioluminescent bioreporter cell populations for quantitative sensing of microbial pathogenic species in raw or minimally processed meats, fruits, and vegetables; and

2. analyzing bacteriophage/bioluminescent bioreporter system with portable field-based photomultiplier unit for the development of a suite of simple, rapid, real-time, on-site testing mechanisms for bacterial pathogens in meats, fruit, and vegetables. This technology could cut pathogen detection time from days to hours.

How serious is the problem?

Of the nearly 2,300 identified serovars of *Salmonella*, 300 have been related to human illness. *Salmonella* most commonly cause gastroenteritis, but can result in severe systemic infections. *Salmonella*-related illness has been linked to cantaloupe, alfalfa sprouts, tomatoes, and watermelon. *Salmonella* has also been isolated from a wide variety of fresh vegetables, including artichoke, cabbage, cauliflower, celery, eggplant, endive, fennel, lettuce, mustard cress, parsley, and spinach.

*Listeria monocytogenes* causes serious human disease manifested by sepsis and meningitis. It is commonly found on vegetables, including lettuce, tomatoes, asparagus, broccoli, and cauliflower. A documented listeriosis outbreak has been associated with cabbage. Growth of *L. monocytogenes* can occur at cool (5-15°C) storage temperatures.

*E. coli* 0157:H7 is an emerging human pathogen first linked to food-illness (i.e., fast food hamburgers) outbreaks in 1982. It produces enterohemorrhagic toxins that can lead to death, particularly in the very young and old. Cattle appear to be a primary reservoir, but *E. coli* 0157:H7 has been
linked to outbreaks from cantaloupe, broccoli, and lettuce. The organism can survive and grow on cubed melon and watermelon, and has been isolated from cabbage, celery, cilantro, and coriander.

*Campylobacter* is an emerging pathogen that causes acute gastroenteritis and has been identified as a common antecedent to Guillan-Barre syndrome, an acute neurological disease. Illness is most commonly associated with consumption of contaminated poultry and raw milk, although *Campylobacter* has been linked to raw fruits and vegetables.

**What was your most significant accomplishment in this past year?**

We obtained a P22 recombination vector to construct a luxI- and ainS-based P22 bacteriophage to use in the *Salmonella* Bacteriophage/Bioluminescent Reporter System to eliminate the use of an antibiotic resistance marker in the phage. The recombination vector was modified to contain luxI (in collaboration with Laszlo Csonka in the Department of Biology and in the Applegate laboratory). The gene was successfully cloned into the vector for construction of the recombinant P22 phage. The impact of the success of this approach is the elimination of the resistance marker for screening the phage, making the system more attractive for use by industry.

We constructed a model *Salmonella* Bacteriophage/Bioluminescent Reporter System to evaluate the luxI constructs in *Salmonella* in a phage-based format. An F-prime *Salmonella enteriditis* was constructed to evaluate a model *Salmonella* Two Component Bacteriophage/Bioluminescent Reporter System (in collaboration with Laszlo Csonka in the Department of Biology and in the Applegate laboratory). The system was evaluated and showed similar results to the *E. coli* model system. The success of the model system validated the approach for utilization of P22 and the expansion of the system into a pathogenic bacterium. We investigated the nature of the quorum-sensing mechanism in the *Vibrio fischeri* strain ATCC 49387 (MicroTox assay strain) due to a mutation discovered in the luxI of this strain inactivating its synthesis of 3-oxo-C6-HSL. A set of *E. coli* expressing various components of the lux-based quorum sensing system were constructed in our laboratory to elucidate the MicroTox quorum sensing system. The MicroTox quorum system was elucidated and it was determined it was based on C8-HSL as opposed to 3-oxo-C6-HSL. The discovery that the MicroTox quorum sensing is based on C8-HSL will allow the substitution of the gene responsible for the synthesis of C8-HSL (ainS) to be utilized in place luxI. This allows the MicroTox strain to be used as the reporter strain and eliminates the use of an antibiotic-resistant strain in the assay.
This technology could cut pathogen detection time from days to hours.

What major accomplishments do you anticipate over the life of the project, and what do you predict their impact will be?

The major accomplishments over the life of the project are summed up in the successful execution of proof-in-principle experiments validating the approach of biological light amplification coupled with phage specificity to allow the use of low-sensitivity light detection. This is fundamental to the assay being developed and will allow the utilization of low-sensitivity light detectors for pathogen detection in this format. The amplification assay also allows for the rapid detection of the pathogens when sensitive light-detection apparatus are utilized, as their threshold detection limits are exceeded very quickly in the process. The assay being developed could have immediate impact in the food safety arena as it can be incorporated with equipment already deployed and used routinely for the ATP hygiene test.

What do you expect to accomplish, year by year, over the duration of the project?

**Year 1**

1. Genetically engineer a lux-based bioreporter cell line responsive to acyl-homoserine quorum sensing signal induction.


3. Test bacteriophage/bioreporter systems in pure culture studies, emphasizing hand-held luminometer format.

4. Lyophilize bacteriophage/bioreporter systems for long-term storage assessment.

**Year 2**

1. Comprehensively test bioreporter systems on lettuce and tomato with introduced pathogens.

2. Compare and contrast pathogen detection parameters utilizing hand-held photomultiplier units and microtiter plate format.

3. Continue evaluating lyophilized bacteriophage/bioreporter systems for long-term storage assessment.

**Year 3** (No cost extension)

1. Evaluate and incorporate MicroTox strain into assay.
Science/Technology Transfer


A private company is currently investigating commercialization of the technology. The technology could be field-ready for *Salmonella* sp. by August 2003. According to interested corporate scientists, the major constraint until recent developments was the use of antibiotic resistance markers in the components of the assay. This now should not be a problem, as shown in the recent accomplishments above.

Presentation

Bioamplification Using Phage Display for the Detection of *Salmonella* spp. and Its Evaluation as a Technology Platform for the Simultaneous Detection of Multiple Pathogens in the Same Sample

**Investigators:** Bruce M. Applegate and Michael R. Ladisch

**What major problem or issue are you resolving, and how are you resolving it?**

Microbial contamination of fresh fruits and vegetables, as well as meat, has become a growing concern due to an increased emphasis on these products in a healthy diet and due to the recognition of new foodborne pathogens. In response to this rising concern, the President announced a Food Safety Initiative in 1997. A joint agency guide on approaches to minimizing microbial contamination of fresh fruits and vegetables was recently released as part of this initiative. While current recommended practices can help minimize risk, we could improve safety if we had rapid detection systems that gave us real-time assessment of risks in the production, processing, and distribution systems.

The goal of this research is to harness the power of bacterial phage display with affinity chromatography in order to develop a biological amplifier that can detect small numbers of pathogenic organisms in foods. The research will generate and purify phages designed to selectively detect *Salmonella* spp. Projects on biochips, antibody-based assays coupled with impedance-based spectroscopy, fluorescence microscopy, and enzyme-linked immunosorbent assays (ELISAs) are addressing the diverse needs of detection methods for food pathogens.

Research underway in our laboratories is addressing the processing of food samples, amplification of organisms using culture or rapid separation techniques, and selective capture of pathogenic organisms from a complex background of other organisms, protein macromolecules, and other substances. That research involves cooperative efforts with investigators in Food Science, ECE, Biomedical Engineering, Agricultural and Biological Engineering, Biology, and LORRE. The proposed project complements these efforts by examining the biological fundamentals of applying a phage display method with the use of affinity (liquid) chromatography to obtain purified phages for infecting *Salmonella* spp. It also separates phages that would be generated in the presence of *Salmonella* spp., thereby indicating the presence of this food pathogen, even if other (non-pathogenic) bacteria are present. If successful, this technology platform could be directly coupled to the existing antibody-based projects mentioned above.

**How serious is the problem? Why does it matter?**

*Salmonella* is one of the top foodborne pathogens, causing 15.1 cases of foodborne illness per 100,000 persons each year in the United States. The National Health objective aims to reduce this rate to 6.8 in 2010 (Food Net, 2001). *C. jejuni* and *Salmonella* spp. are the most common causes of foodborne illness associated with meat and poultry products, and they cause an estimated 4 million cases per year.

**What were your most significant accomplishments in this past year?**

We obtained a P22 recombination vector to construct the modified P22 tail-spike protein that can eliminate the use of an antibiotic resistance marker in the phage. In collaboration with Laszlo Csonka in the Department of Biology and in the Applegate laboratory, we modified the recombination vector to allow insertion of the second tail-spike copy. The vector was successfully modified for construction of the recombinant P22 phage. This approach can eliminate the resistance marker for
screening the phage, making the system more attractive to industry. During previous construction of the recombinant M13 to be utilized in the assay, numerous mutations were consistently found in the modified GIII gene, so another strategy was pursued to construct the modified protein. In my laboratory, an M13 GIII phage display library was panned and a streptavidin-binding epitope was isolated, the gene subsequently amplified, and cloned into an E. coli vector. The result will allow the development of purification protocols and proof in principle assays for the ultimate success of the project.

What major accomplishments do you anticipate over the life of the project, and what do you predict their impact will be?

The anticipated major accomplishment of this project will be the incorporation of the specificity of bacteriophage with established antibody detection methods. This technology platform can then be integrated in the future with antibody-based assays coupled with impedance-based spectroscopy, biochips, fluorescence microscopy, and enzyme-linked immuno-assays (ELISAs) which address the diverse needs of detection methods for foodborne pathogens.

What do you expect to accomplish, year by year, over the duration of the project.

Year 1
1. Construct and propagate modified bacteriophages (MB) in E. coli and Salmonella spp. host cells.
2. Purify modified bacteriophages using affinity chromatography.

Year 2
1. Develop an assay using the modified bacteriophages to detect food samples infected with Salmonella and/or non-pathogenic organisms. Capture, concentrate, and detect the organisms using affinity chromatography and other forms of liquid chromatography.

Science/Technology Transfer
A Provisional Patent Application was filed in March covering the current technology of the Bioamplification/Phage Display technology with modifications.
What major problem or issue are you resolving, and how are you resolving it?

Microbial contamination of food has become an increasing concern during the last decade. Keeping fresh vegetables free of contamination is particularly important, due to an increased emphasis on these products in a healthy diet and the recognition of new foodborne pathogens such as *Campylobacter jejuni*, *Escherichia coli* O157:H7, and *Listeria monocytogenes*. Current detection methods are not effective at quickly, economically, and specifically identifying disease-producing microbes.

The proposed work will focus on improving our ability to identify four pathogens of significant concern to the food industry: *Salmonella* species, *Listeria monocytogenes*, *Escherichia coli* O157: H7, and *Campylobacter*.

The rationale for this research is based on the specificity of DNA probes for the detection of specific target DNA sequences associated with the virulence of the foodborne pathogens to be detected. DNA hybridization analysis in its various applications is a powerful tool and has various advantages over other identification methods. The current culture-based methods are time-consuming and can require long incubation times to obtain results. Antibody methods rely on the detection of surface antigens but have numerous drawbacks, including cross-reactivity and antigen expression, which can be affected by environmental conditions. The use of multiplexed PCR offers an alternative approach to current antibody methods, allowing detection of a suite of genes present in a pathogen and providing redundancy and elimination of false negatives and positives. Multiplexed PCR relies on the amplification of multiple target sequences that are pathogen specific. Once amplified, the fragments are analyzed by electrophoresis for the indicative banding pattern, which is definitive. The technology being developed is a fluorescence-based wavelength shift assay utilizing FRET (Flourescence Resonance Energy Transfer) to detect hybridization of a set of target DNA sequences associated with identified pathogens. The use of this spatial detection format (arrayed probes) will allow for the simultaneous detection of products from multiplexed PCR reactions more rapidly than could be achieved with electrophoresis. The spatial isolation of the probes allows for the simultaneous detection of a larger number of targets than would be feasible with current real-time PCR assays. Real-time multiplexed PCR relies on the use of a different pair of fluorophores for each molecular beacon due to the detection format. Currently there are few fluorophore pairs with distinct emission wavelengths to facilitate detection of large numbers of target DNA molecules. The proposed technology will allow the construction of all of the beacons with a single fluorophore pair. The use of the same fluorophore pair for each beacon simplifies the design of the probes.

How serious is the problem? Why does it matter?

Based on recent estimates, *Salmonella* species, *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Campylobacter* cause two-thirds of all food-related deaths in the United States, and greater than 95% of food-related deaths caused by bacteria. In association with illness, the economic impact is also serious. *L. monocytogenes* was associated with a recent recall of 27.4 million pounds of deli meats by Pilgrim’s Pride Corporation (Associated Press http://www.chicagotribune.com/business/sns-ap-meat-recall1014oct14.story).
What were your most significant accomplishments this past year?

The core of the project is the wavelength shift format using the FRET approach, which requires the identification of a pair of fluorophores with appropriate overlap of emission and excitation spectra. A review of the commercially available fluorophores was undertaken to identify a suitable pair of fluorophores by the two project principle investigators. The review resulted in the choice of the fluorophores: fluorescein and JOE, (2,7-dimethoxy-4, 5-dichloro-6-carboxyfluoroscein) which provide emission and excitation overlap and a large wavelength difference in overall excitation and ultimate emission wavelength. The determination of the fluorophore pair is crucial to the success of the project and their characteristics will provide system robustness.

To facilitate the construction and analysis of the genes included in the detection format, it was necessary to clone the full-length target gene for each of the identified target sequences. Primers were designed to amplify the target sequences and used in a PCR reaction with the targeted pathogen. Amplicons were cloned into an E. coli cloning vector in my laboratory. To this date, seven of the targeted genes have been cloned, and five of these clones have been subjected to large-scale preparation. Aliquots have been sent to Dr. Pina Fratimico for sequencing. This cloning will facilitate the long-term goals of the project by providing a standard source DNA for evaluation of the developed assay.

What major accomplishments do you anticipate over the life of your project, and what do you predict their impact will be?

Using FRET in a spatial application will allow the multiplexed detection of pathogens using a group of target genes per organism allowing increased reliability by decreasing the number of false positives and false negatives. The developed platform will also move laterally into other detection formats utilizing nucleic acid probe technologies.

What do you expect to accomplish, year by year, for the duration of your project?

**Year 1**

1. Design of a molecular beacon incorporating appropriate FRET pairing to provide maximum emission separations with excitation overlap.
2. Design of molecular beacon target sequences and corresponding amplicon primer pairs for identified target sequences to use in a multiplex PCR.

**Year 2**

1. Construct the molecular beacon array and optimize hybridization conditions with the PCR amplicons.
2. Construct the field-deployable detection systems and validate the integrated system.
What major problem or issue are you resolving, and how are you resolving it?

The presence of a few *Listeria monocytogenes* cells in processed ready-to-eat products can be a serious threat to susceptible consumers with compromised immune systems. Therefore, sensitive and specific detection of this pathogen is essential. Conventional microbiological methods take too long (2-7 days) to detect and identify pathogens in food. A biosensor-based approach presents a promising and sensitive alternative for detection of a few bacterial cells in hours instead of days.

In this project, our goal is, after initial selective enrichment, the capture and concentration of *Listeria* cells from enriched meat samples using immunomagnetic beads or other immunobeads. Biosensor-based probes will be used to detect viable *L. monocytogenes* cells. Two approaches are currently being pursued: (a) an antibody-coupled fiber optic biosensor, and (b) a cell-based sensor to measure *Listeria* interaction with animal cells.

How serious is the problem? Why does it matter?

*Listeria monocytogenes* has caused multiple recent outbreaks. The CDC estimates that there are more than 2,500 annual cases of foodborne *Listeria* infection with mortality ranging from 20 to 28 percent. An investigative report of a recent major outbreak indicated that *L. monocytogenes* at levels below one colony-forming unit per gram of meat was fatal to consumers. Therefore, the technologies must be sensitive and robust to detect such low levels of *L. monocytogenes* in foods.

What were your most significant accomplishments this past year?

Single Most Significant Accomplishment During FY 2003

A cell-based sensor was developed to detect viable pathogenic *Listeria monocytogenes* in food. An adherent RAW (macrophage) cell line and a Caco-2 cell line were used for cytotoxicity and adhesion/invasion analyses for potential use in the cell-based sensor. Also, the ability of these cell lines to attach to an interdigitated electrode chip was investigated by using fibronectin adhesion-promoting peptides (collaborators: Mark Morgan and Rashid Bashir). Data showed that these cell lines are sensitive to *L. monocytogenes*, and the cytopathic effect could be assayed in 2 to 4 hours, depending upon the initial *L. monocytogenes* concentrations on the chip. Adhesion and invasion analysis of 25 different *L. monocytogenes* strains representing 13 serotypes indicates that most of them can infect Caco-2 cells. This study indicated that RAW cells could be grown on the chip and the cell cytotoxicity could be measured by a colorimetric assay in 2 to 4 hours. This assay will be able to detect viable pathogenic *L. monocytogenes* in 2 to 4 hours.

Other Significant Accomplishments

An appropriate interdigitated electrode biochip is needed to improve the impedance reading of cell cytotoxicity induced by pathogenic *Listeria* cells. A new interdigitated electrode with a width of 50 µm and a center-to-center separation of 100 µm was fabricated on a silicon substrate with 0.6 µm thermally grown SiO₂. This would allow 70 percent of current flow through a layer 20 µm thick above the substrate, making it suitable for extracting impedance information about the whole cell.
Arun Bhunia (left) and Mark Morgan (not pictured: Rashid Bashir)

(conducted in R. Bashir’s Lab in the Computer and Electrical Engineering Department). The area-of-impedance measurement was also significantly reduced by covering the glass substrate with 0.3 µm PECVD oxide. These chips are currently being used for RAW cell growth and cytotoxicity analysis as indicated above.

We are developing a fiber-optic-based detection sensor for low levels of *L. monocytogenes* from enriched food samples. A fiber-optic biosensor prototype was developed using a polystyrene wave-guide and appropriate capture and detection antibodies. This sensor was able to detect a cell concentration of $4.3 \times 10^3$ cfu/ml of pure cultures of *L. monocytogenes* in 2.5 hours of sampling. It was able to detect *L. monocytogenes* even in a food sample or in the presence of *Listeria* species or other common foodborne microorganisms such as *E. coli*, *Enterococcus faecalis* or *Salmonella Typhimurium*. This sensor shows a great promise for rapid (24 hour) detection of *L. monocytogenes* in food.

**What major accomplishments do you anticipate over the life of the project, and what do you predict their impact will be?**

We plan to develop rapid and sensitive methods (two-step detection methods) for low levels (1-100 CFU) of *L. monocytogenes* contamination in ready-to-eat food. A two-step method was developed to detect viable cells and to reduce false-positive or false-negative results. The first step of this detection method involves capture and concentration of *Listeria* cells from food products. For that, we have developed an immunobead separation method that is capable of capturing cells from food with initial cell populations of approximately 1 to 100 CFU/100 ml in 12 to 16 hours. In the second step, the viable captured *Listeria* cells are detected by the cytotoxicity-based assay in 1 to 2 additional hours. Thus, our current two-step method could detect viable *L. monocytogenes* cells as few as <1 CFU/ml of product extract in 14 to 16 hours. In the second step of the two-step method, we have used a fiber optic immunosensor and showed that this sensor can detect *L. monocytogenes* from food. A large number of naturally contaminated, ready-to-eat meat products are currently being tested for validation. We anticipate that, once developed, this method could be used by meat processors to test for the presence of any viable, harmful *L. monocytogenes* in processed ready-to-eat products less than 24 hours before the products are sold for consumption.

**What do you expect to accomplish, year by year, for the duration of the project?**

With the first three years of support, we believe we have accomplished a major milestone, because already we can capture low numbers of viable *Listeria* cells from hotdogs or other ready-to-eat meats in 18 to 24 hours. An immunobead developed in our laboratory captures only *L. monocytogenes* and *L. innocua* cells, not other *Listeria* species. In subsequent cytotoxicity assay, only
L. monocytogenes showed positive cytotoxicity effects, making this assay a specific one for L. monocytogenes. We also have examined different selective enrichment broths that would improve resuscitation, capture, and concentration of Listeria cells from enriched food samples.

In the next phase of this project, our primary focus would be to develop biosensor kits that could be used for field trials. In the cell-based sensor, a miniature hand-held, self-contained device will be built that will have a chamber for cell growth, a sample injection port and a detection window. Initially, an optical sensor (miniature spectrophotometer) will be used to measure the cell cytotoxicity. This system will be tested with immunobead-captured Listeria cells from naturally contaminated, ready-to-eat meat products.

Science/Technology Transfer
It will be at least another year before the technology will be available for end users.

Popular Press


“Researcher aims to eradicate deadly bacteria.” The Exponent, Purdue University, July 25, 2003.

Scientific Publications


**Abstracts**


**Book Chapter**

What major problem or issue are you resolving, and how are you resolving it?

Most *Fusarium* species produce mycotoxins in cereal grains and foods, and three of those mycotoxins — fumonisins, trichothecenes, and zearalenone — are linked to potential human health problems. These mycotoxins are produced worldwide in grains growing in the fields, during storage in grain elevators, and during processing of some foods. They are difficult to destroy in grains and foods because they resist most food processing operations. However, we need to detect *Fusarium* species in grains and foods before they can grow and produce mycotoxins. Currently, there are no rapid methods for detecting *Fusarium* species.

A previous project in which we were involved was designed to develop rapid methods to detect *Fusarium* species in grains and foods by using techniques based on enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR). In the previous project, an ELISA and a PCR assay were developed that could detect *Fusarium* species as a general group. Also, a multiplex PCR was developed to detect the major species of *Fusarium* that produce three major mycotoxins: fumonisins (produced by *F. verticillioides* and *F. proliferatum*), and trichothecenes and zearalenone (produced by *F. graminearum*, *F. culmorum*, and *F. sporotrichioides*).

This project continues our previous research by using antibodies for immunocapture of the *Fusarium* species that then can be detected by multiplex real-time PCR. Our overall goals are 1) to develop a biosensor protocol based on the antibodies that were produced to capture antigens of mycotoxin-producing *Fusarium* species and 2) to combine these antibodies with the real-time PCR assay to rapidly identify *Fusarium* species.

Our specific objectives are to

- develop a method to capture the antigens of mycotoxin-producing *Fusarium* species using the antibodies produced against *F. graminearum* and *F. verticillioides*;
- develop real-time PCR assays to primers that are specific for the *Tri6* gene involved in trichothecene biosynthesis and to *Fum5*, the gene involved in fumonisin biosynthesis; and
- use this immunocapture-real-time PCR assay to detect *Fusarium* species in foods and grains.

How serious is the problem? Why does it matter?

The extent of exposure of humans to mycotoxins produced by *Fusarium* species is not fully understood, because few studies have been done on dietary intake and risk assessment. Also, the true health effects of mycotoxins produced by *Fusarium* species are not fully understood because researchers have not conclusively shown the links in humans to effects on the endocrine and immune systems; potential cancers or disruption of functions of the esophagus, liver, or kidney; and gastrointestinal pain leading to further complications. There are some published reports that low levels of *Fusarium* mycotoxins are found in many ready-to-eat foods, especially those made from barley, corn, and wheat. Although the USDA “Food Guide Pyramid,” which recommends consuming 6 to 11 servings of cereal or grain-based foods daily, is under reconsideration, there still is a recommendation for the population of the United States to consume more whole-grain foods. It is not known whether consuming these whole-grain foods will lead to greater health risks from long-term...
consumption of low levels of *Fusarium* mycotoxins, especially fumonisins, trichothecenes, and zearalenone. Since there are no rapid methods for detection of *Fusarium* species in grains or foods, it is difficult for grain-storage operators or food processors to rapidly detect *Fusarium* species and alter conditions to prevent the further production of mycotoxins. The development of a new rapid biosensor-based method for detection of mycotoxigenic *Fusarium* species will aid food processors in deciding if a specific load of grain can be used for food production.

**What were your most significant accomplishments this past year?**

*Single Most Significant Accomplishment During FY 2003*

This research was done to develop a 5’ fluorogenic real-time PCR-based assay to detect trichothecene- and fumonisin-producing *Fusarium* species and to use the assay to detect *Fusarium graminearum* and *Fusarium verticillioides* in field-collected barley. A multiplex PCR assay was developed in the laboratory of Dr. C. P. Woloshuk to detect: 1) the genus level *Fusarium* species using an internal transcribed spacer (ITS) region by aligning rDNA sequences from several *Fusarium* species, 2) trichothecene-producing *Fusarium* species using the *TRI6* genes, and 3) fumonisin-producing *Fusarium* species by using the *FUM1* genes. The real-time PCR assay was able to amplify all three products from distilled water and naturally contaminated barley in a single format with detection over four orders of magnitude of the template at 5 pg to 5 ng of genomic DNA/reaction. This multiplex real-time PCR assay, which was the first assay to detect three fungal products simultaneously, provides a basis for developing a sensitive, accurate, and differential assay to detect trichothe-
What major accomplishments do you anticipate over the life of the project, and what do you predict their impact will be?

The three primer sets that were developed to detect *Fusarium* species in grains and foods were: 1) the rDNA for the general detection of *Fusarium* species with a product size of 431 base pairs (bp), 2) the *TRI6* gene involved in trichothecene biosynthesis by *Fusarium graminearum* with a product size of 131 bp, and 3) the *FUM1* gene involved in fumonisin biosynthesis by *Fusarium verticillioides* with a product size of 183 bp. Probes were designed using labels at the 5’ ends with three reporter dyes that had different emission spectra that enable detection of the three products simultaneously in the same reaction. When DNA from 42 mold species were analyzed, only the 17 *Fusarium* strains were positive for the ITS products; the three trichothecene-producers (*F. culmorum*, *F. graminearum*, and *F. sporotrichioides*) were positive for the *TRI6* products; and the four fumonisin-producers (*F. proliferatum*, *F. subglutinans*, *F. thapsinum*, and *F. verticillioides*) were positive for the *FUM1* product. These results show that these primers were genus- and mycotoxin-group-specific. The detection limit was 5 pg DNA/reaction for *F. graminearum* and *F. verticillioides*, either individually or in mixed reactions. In addition to finding *Fusarium* in the barley, this multiplex PCR assay detected the ITS, *TRI6*, and *FUM1* products in contaminated corn and inoculated corn meal.

In corn and barley samples that tested positive by the multiplex assay, *F. graminearum* and *F. verticillioides* were isolated by traditional plating methods. Also, deoxynivalenol and/or fumonisins were detected in all but one sample, in which the molds were isolated but not enough toxin was produced. This assay could be used to detect the *Fusarium* species in either commingled samples or individual kernels. This latter detection is important because mycotoxins are usually not evenly distributed and may be in only a few kernels.

The antibodies that were produced to *F. graminearum* and *F. verticillioides* currently are being used to develop an immunocapture method for *Fusarium* species in foods. Methods were developed to capture *Fusarium* species in tubes and with immunomagnetic beads. These methods are now being optimized to determine the best conditions for the most efficient capture.

What do you expect to accomplish, year by year, for the duration of the project?

For the remainder of the project, the continuation of the immunocapture procedures and optimization will continue. Once these methods are optimized with buffer systems, research will be done with the grains and foods. The final part of the research is to combine the immunocapture with the multiplex PCR to have an Immunocapture-Real-Time PCR assay for the rapid detection of both *Fusarium* species and specific mycotoxin-producing *Fusarium* species, such as those that produce trichothecenes and fumonisins.
Science/Technology Transfer

With further research, companies that manufacture diagnostic test kits will be able to use this information. One constraint for the acceptance of this method will be to test it in an industrial food processing operation, such as the brewing industry, where the detection of these molds in raw ingredients will allow the company to make early decisions on whether the ingredients can be used in further processing. The ability to transfer this method to a commercial food processing operation will be important to show the diagnostic companies that there is market potential and that they will have customers for the rapid test developed from this research.

Presentations


Scientific Publications

Journal Articles


Thesis

What major problem or issue are you resolving, and how are you resolving it?
In recent years, there have been multiple outbreaks, product recalls, illnesses, and loss of lives resulting from the association of pathogens in processed ready-to-eat food products. Rapid identification and isolation of organisms that cause many of these problems in food — pathogenic bacteria such as *Listeria monocytogenes* and *Escherichia coli* — are imperative to reduce the health hazards to the general population. Development of selective enrichment and plating methods has significantly reduced time needed for identification, but quicker methods are highly sought after by industry.

Our objective is to develop a remote and rapid light-scattering sensory method for detection, characterization, and discrimination of strains of *Listeria* and enterohemorrhagic *E. coli*.

How serious is the problem? Why does it matter?
Problems caused by pathogens in processed, ready-to-eat food products not only put the public at risk, but are costly to companies due to the loss of production time, product recalls, and liability. The CDC estimates that there are more than 2,500 annual cases of foodborne *Listeria* infection with mortality ranging from 20 to 28 percent. *E. coli* O157:H7 causes 1.6 cases of foodborne illness per 100,000 persons each year in the United States.

What were your most significant accomplishments this past year?
**Single Most Significant Accomplishment During FY 2003**
With the knowledge and experience gained last year in preliminary experiments with our vacuum scatterometer, we have designed and fabricated another scatterometer optimized for bacterial detection. The new scatterometer uses visible light and has integrated imaging capability. It already is yielding experimental results. Both scatterometers are located in the Mechanical Engineering Micro/Nanosystems Diagnostics Laboratory at Purdue University.

**Other Significant Accomplishments**
*Listeria innocua* and *E. coli* DH5alpha were tested at different time intervals to determine if the scatterometer could detect micro colonies. In the Molecular Food Microbiology Laboratory, *Listeria innocua* and *E. coli* DH5alpha were plated at intervals ranging from two hours to twenty hours. The samples were run on the scatterometer in the Micro/Nano Diagnostic Laboratory, and light-scattering characteristics were recorded. The scatterometer appeared to detect bacterial colonies as early as 8 to 10 hours after initial inoculation. This is earlier than the human eye can detect a colony.

Light-scattering experiments were done on *Listeria innocua*, *Listeria ivanovii*, and *E. coli* DH5alpha. The colonies were illuminated and the light-scattering characteristics were recorded. Substantial experimental results also show that light scattering can be used to discriminate among bacterial colonies of different genera, and even among different species from the same genus.

We also measured some properties of agar and bacterial colonies, such as agar’s refractive index; agar surface roughness; and transmittance and
reflectance of agar, *Listeria innocua*, *Listeria ivanovii*, and *E. coli* DH5alpha. This will contribute to further research in optical scattering applications in bacteria colonies.

**What major accomplishments do you anticipate over the life of the project, and what do you predict their impact will be?**

We have developed a scatterometer that uses visible light and has integrated imaging capability to help the food industry detect pathogenic organisms before they can become a problem in food. The new scatterometer will be able to detect bacterial colonies as early as 8 to 10 hours after initial inoculation — earlier than the human eye can detect a colony.

**What do you expect to accomplish, year by year, for the duration of the project?**

**Year 2**

1. Complete construction and validation experiments with the new scatterometer.

2. Continue collection and analysis of different bacterial light-scattering characteristic and determine if the characteristics are unique to a bacterial genus and/or species.

3. Compare experimentally attained data and the numerically predicted light-scattering characteristics. From this information, the level of modeling accuracy of the light scattering codes will be determined.

4. Use light-scattering sensors to detect and characterize *Listeria* species and *E. coli* in artificially or naturally contaminated meat samples. The objective of these experiments is to determine if a viable target organism can be identified in the presence of background microflora, which may grow on the selective agar plates.

5. Determine the accuracy of the light scattering method, by using standard microbiological and molecular-biology-based (RiboPrinter) assay methods to confirm bacterial colonies.

**Scientific Publications**

What major problem or issue are you resolving, and how are you resolving it?

Pathogenic bacteria in foods cause 90 percent of reported foodborne illnesses. Of these, *Listeria monocytogenes* has emerged as one of the most important food pathogens with a “zero tolerance” in ready-to-eat processed (lunch) meats and dairy foods. This bacterium not only causes serious illness but also is also lethal in infants, people over 60, and immune-compromised individuals.

Current methods of detecting this bacterium rely upon enrichment of the numbers of bacteria present in a sample by incubating a food or food extract in special growth media for 12 to 24 hours. The resulting culture is tested for *L. monocytogenes* using analytical procedures that require an additional 3 to 24 hours to complete. The food industry includes many small food processors and producers that do not have in-house microbiological laboratories for the purpose of testing for food pathogens. Many companies, therefore, send out samples for analysis. This can add 24 hours to the time between food sampling and bacterial detection. An overall time of 2 to 3 days is typical of the time that elapses between when the food is sampled and the test results are available. The elapsed time, referred to as “time to result” or TTR, is problematic since some foods are consumed before test results would be available.

Rapid and affordable technologies to detect low numbers of *L. monocytogenes* cells directly from food, and which distinguish living from dead cells, are needed. This multi-disciplinary, multi-departmental research project is addressing the fundamental engineering and science required for development of microchip, bio-based assays that are transportable to the field, useable in a manufacturing plant environment and capable of rapidly detecting *L. monocytogenes* at the point of use. This research has the goal of microscale detection of *Listeria monocytogenes* on a real-time or near real-time basis.

The system that we are developing and bringing into testing stages concentrates 100 mL samples from a stomached hotdog into a 10 micro-liter volume in 30 min. The 10 micro-liter sample contains the cells to be processed further and interrogated for presence of *L. monocytogenes* using a microchip based systems. Several approaches are under study for processing the concentrated liquid sample. These include capture of the cells on 1 micron-sized beads and their transport into microwells for testing; capture of cells directly on antibody-derivatized fibers or on microchip surfaces followed by measurement of living cells; and use of dielectrophoretic forces to concentrate organisms captured on antibody-labeled microbeads. The rapid concentration of cells, which we call CCR (for cell concentration and recovery) supplants the more lengthy culturing techniques, and is made practical by a combination of membrane filtration techniques that enable rapid recovery of living organisms in small volumes, coupled with the small volumes of microchips that detect organisms through impedance measurements, and which require as little as 1 micro-liter to carry out an analysis in less than 3 hours.

Our multi-disciplinary research team is addressing the development, engineering and validation of such a microchip system that combines bioseparations technology for rapid concentration and recovery of microbial cells and bionanotechnology to construct postage-stamp-size devices capable of interrogating fluids for pathogens. We believe our approach will result in a technology platform capable of detecting other types of foodborne and medically relevant pathogens, although the focus of the research is on rapid detection of *Listeria*.
monocytogenes through a combination of technologies that will ultimately give a time to result of hours. The team approach of the research has resulted in pooling of resources from individual team members into single locations in a manner that maximizes the progress of the research. Research progress is summarized in the listing of accomplishments below.

How serious is the problem? Why does it matter?

The Centers for Disease Control and Prevention estimate that there are 76 million cases of foodborne illness/year in the U.S. resulting in hospitalization of 325,000 people, 5,500 deaths, and an annual cost of $7 to 23 billion. E. coli O157: H7 and Listeria monocytogenes are the pathogens of most concern. Ground meat containing E. coli O157:H7 is now considered to be an adulterated food while Listeria monocytogenes has emerged as one of the most important food pathogens with a “zero tolerance” for it in ready-to-eat processed (lunch) meats and dairy foods. Consumption of contaminated food may cause meningitis, encephalitis, liver abscess, headache, fever and gastroenteritis (diarrhea) in immunologically challenged individuals and abortion in pregnant women. Early and rapid detection of pathogenic bacteria in foods is a necessary condition for preventing the food from reaching the consumer, being eaten, and causing illness. A rapid and facile detection system that can be used by scientists and non-scientists alike is needed to ensure a safe food supply. Our research targets the pathogenic bacterium Listeria monocytogenes.

The genus Listeria is comprised of six species, L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri and L. grayi. However, only L. monocytogenes is harmful to humans. L. monocytogenes is ubiquitous in nature and can be found in meat, poultry, seafoods and vegetables. Occurrence of this organism in food could be as high as 32 percent. In a food sample, L. monocytogenes is often present in close association with other nonpathogenic Listeria species, thereby complicating the specific detection procedures.

Industry needs biochips that are affordable, capable of rapid detection of food pathogens, and easy to use by small food processors as well as major food companies. Achieving such devices requires research on methods for:

- rapidly concentrating and recovering microorganisms from food samples for subsequent interrogation of pathogens;
- sampling and conditioning fluids that contain the cells while maintaining their information content (i.e., the molecules or cells that represent possible targets of the chip);
- transporting sample fluids on and/or off the chip so that target microbes are captured and retained so that they can be probed for the presence of pathogens;
- interfacing biological molecules (i.e., biomolecules) with electronic components;
Research in these areas will contribute to improved food safety through better diagnostic technology for food pathogens. It will also contribute to the fundamental knowledge base and a technology platform that will help us use this approach to detect multiple foodborne pathogens and other targets. The research during fiscal year 2003 addressed all of these areas, with focus on measuring the concentration of the microbial cells in the sample (both on the chip and off the chip) and the design of a revised microchip format that couples cell capture with electronic measurement.

What were your most significant accomplishments this past year?

**Single Most Significant Accomplishment During FY 2003**

**Cell Concentration and Recovery.** Rapid concentration and recovery of cells from foods is needed to reduce the time for sample preparation from the current 1 to 3 days to less than an hour. A systematic study of membrane separations that not only concentrates cells, but also enables their recovery in sub-microliter volumes was carried out as a collaborative effort between laboratories in LORRE (Ladisch), Food Science (Bhunia), ECE (Bashir), and BME (Robinson). In 30 minutes, we increased concentration of *E. coli* and wild-type *L. monocytogenes* from an initial 20 to 30 cells per mL in 100 mL hotdog meat broth to 20,000 cells/mL. Rapid concentration and recovery methods will supplant the more time consuming, one to three day cell enrichment methods. The new methods use membrane-based filtration that is much faster. This method also results in a sample volume compatible with the nanoliter/micron-sized channels that make up the microfluidic-based pathogen detection system.

**Other Significant Accomplishments**

**On-Chip Cell Capture.** The processing of fluid that contained microbial cells recovered from 100 mL and was concentrated into 10 μL required that cells be retained on the chip for further measurement. This work was done in ECE (Bashir) in collaboration with Food Science (Bhunia), BME (Robinson), and LORRE (Ladisch). The application of a small electrical force field through electrodes (dimensions 3 to 5 microns) captured and retained cells on the chip and prevented cells from being washed away by fluid flow. The capture of cells in biochips with wells whose volume is 50 nanoliters (1000 nanoliters = 1 microliter) is essential if electronic means of probing the cells for pathogens is to be successful.

**Food Pathogen Biomarker Response to Processing and Storage Conditions.** Antibodies that select pathogenic microbes by binding with proteins displayed on the surface of those microbes, but absent from surfaces of innocuous bacteria, are
ineffective when these proteins are missing. Research on the stealthiness of *L. monocytogenes* has been led by Food Science (Bhunia) in cooperation with BME (Robinson), ECE (Bashir) and LORRE (Ladisch). *L. monocytogenes* that is exposed to high temperature or low pH has been found to temporarily lose its distinctive biomarkers. However, the biomarkers return when the organism is cultured. Consequently, multiple antibodies may be necessary to increase probability of the organism’s detection, independent of its history.

**Imaging of Fluorescent Cells.** Rapid validation of the presence, capture, or analysis of cells requires fluorescent microscopy, since culture techniques require about a day’s time to indicate the development of visible, colony-forming units. Microscopy of fluorescently labeled *E. coli* or *Listeria* spp. is being carried out in BME (Robinson) in cooperation with laboratories in Food Science (Bhunia), ECE (Bashir) and LORRE (Ladisch) who have provided various samples and experimental conditions where visual confirmation at a micron scale is an important part of the research. Careful microscopy coupled with microbiological techniques has shown that derivatization of bacteria using fluorescein dyes enables them to be detected without killing the cells. This result is central to rapid development of cell recovery, pathogen detection, and integration of biological with electronic and software components being pursued on a topical basis, the integration of these components into a functioning system for pathogen detection is the ultimate metric of success. Overall systems integration for cell concentration and recovery is being led by LORRE/ABE (Ladisch) and for the microchip by ECE (Bashir) in close cooperation with Food Science (Bhunia) and BME (Robinson). Engineering achievements in this part of the project are the development of cell concentration and recovery kits, and packaging of the accompanying detection system into a transportable form. While subsystems integration is a work-in-progress, the first steps are underway toward the ultimate goal of achieving an on-site detection system for food pathogens.
What major accomplishments do you anticipate over the life of the project, and what do you predict their impact will be?

The project’s goals are to contribute to the fundamental scientific and engineering knowledge base for rapid detection of microbial pathogens, as well as to deliver a system that can be used in the food processing industry for rapid detection of *L. monocytogenes*. Significant progress has resulted from fundamental studies on the microbiology of *L. monocytogenes*, the behavior of microorganisms at inorganic and organic interfaces, design and fabrication of micro-fluidic chips for detection and quantitation of bacteria, and processing of chemically complex and biologically heterogeneous meat samples. Fundamental advances and basic research findings have been submitted to and published in refereed journals. They also have been presented as papers at national professional society meetings, seminars at universities, and cooperative research meetings.

FY 2003 has resulted in integration of the fundamental science into prototype devices, with plans for alpha-site testing of selected components for detection of *L. monocytogenes* by the end of FY 2003. The predicted impact is development of a practical micro-chip-based method for detection of *Listeria* for β-site testing by the end of FY 2004. The availability of such a test will contribute to rapid testing for food pathogens so that the time-to-result will be measured in a matter of hours. This will enable corrective actions at the food processing site to be taken more quickly and will help to ensure food safety. The microchip technology that results from this project will also provide a platform for development of protein-based chips that can be used to detect other types of pathogens in other food applications.

What do you expect to accomplish, year by year, for the duration of the project?

The goal of this project is to engineer a biosystem that can rapidly detect *Listeria monocytogenes* and other pathogens in foods. The biosystem being developed by our multi-disciplinary team combines protein chemistry, rapid cell concentration, and recovery with a microchip. The result is anticipated to be a postage-stamp-size biosensor that will detect the presence of *Listeria monocytogenes* in a matter of minutes. This biosystem could be coupled to a transportable device capable of reading and interpreting the signal from the chip, and thereby facilitate rapid, in-plant detection of a pathogenic organism. Such a configuration will enable rapid transmission of the results to a computer and ultimately through the Internet to remote locations, as needed.

This biosystem is based on the concept of binding a protein (antibody) on electrically conductive surfaces placed on a non-conducting surface (such as silicon dioxide or plastic) of a microchip. The protein (antibody) will selectively bind with the pathogenic organism, if present, in a liquid sample passed over the chip. Microchip users will know when a pathogenic organism is present, because the electronic signature will change when the protein (antibody) on the surface of the chip, or on beads transported into the chip binds with its antigen (a protein on the surface of the cell). Electronic detection is intended to supplant the more expensive optical methods that are currently available and in use (including in our laboratories).

The biosystem will incorporate bioseparations technology to rapidly concentrate and recover microorganisms from food samples while avoiding...
time-consuming culturing techniques. The quantities of sample to be processed are large, since the cells are at low concentration in the foods. However, the volume processed on-chip is small, since the volume of the channels on the chip is on the order of 100 to 500 nanoliters. Hence, the cell concentration must be in the range of 10 to 100 cells per microliter to enable sufficient cells to be transported through the chip at flow rates of microliters/min., for detection purposes. Hence, rapid concentration of the cells in a large volume (about 100 mL) into a very small volume (about 10 μL) compatible with the biochip is needed.

Objectives of the proposed four-year, USDA-Purdue University cooperative project are to

- characterize binding of polyclonal and monoclonal antibodies with *Listeria monocytogenes* cells at silicon dioxide/platinum surfaces, beads and/or membrane surfaces that will be representative of microchip-based sensing systems;

- carry out fundamental research on detecting the binding and state of cells (living or dead) using electrically based sensing systems on a microchip; and

- develop methods for sampling, cell concentration, and sample delivery for a microchip-based system.

The significant results from FY 03 set the stage for a focused effort on integrating the various components into a functioning system for evaluation at USDA laboratories as well as at Purdue. The aims for the coming year will focus on fielding an integrated system that interrogates microliter and sub-microliter samples for the presence of *Listeria monocytogenes* and whose design can be extended to test other microbial pathogens in food samples. The system’s performance will be gauged based on accuracy and the ability to complete the test in less than 4 hours, as well as its capability to perform rapid cell concentration, recovery, fractionation of pathogenic microbes from other non-pathogenic ones, and detection in a contiguous series of operations. The goal will also be to engineer and deliver a complete system suitable for evaluation at USDA and in an industrial setting, if possible. Specific fundamental research aims for FY 04 are to:

1. Carry out the necessary engineering to integrate the individual functioning components into a single operational system;

2. Carry out, concurrently, fundamental multidisciplinary research on:

   a. Engineering media designed to support and promote growth of viable organisms in order to increase pathogen population after the sample is placed on the biochip.

   b. Improving avidity and selectivity of antibodies designed to capture the target organism.

   c. Utilizing transformed *E. coli* and *L. monocytogenes* that express either green or red fluorescent protein as test organisms in order to speed development and evaluation of improved capture methods. A green fluorescent protein *E. coli*, cloned in our laboratory, was instrumental in accelerating the pace of validation of cell concentration and recovery, and development of a test kit, which is now being evaluated.

   d. Redesigning and fabricating microchips that combine on-board capture and detection of pathogens, and integrate dielectrophoretic methods of cell capture, and impedance measurements for detection.
e. Rapidly prototyping various configurations and chip modifications in order to improve performance of system components as needed, based on results of evaluation of the system by USDA, industry, and Purdue.

f. Automating cell concentration and recovery (abbreviated CCR) as appropriate, in a manner that will integrate practical considerations for improvements as indicated by evaluation with various food samples. If the evaluations prove successful, integration of CCR with the detection system will need to be addressed next. Integrate CCR with detection system.

g. Enhancing imaging capabilities so that rapid validation of bacterial counts, bacterial capture, and microfluidic properties can be carried out. This validation effort is a key to rapidly integrating and improving the various system components, and is tied to aim 2(c), since the availability of green and red fluorescent microbes will enable sorting, counting, and imaging to be done quickly using the microscopes and cytometry instruments available in the imaging facility.

3. Develop components of the detection system to form a technology platform that can be extended to detection of other individual or multiple pathogens. The focus of FY 04 will be on *Listeria monocytogenes*. However, we expect the fundamental research will yield knowledge that applies to other pathogens that impact food safety. In FY 05 and afterward, components of the chip will be structured to allow transition to other pathogens, and to allow transition of the biosystem to different foods and agricultural products in the food chain.

Science/Technology Transfer

The science and technology has been licensed to a start-up company, Biovitesse, of which Rashid Bashir and Michael Ladisch are two of the co-founders. The third co-founder is Dr. Laila Razouk, who is CEO, and who has led development of a business plan.

**When is the science and/or technology likely to become available to the end-user (industry, farmer, other scientists)?**

The end-user is intended to be industry, where there are unmet needs for rapid methods for detection of pathogens. The goal is to make technology available that combines rapid cell concentration and recovery with microfluidic biochip detection.

**What are the constraints, if known, to the adoption and durability of the technology products?**

Biovitesse is examining the needs for “productizing” the biochip technology. Specific technical constraints imposed by potential users are being identified and market needs analyzed. One constraint is lack of resources needed for fielding the first prototype systems for β-site testing. This is being addressed by the CEO, as she attempts to raise funds for commercializing this technology.

Popular Articles

Preventing and Detecting Food Contamination: Purdue University’s Center for Food Safety Engineering, *IIE Solutions*, December 1, 2002.

Biochips that are affordable, capable of rapid detection of food pathogens, and easy to use by small food processors as well as major food companies are needed.


**Journals and Proceedings**


**Posters**


A rapid and facile detection system that can be used by scientists and non-scientists alike is needed to ensure a safe food supply.


Abstracts (major papers presented)


**Invited Lectures and Seminars**


What major problem or issue are you resolving, and how are you resolving it?

To keep the food supply safe, food production, processing, and retail establishments must be able to identify and then get rid of microbial foodborne contaminants such as pathogenic *Salmonella*, *Campylobacter jejuni*, and *Escherichia coli* O157:H7. The CDC estimates that annual foodborne-related outbreaks result in 76 million cases of illness, 325,000 hospitalizations, and 5,000 deaths (Mead et al, 2000).

Conventional detection methods take at least 24 to 48 hours to differentiate and identify microorganisms; therefore, measures taken to counteract food contamination must wait at least that long. In order to facilitate timely intervention measures, the food industry needs more rapid detection methods and a sensor able to accurately and rapidly identify low levels of microbial foodborne contaminants within food systems or cultural media. We are investigating the efficacy of infrared (IR) technology as a means of rapid detection of select bacterial pathogens. To accomplish this goal, we plan to:

- create a library of Fourier-transform infrared (FT-IR) spectra of bacterial cell wall components and whole cells needed for pathogen identification and differentiation;
- develop FT-IR methods for identification and quantification of these pathogens from water, cultural media, and select foods. This will include standardizing sampling procedures, quantification methods, spectral analysis procedures, and developing an overall chemometric approach for the analysis of FT-IR data; and
- design an IR sensor based on the most promising few-wavelength algorithms developed using FT-IR data generated from research activities in the first two objectives.

What was your most significant accomplishment this past year?

The most significant accomplishment in the early stages of our project has been development of a preliminary experimental approach using FT-IR bench-top equipment that is appropriate for later use in a portable, rapid detection instrument. The method has been successful at monitoring growth of bacteria over time in cultural media, and work on defining

How serious is the problem? Why does it matter?

The estimated cost from illness, hospitalization, and lost productivity due to *Salmonella*, *Campylobacter* is $4.3 billion annually in the United States (CDC, 2001). Two to four million cases of salmonellosis occur annually in the United States, and cases have been linked to raw meats, poultry, eggs, milk and dairy products, sauces, and salad dressing (FDA, 2002c). Campylobacteriosis is the most common bacterial diarrheal illness in the United States, and outbreaks have been associated with unchlorinated water, under-pasteurized and raw milk, and undercooked chicken (FDA, 2002a). Outbreaks of *E. coli* O157:H7 have been associated with unpasteurized fruit juices, raw milk, and undercooked ground beef (FDA, 2002b). The ability to rapidly and accurately monitor the food supply (at processing and/or retail facilities) for contamination by these pathogens could likely reduce both the number of associated outbreaks and the impact of recalls, lost product, and law suits in the food industry.

Researchers in Mauer’s lab find that when they pass an infrared laser through samples of pathogenic bacteria, each has a unique spectrum which can be used to quickly identify the bacteria.
detection limits is underway. Further development of this approach will allow for the design of a sensor that can be used in a production or retail facility to characterize a food sample as contaminated or free of select pathogenic bacteria in less time than current methods for detection.

In our work to develop an infrared sensor that can be used for rapid detection of select pathogenic bacteria, bacterial and cell wall component samples have been prepared by Dr. Maribeth Cousin and Dr. Brad Reuhs in the Department of Food Science at Purdue University and by Dr. Jean Guard-Petter at USDA-ARS Southeast Poultry Research Laboratory. Also, spectra of a combination of three different microorganisms, select foods, and foods contaminated with bacteria have been measured and analyzed using a Thermo-Nicolet Nexus 670 mid-infrared FT-IR spectrometer and Continuum IR microscope in Dr. Lisa Mauer’s laboratory in the Food Science building at Purdue and in Dr. Jay Gore’s Mid-IR Consortium laboratory in the Mechanical Engineering building at Purdue.

What major accomplishments do you anticipate over the life of the project, and what do you predict their impact will be?

We hope to develop an infrared (IR) sensor able to accurately and rapidly identify low levels of certain microbial foodborne contaminants within food systems or cultural media. If we are successful, such a sensor would decrease the time needed for microbial detection.

What do you expect to accomplish, year by year, for the duration of the project?

**Year 1**

1. Create a library of FT-IR spectra of bacterial cell wall components and whole cells needed for cell identification and differentiation.
2. Develop FT-IR methods for cell identification and quantification in water, cultural media, and foods.

**Year 2**

1. Continue creation of spectral library and development of FT-IR detection methods initiated in year one.
2. Build and validate an IR sensor based on the most promising few-wavelength algorithm developed using FT-IR techniques in year one.

**Science/Technology Transfer**

Although the limited wavelength infrared sensor shows great potential, this technology will likely not be available for use in industry until after the project is completed in 2005.

**References**


Rapid Detection of PCBs and Toxicity Equivalence Quotient (TEQ) in Fish Tissue from Indiana Waters and Use of a Novel Device to Predict Contaminant Load in Fish

Investigator: Charles R. Santerre

What major problem or issue are you resolving, and how are you resolving it?

The cost to develop fish consumption advisories is prohibitive for health departments. In the past 10 years the State of Indiana has tested fewer than 700 carp and 200 largemouth bass samples. Other species were analyzed even less frequently and all of these fish were collected over a wide geographical area. We are testing new technologies that will reduce or eliminate the need to directly sample fish for determination of residue levels. As a part of this effort, we are developing lower-cost, more rapid methods for measuring contaminants (PCBs and mercury). By reducing the cost for analysis, federal and state agencies will have the ability to collect and analyze a greater number of samples and improve the accuracy of their advisories or surveys.

We also need to inform anglers and their families of the importance of fish consumption advisories and to encourage greater compliance. It has been estimated that 38% of anglers do not do so in Indiana. This extrapolates to a possible 10% of the population, or around 600,000 people, that may be exposed to high levels of PCBs and mercury because they do not follow the advisory.

Our overall objective is to protect people from exposure to harmful chemical contaminants in fish and fish oil supplements. Specifically, we are developing rapid methods to measure total PCBs and mercury in fish tissue so that health departments can conduct more complete risk assessments related to chemical contaminants in sportfish and commercial fish. To achieve this objective, our goals are to

- finalize the validation of a lower cost, rapid assay for the measurement of PCBs in fish tissue that could be used by states when developing fish consumption advisories. This assay would allow states to analyze more fish samples and develop better advisories.
- measure the levels of PCBs and omega-3 fatty acids in fish oil dietary supplements using the ELISA assay. Since consumers are turning more to dietary supplements, we wanted to assess the exposure to PCBs when taking a fish oil supplement.
- compare mercury residues in fish tissue measured by a standard method to residues measured using a new analytical instrument. Improving the throughput and lowering the cost for mercury testing can help in the development of fish consumption advisories and improve oversight of commercial fish that may contain high levels of mercury.

How serious is the problem? Why does it matter?

PCBs are highly toxic and production was banned by the United States in 1977. PCBs are also “reasonably anticipated to be human carcinogens” according to the 1998 summary report on carcinogens (NTP 1998). The major route of exposure to PCBs and mercury is through fish consumption. Currently across the United States, 679 fish consumption advisories have been issued by agencies in 35 states due to the presence of PCBs in wild fish. The Environmental Protection Agency (EPA, 1997) estimates that 43 States have issued sportfish consumption advisories due to the presence of mercury. In addition, the FDA has issued an advisory due to the presence of mercury in selected commercial fish species (i.e., tilefish, swordfish, king mackerel and shark).

Yet, the omega-3 fatty acids in fish are good for us, so we need to know which fish to avoid to reduce our exposure to PCBs and mercury and which to eat to get good fats in our diets.
The populations that are most at risk from PCBs are developing fetuses and nursing infants that can receive PCBs from their mothers through the placenta or her breast milk. A meal of contaminated fish consumed by the mother can result in a larger dose of contaminants being passed to the fetus or infant. PCBs are believed to alter reproductive function and to delay neurobehavioral and physical development in perinatal and school-aged children. It has been estimated that a two- to eight-point reduction in IQ results from in-utero exposure to PCBs. In addition, PCBs are believed to adversely affect the liver, thyroid, and immune systems, and to increase cancer risk from non-Hodgkin’s lymphoma.

What were the most significant accomplishments this past year?

Single Most Significant Accomplishment During FY 2003

Our research group validated the extraction and cleanup methodology prior to analysis with a commercial ELISA kit for the measurement of PCBs in fish tissue. This technique reduces analysis time, produces less solvent waste, and can be performed at a lower cost than the conventional GC/ECD method.

PCBs in fish tissue were analyzed using the ELISA. Standard curves for Aroclor 1248, 1254 and 1260 were obtained by spiking catfish tissue samples in the ranges of 0.05 to 0.5 ppm and 0.5 to 5.0 ppm. A sulfuric acid/silica gel column was used for the extraction and cleanup of the fish tissue sample. Thereafter, forty wild fish samples (0.05 to 0.5 ppm total PCBs) and twelve wild fish samples (0.5 to 5.0 ppm total PCBs) were obtained. The concentration of PCB residues obtained from the ELISA were not significantly different (p=0.05) when compared with the residues obtained using GC/ECD.

The analysis was carried out at the Department of Foods and Nutrition, Purdue University, West Lafayette, IN 47906. Other collaborators included J.L. Zajicek and D.E. Tillit from U.S. Geological Survey, Columbia Environmental Research Center, Columbia, MO 65201 and D.C. Deardoff of Strategic Diagnostics Inc., 111 Pencader Drive, Newark, DE 19702.

Other Significant Accomplishments

Our research group measured PCB residues and fatty acids in 26 omega-3 dietary supplements. We tested for mercury in 216 cans/pouches of tuna, 16 cans of canned mackerel, 16 cans of canned/pouched salmon, and 180 fish sandwiches from six retail chains. Our group also compared mercury residues in fish tissue measured by a standard method to residues measured using a new analytical instrument.

Polychlorinated biphenyls (PCBs) in fish tissue were analyzed using enzyme-linked immunosorbent assay (ELISA) and gas chromatography/electron capture detector (GC/ECD) methods. Fish tissue samples were collected in 2000-2001 during an Indiana fish survey. Extracts derived from fish tissue samples were analyzed by the GC/ECD. The extracts and fish tissue samples were thereafter divided into two groups such that they fell into either one of two ranges 0.05 to 0.5 ppm total.
We anticipate that the impact that our project could have is that the fish consumption advisories could be built on the analysis of larger number of fish samples. Thus, advisories would be more accurate and helpful to anglers and their families.

PCB and 0.5 to 5.0 ppm total PCB. The extracts were exchanged into methanol and ELISA diluent and analyzed by ELISA. Fish tissue samples were analyzed by ELISA after a sulfuric acid/silica gel extraction and clean-up. The data obtained in the two ranges from the analysis of extracts and fish tissue by ELISA were compared with the GC/ECD data. From 0.05 to 0.5 ppm range (n=40), ELISA analysis of fish tissue was as precise as the GC/ECD, but they were significantly different from the Soxhlet’s extracts (p<0.05). However, from 0.5 to 5.0 ppm range (n=12), the results obtained from the GC/ECD of fish tissue and ELISA analysis of the extracts were not significantly different, but were found to be different from the ELISA of fish tissue (p<0.05).

Our results showed that the fatty acids EPA and DHA ranged from 51-92% and 61-97% of the amounts stated on the product labels in the 24 fish-derived supplements, respectively. The algal oil supplements contained only DHA which was measured at 128% and 162% of the stated label concentrations. Our group also found that if a consumer takes the amount of supplement that is recommended on the label (up to 6 capsules per day) that they may receive up to 40% of the RfD for intake of PCB. The RfD is the maximum amount that a person should receive in a single day. Receiving a dose that is higher than the RfD increases the risk for cancer. However, if the person only takes enough of the supplements (generally 1 capsule per day) to reach the National Academy of Sciences’ Adequate Intake, then the exposure to PCBs is less than 12% of the RfD. Our research demonstrates that individuals who take an omega-3 dietary supplement as recommended on product labels are likely getting adequate intakes of the long-chain omega-3 fatty acids, but may also be increasing their risk due to increased exposure to PCBs.

We found excellent correlation between mercury residue data collected using the new analytical instrument when compared to data collected using a standard method. It was also faster. From a ground fish sample, we could obtain a value in 6 minutes. This is much more desirable than the current method which requires sample digestion prior to analysis.

Our results indicate that some canned tuna products are very low in mercury while others have moderate levels. Since tuna is the second most popular fish in the U.S. and since one woman out of every 12 has elevated levels of mercury in her blood, it is likely that tuna is the food that contributes the most mercury in the diet. Tuna is served in the School Lunch Program and is available through the Food Stamp Program. Our research also demonstrated that retail fish sandwiches have lower levels of mercury than one might expect. Concentrations of mercury ranged from 48 to 340 ppb in canned tuna; 50 to 61 ppb in canned mackerel; and 20 to 70 ppb in canned salmon. Mercury in retail fish sandwiches ranged from 5 to 132 ppb.

What major accomplishments do you anticipate over the life of the project, and what do you predict their impact will be?

This project has shown that it is possible to simplify, reduce the cost, and increase the throughput when analyzing PCBs and mercury in fish tissue that is analyzed for fish consumption advisories or for monitoring fish entering into interstate trade. This technique simplifies clean up and extraction, which makes the ELISA more time-efficient and economical. It also produces less waste than the conventional GC/ECD method.

The overall impact is that the fish consumption advisories could be built on the analysis of a larger number of fish samples. Thus, advisories would
be more accurate and helpful to anglers and their families. It will better protect consumers’ health. In addition, it would help to restrict fish that has elevated levels of contaminants from entering the marketplace.

After finding that some tuna products are moderately high in mercury, our group also has proposed that FDA develop a ‘kid-safe’ label that can be used on canned tuna products shown to have low levels of mercury.

**What do you expect to accomplish, year by year, over the duration of the project?**

The objective of the project is to show that the ELISA is a feasible method to replace the GC/ECD method for the analysis of total PCB in fish tissue samples.

For the remainder of the project, we are attempting to use statistical reduction of data collected by state agencies over the past decade to build regression equations for predicting PCB residues in fish tissue. We have had some success developing a regression equation to predict PCBs in carp.

We are attempting to determine whether a total PCB measurement correlates with toxicity. Since PCBs in fish include a possible 209 congeners, reporting of total PCB concentration may not be predictive of toxicology, since current estimates suggest that toxicity for the congeners may vary by a factor of 5. Thus, we are statistically analyzing EPA fish tissue residue data to see if a total PCB correlates with a standardized toxicity index.

As a part of this research, we are attempting to improve the process by which fish consumption advisories are developed by statistically analyzing data with regression equations to predict residue levels. We hope that a simplified advisory will increase angler compliance and reduce exposure to mercury and PCBs in the diet.

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**Science/Technology Transfer**

In addition to technical presentations, we have developed a Web site to inform anglers and their families of the Indiana fish consumption advisories. The Web site provides information on fishing locations that may have contaminated fish, the safety of farm-raised fish, the proper preparation techniques for reducing contaminants, the nutritional value of fish and information on the dangers of consuming fish that are contaminated with PCBs or mercury. In addition, we have had several press releases to reach out to consumers in Indiana and nationally. (http://fn.cfs.purdue.edu/anglingindiana/)

We are working closely with staff at the Indiana State Department of Health and the Indiana Department of Environmental Management. We hope to demonstrate to these agencies the benefits of using rapid assays in the development of fish consumption advisories.

**Popular Press**

Dr. Santerre has shared scientific information on seafood safety in the Philippines, Holland, Canada, Honduras, and Norway. Using media contacts and press releases he has informed national and state audiences of issues surrounding food safety and nutrition. These media sources reach virtually every American and Canadian resident. Press releases involving his efforts have been circulated
We are testing new technologies that will reduce or eliminate the need for direct sampling of fish for determination of residue levels, and we are developing new methods for measuring contaminants at a lower cost and with a rapid throughput.

by the Associated Press, United Press International, Reuters and Scripps Howard News Service and highlighted in stories in the following media outlets:

- **Newspapers** - USA Today; Wall Street Journal; Chicago Tribune; Newsday; The New York Times; The Oregonian; The Detroit News; The Indianapolis Star; Hartford (Conn.) Courant; Pittsburgh Post Gazette; The Columbian (Portland, Ore.); Toledo Blade; The Muncie Star Press; The Globe and Mail (Canada); La Presse (Canada); The National Post (Canada); The New Brunswick (Canada) Telegraph Journal; and The Purdue Exponent

- **Magazines** - Reader’s Digest; Popular Science; Scientific American; Good Housekeeping; Fitness; Business Week; Health; Shape; Self; Glamour; Allure; Oxygen; Men’s Health; Progressive Farmer; Bottom Line Personal; Environment News Service; Environmental Nutrition; Food Quality; Hatchery International and Northern Aquaculture Magazine; and IntraFish

- **Web** - CBS Health Watch; Health Scout News Service; and Food Chemical News

- **Radio** – WGN Radio & Extension 720 with Milt Rosenberg; WFYI Sound Medicine; CKNW AM980 Rafe Mair Show; Great Lakes Radio Consortium; WBEZ-FM in Chicago; USA Radio; WAFK Radio; WBAA Radio in West Lafayette; and WFHB Radio in Bloomington

- **Television** - Fox News (Chicago); CBS News; KABC News in Los Angeles; WTOP Radio; WPEC-TV CBS in West Palm Beach, Fla.; and WLFI in West Lafayette.

On the same day that we were cited on a front-page of USA Today calling for more FDA action to protect at-risk populations, the FDA announced that it would reconvene its advisory committee to review the existing fish consumption advisory.

**Presentations**


Shim, S.M., Santerre, C.R., Dorworth, L.E., Miller, B.K., Stahl, J.R. , and Deardorff, D.C., 2003. Semipermeable Membrane Devices (SPMDs) to Predict Total PCB in Fish Tissue. ESEI (The Environmental Sciences and Engineering Institute), Environmental Symposium, April 11. Lafayette


**Scientific Publications**


Biosensor Processes for Detecting Pathogenic Bacteria in Foods

Eastern Regional Research Center, Wyndmoor, Pennsylvania

Investigators: S. Tu, J.D. Brewster, P.L. Irwin, A.G. Gehring

1. What major problem or issue is being resolved and how are you resolving it?

The presence of food-borne pathogenic bacteria must be quickly determined at every stage of food production, processing, and distribution in order to achieve proper treatment before consumption. Farmers, processors, retailers, and researchers need rapid, sensitive tests to detect pathogens in foods. Effective screening procedures must meet a number of criteria. Speed is desired since modern processing and distribution systems operate rapidly. High sensitivity is required since an infectious dose may be only one organism. Lastly, selectivity is essential because pathogenic bacteria comprise only a small fraction of an otherwise benign population of microorganisms. Because the time and skills required for traditional microbiological protocols limit their general adoption, alternatives such as biosensor-based processes should be developed.

Pathogen detection methods used by food producers, processors, retailers and regulatory agencies vary widely because of differences in their practices and due to their wide-ranging economic circumstances. Until universally accepted approaches are found, research is needed to develop new, as well as modify existing, methods to meet the needs of our customers. Thus, we seek to develop and improve processes to separate and concentrate pathogens obtained from food, to enhance detection procedures using existing equipment, and to conceive simple, inexpensive detection techniques for potential adoption by stakeholders.

2. How serious is the problem? Why does it matter?

Foodborne illnesses caused by pathogenic microorganisms pose a threat to public health. Over 5 million cases of foodborne bacterial diseases occur annually in the U.S. The economic impact of foodborne illnesses is significant in that time lost from work, medical care, and the costs of recalling and destroying contaminated products amount to billions of dollars a year. Traditional microbiological methods require days to detect pathogenic bacteria such as E. coli O157:H7, Campylobacter, Salmonella, Listeria monocytogenes, etc. in foods. New approaches must be developed to allow detection of low levels of specific pathogenic bacteria within a standard 8-hour shift.

3. What were the most significant accomplishments this past year?

A. Single Most Significant Accomplishment during FY 2003 (one per Research Project).

Lacking of high quality antibody for many important pathogens hinders the potential applications of highly sensitive biosensor methods to detect pathogenic Listeria monocytogenes in foods. To overcome this barrier, scientists at ERRC have developed genetic engineering approach to generate antibody-like proteins (scFv) from virus-infected bacteria. The efforts resulted in the production of a scFv highly specific only to pathogenic strains of Listeria monocytogenes but not other varieties of Listeria. The availability of this scFv will significantly improve the applicability of many rapid and sensitive biosensor methods to the detection of Listeria monocytogenes in foods and the developed genetic engineering approach may be extended to the production of antibodies against other difficult to detect pathogens.

B. Other Significant Accomplishments during FY 2003.

1. Recently, raw alfalfa sprouts have emerged as a recognized source of foodborne illness caused by Salmonella and E. coli O157 outbreaks. To minimize the possibility of having the similar outbreaks in the future, scientists at ERRC have developed a new approach including the use of magnetic immuno-beads to capture and concentrate targeted bacteria and the application of time-resolved fluorescence for bacteria detection. With this approach, E. coli O157:H7 and Salmonella in the sprouts germinated from seeds contaminated by as low as 4 CFU of the
CFSE Research Reports

4. Describe the major accomplishments over the life of the project, including their predicted or actual impact.

To lower the high costs associated with biosensor detection methods, there is a need to develop procedures capable of simultaneous detection of multiple pathogens. Taking the advantage of easily differentiated time-resolved fluorescence properties among lanthanide metals, researchers at the Eastern Regional Research Center in collaboration with PerkinElmer Life Science, utilized specific antibodies conjugated with europium (Eu) and samarium (Sm) to label \textit{E. coli} O157:H7 and \textit{Salmonella Typhimurium} captured by proper immunomagnetic beads. The quantities of each pathogen were then simultaneously determined from the fluorescence associated with Eu and Sm, respectively. With this approach, 1 cfu of each pathogen per gram of ground meats (beef, turkey and pork) can be detected after a 4.5-hour enrichment. Our collaborator, PerkinElmer Life Science, Inc, has evaluated the potential of this methodology for commercial applications.

The immuno-magnetic bead (IMB) capture and concentration of non-target species interferes with the sensitive detection of the target pathogen. Nu-
merous chemical substances were tested as potential blocking agents against the non-specific capture of background microflora in the detection of *Salmonella* or *E. coli* O157:H7. Successful blocking buffer was developed for a particularly problematic *E. coli* K12-type isolates and was further studied from the standpoint of its mechanism of action. The successful development of an effective blocking buffer could greatly improve the impact of IMB utilization.

There is a need for developing rapid methods with lower detection limits for the assessment of pathogenic microorganisms in foods. The formerly cost-prohibitive detection technique, chemiluminescence, has recently become affordable and portable. Chemiluminescent detection holds promise for increasing the sensitivity of a variety of rapid assay formats. ARS scientists at ERRC and their industrial collaborator have specifically developed a rapid, sandwich enzyme-immunoassay with chemiluminescent detection for the pathogenic bacteria, *E. coli* O157:H7. This assay may be used by food manufacturers, clinical labs, food distributors, consumers, etc. for determining the presence of potentially life-threatening bacteria in foods.

Detection of low levels of pathogens requires either a lengthy pre-enrichment step or a means of isolating and concentrating the target organism from a large volume of food sample. Research at ERRC led to significant improvements in micro-immunoadfinity columns for isolation/concentration of *E. coli* O157:H7. Using a new linkage method, stable, reproducible columns were prepared and studied to determine optimum operating conditions. This approach will allow detection of *E. coli* O157:H7 without prior enrichment, reducing detection time to less than 4 hours.

5. **What do you expect to accomplish, year by year, over the next 3 years?**

The current project will expire in 2005. Thus, we will have to start to develop new research plan for the future beyond 2005. For objectives of current project, we intend to accomplish the following:

1. to develop IMB capture model and effective blocking reagents to minimize non-specific binding of non-target bacteria with a milestone as: Year 1, starting the studies on the selection of blocking agents to minimize cross reactivity, and Year-2, to complete the cross reactivity research.
2. to develop effective non-culture enrichment methods to increase pathogen concentration with a milestone as: Year 1 and 2, to characterize interactions between pathogens and immunomagnetic beads (IMB) and to establish conditions for effective applications of IMB for pathogen capture and concentration in food matrix.
3. to develop effective biosensor processes for pathogen detection with a milestone as: Year 1 and 2, to continue the optimization of conditions for the applications of time–resolved fluorescence (TRF) methods for multiple pathogen detection in different foods and to integrate automated IMB processing into TRF processes for potential adoption by stakeholders.
4. to develop multiplexed immunoassays for foodborne pathogens in microarray format with a milestone as: Year 1 and 2, to test novel simplified and rapid protocols based upon either microscope slide or microtiter plate format.

6. **What science and/or technologies have been transferred and to whom? When is the science and/or technology likely to become available to the end-user (industry, farmer, other scientists)? What are the constraints, if known, to the adoption and durability of the technology products?**

In FY 2003, there are a few technology transfer activities associated with this CWU worthy of mention. A major IMB producer showed considerable interests to these efforts and entered a formal reimbursable cooperative agreement.
with ARS to co-develop a physical model for the capture. Our past accomplishment in the use of bioluminescence for the detection of viable pathogens in foods attracted the interests of private scientific development companies. A formal reimbursable cooperative agreement has been established between one company and ARS to develop specific luminescence instrumentation for food pathogen detection. The method, enzyme-linked immunomagnetic chemiluminescent detection has been developed and related findings during the project include understanding of control of background response due to identified enzyme contamination of commercially available antibody-coated particles and optimization of selected chemiluminescent substrates.

7. List your most important publications in the popular press and presentations to organizations and articles written about your work (NOTE: This does not replace your peer-reviewed publications listed in Question 9).

None.

8. Scientific Publications


Efficacy of Chlorine Dioxide Gas in Reducing Pathogens on Meat Products and Poultry and Its Effects on Meat Quality

Investigators: David E. Gerrard and Yingchang Han

What major problem or issue are you resolving, and how are you resolving it?

Foodborne pathogens in meat and poultry products continue to be a serious health threat that causes an estimated 5 million illnesses and 4000 deaths each year. *Salmonella* spp., *C. jejuni*, and *E. coli*, are often implicated as causative bacterial agents. Aqueous chemical treatments, such as chlorinated water, organic acids, and trisodium phosphate reduce pathogen levels on the surface of raw meat products; however, they don’t cut pathogen levels enough to meet rising standards. More effective sanitizers must be developed.

Chlorine dioxide (ClO$_2$) gas may be an alternative antimicrobial agent for reduction of pathogens on meat products. Results from our laboratory have demonstrated effective use of ClO$_2$ gas for reducing pathogen levels on food and food-contact surfaces. We propose to extend our efforts to study efficacy of ClO$_2$ gas for control of *Salmonella* spp. and *E. coli* O157:H7 on different tissue surfaces of beef (lean, fat), pork (skin, lean, fat) and broilers (skin, lean);

- determine the efficacy of ClO$_2$ gas treatments for control of *Salmonella* spp. and generic *E. coli* on different tissue surfaces of beef (lean, fat), pork (skin, lean, fat) and broilers (skin, lean);
- identify the most resistant tissue surfaces (from objective 1) and optimize ClO$_2$ gas treatments (concentration and contact time) for the most efficient reduction of *Salmonella* and generic *E. coli*;

- determine pathogen reduction of *Campylobacter jejuni* (beef, pork, and boilers) and *E. coli* 0157: H7 (beef) using optimized ClO$_2$ gas treatment parameters (from Objective 2) for its application in meat processing; and

- determine the effects of ClO$_2$ gas treatments on the chemical and physical qualities of the meats (residual ClO$_2$, chlorite, free available chlorine, and chloramines).

Results from this research will help improve the safety of meat and poultry products. Furthermore, it will enhance our understanding of the potential benefits and feasibility of using ClO$_2$ gas as a new antimicrobial treatment for the meat industry.

How serious is the problem? Why does it matter?

*Salmonella*, *C. jejuni*, and *E. coli* O157:H7 are among the top foodborne pathogens with an incidence rate of 15.1, 13.8 and 1.6 cases per 100,000 persons each year in the United States, respectively. The government has set a National Health objective to reduce these rates to 6.8, 12.3 and 1.0 by 2010 (Food Net, 2001).

*C. jejuni* and *Salmonella* spp. are the most common causes of foodborne illness associated with meat and poultry products. They lead to an estimated 4 million cases per year. *C. jejuni* is present on 80-100% of raw poultry, 30% of raw pork, and 4% of raw beef (USDA, 1999). Significant reduction of contamination by these microorganisms has been made through the implementation of HACCP systems. “However, we still have work to do. Foodborne diseases remain a substantial public health burden that affects millions of people each year.” (HHS and CDC, 1999)

In an effort to reduce the incidence of foodborne illness related to meat and poultry products, the USDA-FSIS initiated the Pathogen Reduction Act (1996) requiring meat processors to construct...
a HACCP control plan, to develop sanitation standard operating procedures, and meet microbial performance standards for generic *E. coli* and the presence of *Salmonella*. The current performance standards for *Salmonella* spp. in raw beef, pork, and poultry are 2.7%, 8.7%, and 20%, respectively. Recommended levels of generic *E. coli* are <100, <1000, <10,000 (cfu/cm²) for raw beef, pork, and poultry.

To reduce microbial contamination in meat products and carcasses, chemical treatments, using chlorinated water, chlorine dioxide solution, organic acids, ozone, and trisodium phosphate, etc., have been widely used and investigated. However, the reported treatments achieve only a 1 to 2 log reduction of pathogens on meat surfaces. Highly effective sanitizers need to be developed for meat and poultry products. ClO₂ gas may be an alternative antimicrobial agent for the elimination of pathogens on meat products.

**What were your most significant accomplishments this past year?**

*Single Most Significant Accomplishment During FY 2003*

The purpose of this research was to evaluate a highly effective sanitizing agent as an alternative means in reducing microbial contamination in meat products and on animal carcasses while maintaining acceptable quality characteristics. Different tissue types from beef (fat and lean), pork (skin, fat and lean) and broiler (skin and lean) were spot-inoculated with generic *E. coli* K12 and a *Salmonella* cocktail (*S. enteriditis, S. serinberg*, and *S. typhi*) to determine the most resistant tissue surface by varying different gaseous ClO₂ concentrations and contact times, using a continuous flow system (Figure 1) developed by Drs. Nelson and Linton’s Laboratories, Department of Food Science, Purdue University. Additional studies were conducted using the most resistant tissue or the tissue type that is the most exposed on animal carcasses. These studies evaluated different concentrations of gaseous ClO₂, contact times, and other pathogenic microorganisms to determine the effectiveness of this alternative sanitizing agent in reducing microbial contamination (Table 1).

Microbial reduction results indicate that ClO₂ can be used as an alternative sanitizer to reduce microbial contamination on meat products and animal carcasses. However, it does not appear to have an advantage over traditional sanitizers, as of yet.

**Other Significant Accomplishments**

While studying the effectiveness of gaseous ClO₂ as an alternative sanitizer in reducing *Campylobacter jejuni* on poultry skin, pork lean, and beef fat, we discovered an alternative method for recovering stressed and injured cells. Originally, the research was conducted to see how effectively gaseous ClO₂ reduced microbial contamination of *Campylobacter jejuni*. Results indicate that the new method is 1.5-3 times more effective at recovering presumably injured or stressed *Campylobacter jejuni* cells. Further results, available in the near future, could have a big impact on recovery of injured *C. jejuni* cells from ClO₂-treated meat samples.
What major accomplishments do you anticipate over the life of the project, and what do you predict their impact will be?

Used as an alternative sanitizing agent, gaseous ClO$_2$ appears to be just as effective as traditional agents for reducing microbial contamination in meat processing or harvesting areas. However, we still need to study how gaseous ClO$_2$ affects quality and physical characteristics of the meat products and animal carcasses. If quality can be maintained at an acceptable level, the gaseous ClO$_2$ sanitizing system will need to be tweaked to achieve higher reductions of pathogenic microorganisms to give this sanitizing system an advantage over traditional sanitizing systems for meat products and animal carcasses.

Science/Technology Transfer

The ability of this technology to be phased into meat processing and abattoir facilities depends on how ClO$_2$ treatments affect the overall physical and quality attributes. The ClO$_2$ treatment technologies will not become available to the end user until we finish evaluating the effects of gaseous ClO$_2$ on chemical and physical attributes of the meat samples. One constraint for the acceptance of this sanitation technology is that gaseous ClO$_2$ treatment appears to alter the color of the meat samples, which could negatively affect consumer appeal.

The newly extended objective dealing with a new method for recovering stressed and injured Campylobacter jejuni cells will be made available to scientists in the near future.
### Table 1. Microbial reductions using different concentrations and contact times of gaseous ClO₂

<table>
<thead>
<tr>
<th>Surface Type</th>
<th>Bacteria</th>
<th>Treatment Conditions</th>
<th>Surface Log Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL</td>
<td>Generic <em>E.coli</em> k-12</td>
<td>500 ppm for 10 min</td>
<td>1.89</td>
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<tr>
<td>PF</td>
<td></td>
<td></td>
<td>2.02</td>
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<tr>
<td>PS</td>
<td></td>
<td></td>
<td>1.46</td>
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<td>BL</td>
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<tr>
<td>BF</td>
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<td>CL</td>
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<td></td>
<td>1.34</td>
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<td>PL</td>
<td><em>Salmonella</em> cocktail</td>
<td>500 ppm for 10 min</td>
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<td>PF</td>
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<tr>
<td>CL</td>
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</tr>
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<td><em>Salmonella</em> cocktail</td>
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<td>Salmonella cocktail</td>
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<td>Generic E.coli k-12</td>
<td>4500 ppm for 30 min</td>
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<td>Campylobacter jejuni</td>
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<tr>
<td>PL</td>
<td>Campylobacter jejuni</td>
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<tr>
<td>BF</td>
<td>E.coli O157:H7</td>
<td>4500 ppm for 10 min</td>
<td>2.30</td>
</tr>
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</table>

*Treatments were conducted using a continuous flow-through system with % relative humidity > 90% at a temperature between 4-10°C

**PL-pork lean, PS-pork skin, PF-pork fat, CL-chicken lean, CS-chicken skin, BF-beef fat and BL-beef lean

***Salmonella cocktail included S. seftenberg, S. enteriditis and S. typhi
What major problem or issue are you resolving, and how are you resolving it?

For health and nutrition reasons, consumers currently eat more fresh vegetables and fruits. In fact, fresh produce consumption rose 27 percent in the United States from 1970 to 1993. Fresh fruits and vegetables supply more than one-fourth of the ascorbic acid and about one-fourth of the vitamin A in the American diet. Most popular among consumers are minimally processed and refrigerated (MPR) fruits and vegetables that satisfy the need for convenience and longevity. However, fruits and vegetables can serve as vehicles for almost any foodborne pathogenic microorganisms. The usual sources of pathogen contamination are irrigation or wash water, fertilizers of animal or municipal-biosolid-origin, infected operators, and operation of facilities with poor sanitation practices. There have been numerous reports of foodborne outbreaks associated with these contaminated foods. Outbreaks and incidence of pathogens, such as enterotoxigenic *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp., *Cyclospora*, and hepatitis A virus, have been attributed to consumption of lettuce, coleslaw, cantaloupe, celery, tomatoes, cabbage, alfalfa sprouts, mushrooms, cucumbers, potatoes, radishes, apple cider, orange juice, raspberries, strawberries and salads that contain onions, carrots, zucchini, peppers, broccoli, mushrooms, and tomatoes.

Our long-term goal for this research effort is to improve the safety of fruits and vegetables by developing and applying novel non-thermal food processing technologies, such as the use of gaseous chlorine dioxide treatment.

The specific objectives were to

- determine the efficacy of chlorine dioxide \((\text{ClO}_2)\) gas in reducing selected pathogenic microorganisms (*Escherichia coli* O157: H7, *Listeria monocytogenes*, and *Salmonella* spp.) on selected fruits and vegetables (including green pepper, lettuce, mushroom, strawberries, cantaloupe, apples and oranges);
- investigate \(\text{ClO}_2\) gas sanitation technologies for these selected fruits and vegetables using a laboratory and pilot scale system; and
- evaluate the effects of \(\text{ClO}_2\) gas treatment on quality and shelf-life of selected commodities.

How serious is the problem? Why does it matter?

Washing with chlorinated water (50-200 ppm chlorine) is widely used to sanitize whole fruits and vegetables as well as fresh-cut produce on a commercial scale. However, its effectiveness is limited in reducing the population of microorganisms (less than 2 log CFU) on fruits and vegetables. Therefore, it is a challenge to attain the 5-log reduction recommendation set by FDA. Moreover, environmental and health organizations...
have expressed concerns with the by-products from chlorine reactions with organic materials. These by-products can include chloroform, carbon tetrachloride, chloromethane and others (generally known as trihalomethanes, or THMs) that have been implicated as carcinogens in the development of kidney, bladder, and colon cancer. The produce industry is concerned about the possibility of future regulatory constraints on the use of chlorine as a sanitation agent. Other aqueous sanitation treatments using hydrogen peroxide, peroxyacetic acid, trisodium phosphate, ozone, chlorine dioxide, and their combinations have also been reported, but are not that effective in reducing pathogens on produce surfaces (<3 log). Therefore, the development of highly effective antimicrobial technologies for the reduction of pathogens from fruits and vegetables is needed.

Chlorine dioxide (ClO\(_2\)) gas will be an alternative antimicrobial treatment for the elimination of pathogens on fruits and vegetables. ClO\(_2\) in both the gaseous and aqueous phase is a strong oxidizing and sanitizing agent that has broad and high biocidal effectiveness. It has been reported to effectively inactivate pathogens, including bacteria, viruses, bacterial spores, and algae. It has about 2.5 times the oxidation capacity of chlorine. ClO\(_2\) maintains its bactericidal activity far longer than chlorine. Because it is also less reactive than chlorine with organic compounds, its use is preferred where high organic loads are encountered.

Aqueous ClO\(_2\) has been approved for use in washing fruits and vegetables in an amount not to exceed 3 ppm residual ClO\(_2\). However, the effectiveness of aqueous ClO\(_2\) in decontaminating fruits and vegetables is limited to less than 2 log reductions.

Gaseous ClO\(_2\) is effectively used as a disinfectant in medical sciences. However, information regarding the usefulness of ClO\(_2\) as a gaseous sanitizer in the food industry is limited. Because gas has greater penetration ability than liquid, ClO\(_2\) gas is likely to be a more effective sanitizer for fruits and vegetables than aqueous ClO\(_2\). Previous results from our group show that ClO\(_2\) gas is effective in inactivating greater than 7 log level cfu spoilage microorganisms inoculated on the surface of a model storage tank for orange juice. Also, using green peppers as a model for vegetables, we demonstrated that we can achieve greater than
a 5 log reduction of pathogens using a gaseous ClO₂ treatment. Therefore, the proposed research provides a promising technology to improve the safety of fruits and vegetables, while maintaining product quality.

What were your most significant accomplishments this past year?

The efficacy of ClO₂ gas in reducing pathogens on selected fruits and vegetables, including strawberries, lettuce, green pepper, mushroom, cantaloupes, apples and oranges, were studied using a laboratory-scale generation system. These studies were carried out in Drs. Nelson’s and Linton’s laboratories in the Department of Food Science at Purdue University. Two chlorine dioxide gas treatment systems, one shown in Fig. 1, were used. These mainly include a CDG ClO₂ gas generator, a treatment chamber, and a monitor for measurement of ClO₂ gas concentration, temperature, and relative humidity. Different pathogenic bacteria were selected as targets, including *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp., and *Shigella* spp., and spot-inoculated on selected surfaces. Efficacy data for both batch and continuous ClO₂ gas treatments are summarized in Table 1. Greater than 3 log reductions of selected pathogens were seen on fruit and/or vegetable surfaces (except lettuce) when using either a batch or continuous treatment system. Therefore, a ClO₂ gas sanitizing treatment system seems to be a promising alternative technology for reducing microbial contamination on fruits and/or vegetables. However, its use for decontamination of lettuce is limited due to rapid discoloration of leaves when ClO₂ gas concentration is over 0.2 mg/l. Besides gas concentration, exposure time, temperature, and relative humidity, surface property of produce is another important factor influencing the efficacy data of ClO₂ gas treatment.

What major accomplishments do you anticipate over the life of the project, and what do you predict their impact will be?

After the studies, ClO₂ gas treatment was shown to be effective at reducing pathogenic microbes by more than 5 log on green peppers, apples, strawberries, cantaloupes, mushroom, and oranges while maintaining acceptable product quality.

### Table 1. Efficacy of ClO₂ gas treatments using different produce surfaces

<table>
<thead>
<tr>
<th>Surfaces</th>
<th>Pathogenic bacteriaₘ</th>
<th>ClO₂ gas treatment conditions (at 22°C, &gt;90% relative humidity)</th>
<th>Log reductions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjured green peppers</td>
<td><em>E. coli</em> O157:H7, <em>L. monocytogenes</em>, <em>Salmonella</em> spp.</td>
<td>Batch, 0.6 mg/l - 30 min, Batch, 0.6 mg/l - 30 min, Continuous flow, 0.6 mg/l - 10 min</td>
<td>7.3, 6.3, 5.5</td>
</tr>
<tr>
<td>Apples</td>
<td><em>E. coli</em> O157:H7, <em>L. monocytogenes</em></td>
<td>Batch, 4.0 mg/l – 10 min, Batch, 4.8 mg/l – 10 min</td>
<td>5.5, 4.8</td>
</tr>
<tr>
<td>Strawberries</td>
<td><em>E. coli</em> O157:H7, <em>L. monocytogenes</em>, <em>E. coli</em> O157:H7, <em>L. monocytogenes</em></td>
<td>Batch, 4 mg/l – 30 min, Batch, 4 mg/l – 30 min, Continuous flow, 0.6 mg/l - 10 min, Continuous flow, 0.6 mg/l - 10 min</td>
<td>5.1, 5.3, 3.3, 4.1</td>
</tr>
<tr>
<td>Oranges</td>
<td><em>Salmonella</em> spp., <em>Shigella</em> spp.</td>
<td>Continuous flow, 0.3 mg/l – 6 min</td>
<td>&gt; 5</td>
</tr>
<tr>
<td>Mushroom</td>
<td><em>E. coli</em> O157:H7, <em>Salmonella</em> spp.</td>
<td>Continuous flow, 1-2 mg/l – 10 min</td>
<td>&gt; 5</td>
</tr>
<tr>
<td>Cantaloupes</td>
<td><em>Salmonella</em> spp.</td>
<td>Continuous flow, 1-10 mg/l – 10 min</td>
<td>2-5</td>
</tr>
<tr>
<td>Lettuce</td>
<td><em>L. monocytogenes</em></td>
<td>Batch, 0.2 mg/l - 30 min</td>
<td>2.0</td>
</tr>
</tbody>
</table>

ₘ Bacteria were spot-inoculated and allowed to dry for 2 hrs prior to storage for 1 day at 4°C before treatment.
Therefore, it has great potential for improving the safety and quality of fruits and/or vegetables.

**What do you expect to accomplish, year by year, over the duration of the project?**

Next year, we plan to finish Objectives 2 and 3 as per Question 1 using a pilot scale ClO$_2$ gas treatment system, which we are currently building.

**Science/Technology Transfer**

After next year’s investigation, the ClO$_2$ gas technology will become available to the produce industry. However, regulatory approval (FDA) is needed prior to adoption for commercial applications in the vegetable and/or fruit industry.

**Patent Disclosed**


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**Scientific Publications**


**Presentations and Popular Articles**

R. Rosentrader, D. Delgasdo, Y. Han, R.H. Linton, and P. E. Nelson. 2003. Inactivation of *Salmonella* spp. on the outer surfaces of whole cantaloupes using chlorine dioxide gas. 2003 meeting of Institute of Food Technologists (IFT), Chicago, IL.

Y. Han and R. H. Linton. 2003. Survival and growth of *Escherichia coli* O157:H7 and *Listeria monocytogenes* on strawberries, in strawberry juice and in acidified media as affected by storage time, pH, and temperature. 2003 meeting of Institute of Food Technologists (IFT), Chicago, IL.

Y. Han, R. H. Linton, and P. E. Nelson. 2003. Comparison of inoculation methods to determine the efficacy of chlorine dioxide gas and chlorinated water treatments to reduce *E. coli* O157:H7 on strawberries. 2003 meeting of International Association of Food Protection (IAFP), New Orleans, LA.

Development of a Two-Step Method for Isolation and Detection of Pathogenic *L. monocytogenes* in Food Samples

**Investigator:** Kristen M. Naschansky

**Introduction**

*Listeria monocytogenes* is an ubiquitous organism that affects about 2,500 immunocompromised individuals a year in the United States. Within the affected group, there is a 20% fatality rate (Weis and Seeliger, 1975; Schuchat et al., 1991; Mead et al., 1999). Less than 1,000 cells may be enough to make these individuals ill (Doores, 1998). Several U. S. governmental agencies recently performed a risk assessment for *L. monocytogenes* in which pâtés, soft cheeses, smoked seafood, frankfurters (hotdogs), as well as deli meats and related ready-to-eat foods, were found to have the potential to cause listeriosis in susceptible individuals (USDA, 2001). A further complication is that the growth requirements of *L. monocytogenes* allow it to multiply in refrigerated foods. Moreover, a study of the incidence of *L. monocytogenes* in beef and poultry samples found that 32.5% of commercially purchased meats were contaminated with cytotoxic strains of *L. monocytogenes* (Amoril and Bhunia, 1999).

The current methods for analyzing food products for the presence of *L. monocytogenes*, are complex and time consuming. Conventional methods for *L. monocytogenes* detection require enrichment, plating and biochemical tests that result in a total identification time of 5 to 7 days (Farber and Petterkin, 1991), often with results termed “presumptively positive” (Schuchat et. al., 1991).

A method that has become readily accepted to aid in pathogen detection techniques is immunoseparation by capture of bacteria from food samples using antibody-coated beads. Although commercially available magnetic anti-*Listeria* spp. beads are available, we developed a more specific, non-magnetic, lower-cost alternative termed Immuno-beads. They could be used in a two-step method of immunoseparation and enzymatic cytopathogenicity analysis, although their development was intended for future use in a biosensor-based detection system.

Recently, various biosensor-based technologies have been developed to sensitively detect foodborne pathogens (Ivnitski et al., 1999) as an alternative to conventional methods. Biosensors take advantage of natural attributes of cell cultures. Specifically, the cell cultures are introduced in a sample to elicit a reaction that targets an analyte, which may be recognized directly or indirectly (Ivnitski et al., 1999). Many of the systems are automated and most are rapid in comparison to conventional methods for pathogen detection. Often these biosensor-based systems offer greater sensitivity than conventional methods.

Many varieties of biosensors rely on impedance technology. This technology already serves as a useful tool in the media-based detection of pathogens (Safarik et al., 1995). Impedance spectroscopy is accomplished by passing an alternating current of varying frequencies through an object to determine its impedance values (Kyle et al., 1999), while basic impedance microbiology relies on changes in a growth medium due to metabolism...
Through the introduction of biosensor-based approaches to pathogen detection, we could replace the time-consuming and tedious conventional methods for Listeria monocytogenes detection.

by the target microorganism. This method can be used in conjunction with an immunoseparation step to initially concentrate the bacteria, resulting in faster results.

Mammalian cells can be used in a variety of biosensor systems targeting cell receptors, channels and enzymes that exhibit a reaction with a sample (Pancrazio et al., 1999). Mammalian cell-based biosensors take advantage of natural cellular fluorescence, metabolism, impedance, intracellular potentials and extracellular potentials to gather data in reference to a test sample. An advantage to biosensors employing mammalian cells is that they provide additional information regarding the possible physiological effects of the sample according to their natural response to experimental conditions.

In relation to tissue and cell cultures, electrical impedance (EI) uses the inherent electrical properties of cells to measure the parameters related to the tissue environment (Kyle et al., 1999). The cell can be equated to a simple circuit, since it is nothing more than conductive fluid encapsulated by a membrane surrounded by another conductive fluid. The conductive fluids make up the resistance elements of the circuit, while the membrane acts as a capacitor.

Impedance methods have been used to monitor tissue cultures on-line and in real-time (Ehret et al., 1996). Changes in impedance relate to changes in cell density, growth or cellular behavior. Changes in the membrane integrity, as well as movement between the electrodes, result in changes in the impedance values. Biosensors are able to provide detailed information about the growth characteristics of a tissue culture, including information on spreading, attachment and cellular morphology.

A rapid cytotoxicity assay for L. monocytogenes was developed that took advantage of the cytotoxic effect of listeriolysin O (LLO)-producing strains of L. monocytogenes that induce membrane damage leading to apoptosis in murine Ped-2E9 and EM-7G1, as well as human RI.37 hybridoma cell lines cells (Bhunia et al., 1994). Previous work found that a 100:1 (L. monocytogenes: hybridoma) multiplicity of infection would result in mammalian cell death in just 4 hours. This was measured by the Trypan blue exclusion assay and was later validated by lactate dehydrogenase (LDH) (Bhunia and Feng, 1999) and alkaline phosphatase (AP) enzyme assays (Bhunia and Westbrook, 1998) as well as through a DNA fragmentation assay using light and scanning electron microscopy. Dithiothreitol (DTT), a reducing agent, was found to activate LLO, which furthered the sensitivity and expedited the speed of the assay producing cytotoxic effects in only 1.5 to 2 hours (Westbrook and Bhunia, 2000).

Rational for the Research
Due to the prevalence of L. monocytogenes in the food supply and the resulting severity of illness, it is apparent that steps must be taken to control its emergence in the food supply. The time-consuming and tedious conventional methods for L. monocytogenes detection must be replaced with more rapid and specific alternative methods. This can be accomplished through the introduction of biosensor-based approaches to pathogen detection.

Research Objectives
The overall objective of this research was to develop tools, techniques and rapid methods for assaying L. monocytogenes in order to meet the goals of the National Food Safety Initiative. Specific objectives included concentration and capture of Listeria cells by immunoseparation, following a selective enrichment step, in order to detect viable L. monocytogenes interaction with mammalian cells (Ped-2E9) by electrical impedance spectroscopy.
Materials and Methods

Bacterial Cultures and Mammalian Cell Lines

The bacterial cultures used are Listeria monocytogenes (F4244), Listeria innocua, Listeria grayii, Listeria seeligeri, Listeria welshemeri and Listeria ivanovii. The mammalian cell cultures used are a murine hybridoma Ped-2E9 cell line and a RAW macrophage cell line.

Two-step method for isolation and detection of L. monocytogenes

An Immunobead conjugated with an anti-Listeria monoclonal antibody was used for isolation from samples using immunoseparation. The Immunobeads were also compared with Anti-Listeria Dynabeads® (magnetic beads) from Dynal (Dynal A.S., Oslo, Norway). The bead-captured bacterial cells could then be used in a mammalian cell assay to assess their cytotoxicity.

Testing of naturally contaminated raw or cooked meat products

Naturally contaminated food samples were analyzed using the two-step method to detect L. monocytogenes, including packages of processed hotdogs, raw ground beef, ground pork, and various pieces of raw chicken samples from whole fryer cuts or individually packaged. Meat samples were enriched in half-Fraser broth, then Dynabeads or Immunobeads were used for immunocapture and directly added to the Ped-2E9 cells suspension for cytotoxicity analysis.

Bead-captured cells were also surface plated on MOX plates, suspected black colonies were picked, and their morphology (rod vs cocci) was microscopically determined. Rod-shaped isolates were examined by the CAMP test on a blood agar plate and those found positive were tested on an automated RiboPrinter® (Qualicon, Inc., Wilmington, DE) for genomic analysis.

Off-chip analysis of RAW cell cytotoxicity

RAW macrophage cells are seeded onto 24-well plates until achieving a confluent layer. With the addition of a bacterial suspension, they can then be analyzed for cytotoxicity using the lactate dehydrogenase (LDH) release assay.

Growth of RAW macrophage cells on biochips

The interdigitated biochips modified with a PDMS well are washed in a series of alcohols and acid and then autoclaved. After rinsing chip surfaces, the chips are incubated with a solution of Arg-Gly-Asp tripeptide and a fibronectin adhesion-promoting peptide. After adsorbing to the surface, the solution is removed and RAW cells are seeded onto the chip. Colorimetric (LDH release assay) or
impedance analyses are done following formation of a confluent layer on the chip surface.

**Results and Discussion**

Immunobeads were analyzed against Dynabeads in terms of bacterial capture ability and specificity. Previous research found the Immunobeads were statistically equivalent (p < 0.05) to the Dynabeads in capture efficiency. However, Immunobeads can be made at a reduced cost. In terms of specificity, the Dynabeads have a capture antibody for all *Listeria* species, while the Immunobeads are conjugated with an antibody which will only capture *L. monocytogenes* and *L. innocua*. The benefit to the more specific antibody is apparent in the Ped-2E9 cytotoxicity analysis following immunocapture from a sample (Fig 1). Since the Immunobeads only specifically capture *L. monocytogenes*, only that sample elicits a positive cytotoxicity response, while the Dynabeads cause a cytotoxicity response when capturing *L. ivanovii* (animal pathogen) and *L. seeligeri* (hemolytic activity). Therefore, the Immunobeads will generate a positive response for pathogenic Listeria when *L. monocytogenes* is present.

Both Immunobeads and Dynabeads were used to test over 70 naturally contaminated food samples, the majority of which were raw foods including ground beef, ground pork, and cut-up chicken pieces. These foods gave unusually high AP readings early in the analysis which is indicative of inherent AP in the food product. False positives were also common in the raw foods indicating either cytotoxicity due to non-specifically bound bacteria or AP products from captured bacteria. Several true positives were confirmed by testing for *L. monocytogenes* through Oxford plating, the CAMP test and Riboprinter analysis.

It is indicated from the testing of cooked or processed food that these types of samples would be more suitable for the two-step method, since initial AP readings are generally close to control levels and no false positives have been found. Food enrichment conditions were created according to the protocol for Dynabeads, which is limited by a suboptimal medium (Half-Fraser) and temperature (30°C). It appears that capture and subsequent cytotoxicity analysis may be better enhanced through enrichment with a less harsh media, like buffered Listeria enrichment broth (BLEB) and incubation at 37°C with light agitation (Fig 2).

While the Ped-2E9 cells used in the AP assay were initially tested for their application in the impedance-based biosensor, it was found that since they grow in suspension and settle on the chip, there were large errors in the impedance readings due to the chip surface being exposed to media through

![Figure 2](image-url). Comparison of cytotoxicity induced following Immunobead-captured *L. monocytogenes* from pure culture in three broths. All samples were tested with 2 x 10^6 hybridoma cells/ml and 9.5 mM DTT. The control was uninoculated. BHI, Brain-heart infusion; BLEB, Buffered Listeria enrichment broth; HF, Half-Fraser. Data represents one trial.
leakage. Therefore, a RAW macrophage cell line which can grow in a confluent monolayer was investigated. This cell line was found to have low AP levels, so a modified LDH release assay was used.

Off-chip analysis of the RAW cells demonstrated they would be ideal for *L. monocytogenes* analysis since they elicited a high cytotoxic response at 2 hours, with a reduced response at a lower concentration at 4 hours (Fig 3). While there is a positive response for *L. ivanovii*, this should not be a concern when combined with the high specificity of the Immunobeads.

It was determined that following cleaning with ethanol and acid, plus autoclaving, that a combination of the tripeptide Arg-Gly-Asp and fibronectin adhesion-promoting peptide needed to be adsorbed to the chip surface in order for RAW cell attachment and subsequent growth. Once a confluent layer was achieved, on-chip colorimetric analysis using the LDH release assay could be done. A strong cytotoxic reaction on-chip has been noted for *L. monocytogenes* (Figure 4). The other *Listeria* spp. are currently being tested on-chip, and results are expected to parallel those off-chip.

**References**


Figure 4. Cytotoxicity analysis of RAW macrophage cells with all L. monocytogenes on-chip. The control was uninoculated, the Lm (L. monocytogenes) sample was estimated to be inoculated at 100:1 MOI and Triton-X was used as the positive control. The experiment was performed in duplicate.


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