6-Months USDA-ARS Project Update Report
(June 1 to November 30, 2011)

BARDOT Project
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USDA Progress Report

**Project Title:** Improved Detection Techniques for Foodborne Pathogens  
**National Program:** NP 108 – Food Safety  
**Objective 2:** Develop, evaluate and adopt novel technologies for detection, identification, and quantification of viable and non-viable target microorganisms. Research areas to be addressed are biochip based technology, optical light scattering technology, fluorescence resonance energy transfer spatial technology, and fourier-transform infrared spectroscopy technology.

**Subobjective 2B:** Develop the BActerial Rapid Detection using Optical light scattering Technology (BARDOT) system to rapidly detect and identify pathogenic bacteria based on unique morphologies generated by concentrated colonies

**Introduction**

The optical light scattering sensor, BARDOT (BActerial Rapid Detection using Optical light scattering Technology (BARDOT) is a noninvasive label-free detection system which allows identification of bacterial colonies in real-time, and the speed of detection is unparallel to any existing detection device. In this semi-automated system, Petri-dish containing bacterial colonies is placed in the plate holder and the colony map of the plate is acquired. Incident laser beam sequentially runs through each preselected colony and generate scatter signature which is collected and compared to the image library for identification. BARDOT was also demonstrated to detect pathogens from inoculated food samples validating the system’s ability to detect pathogens from food. During this reporting period, improvement in BARDOT instrument was made to detect smaller sized colonies. In addition, experiments were done to generate scatter signature library for serovars of *Salmonella* and Shiga-toxin producing *E. coli*, and *Staphylococcus*.

**Reporting on Project Milestones**

1) Automation of the BARDOT system: Milestone fully met
2) Capture of scattering images from different bacterial species and expansion of the database: Milestone substantially met
3) Begin studies with the Immunomagnetic separation (IMS) and Pathogen enrichment detection device (PEDD): Milestone substantially met.
4) Integration of IMS and PEDD to BARDOT system: Milestone fully met
5) Capture of scattering images from *E. coli*, *Salmonella* and *L. monocytogenes* grown in meat samples: Milestone fully met
6) Improvements in rapid detection by scattering small (<1 mm) bacterial colonies and identification to the genus, species and strain level: Milestone partially met
7) To improve and automate the image processing and analysis software: Milestone fully met
8) Integration of image analysis software into the packaged BARDOT system: Milestone fully met
9) Prototype will be sent to validation by other laboratories: Milestone substantially met

Progress Report:

Previously, we showed that BARDOT can detect and identify colonies of *E. coli* O157:H7, *L. monocytogenes*, *Salmonella* and *Vibrio*. During this reporting period, we show that BARDOT is able to differentiate and classify 26 different serovars of *Salmonella* when cultured on selective xylose-lysine deoxycholate (XLD) agar plate (*Manuscript in preparation*). Similarly, BARDOT was evaluated for its ability to distinguish seven common Shiga-toxin producing *E. coli* (STEC) serovars cultured on 4 commonly used chromogenic/selective media. BARDOT efficiently differentiated all seven serotypes when grown on SMAC (Sorbitol MacConkey) and Rainbow media suggesting it could possibly be used for initial typing and screening of suspect *Salmonella* and *E. coli* colonies (*Manuscript in preparation*). BARDOT was successfully used with samples that were either prepared by IMS or through PEDD device (*published in early 2011*).

Furthermore, to reduce BARDOT-based total detection time for food samples; approach to detect micro-colony was initiated. Data show that BARDOT could differentiate micro-colonies (100 – 150 µm) of *Salmonella*, *E. coli*, *Staphylococcus*, and *Listeria* after 8-12 h of growth (*one manuscript published and one submitted*). A prototype of portable mini-BARDOT system is now under construction for use onsite for pathogen detection. BARDOT units have been delivered to Dr. John Thomas at West Virginia University, Prof. Dan Hirleman at University of California, Merced: and Battelle Laboratories, Columbus, Ohio.

Project accomplishments

*Detection of bacterial micro-colonies using elastic light scattering*

Forward light scattering patterns from bacterial colonies have been successfully used for bacterial rapid detection. To understand the biophysics behind this phenomenon, the scalar diffraction theory was employed: a bacterial colony is considered as a biological spatial light modulator with amplitude and phase modulation to the incoming light, which continues to propagate to the far-field to form a distinct scattering ‘fingerprint’. An integrated colony morphology analyzer (Fig 1) was built to explore the amount of phase and amplitude modulation of micro-colonies (100~500 pm).
µm) due to their homogeneity. The instrument consists of a confocal displacement meter for profile measurements of the bacterial colonies, and a photodiode for simultaneous transmittance measurement. The integrated system was calibrated and tested on two bacterial species: *Escherichia coli* O157:H7 EDL933 (*E. coli* O157:H7) and *Staphylococcus aureus* ATCC 25923 (*S. aureus*). The developed system was able to clearly distinguish the selected bacteria species based on the profile and transmittance measurement (Fig 2), indicating their distinctive scattering patterns. Moreover, growth rates of the two colonies within the observation period were estimated from the experimental data to help us to understand the different growing dynamics of the two species. As colonies keep growing, complex phase structures are formed within. Phase modulation cannot be obtained through profile measurement only. Therefore, a quantitative phase imaging technique particularly with non-interferometric nature was explored. Two solvers based on finite element method and Fast Fourier Transform were developed. An optical fiber with known refractive index profile was used for calibrating phase measurement. The next step is to apply this technique for bacterial colonies phase imaging.

![Figure 2](image.png)

**Figure 2.** (a) Microscopic images of *S. aureus* (top) and *E. coli* O157 H7 (bottom) at ~150 µm; (b) profile comparison of *S. aureus* and *E. coli* O157 H7 at ~150 µm; (c) transmittance comparison of *S. aureus* and *E. coli* O157 H7 at ~150 µm; (d) microscopic images of *S. aureus* (top) and *E. coli* O157 H7 (bottom) at ~180 µm; (e) profile comparison of *S. aureus* and *E. coli* O157 H7 at ~180 µm; (f) transmittance comparison of *S. aureus* and *E. coli* O157 H7 at ~180 µm; (g) microscopic images of *S. aureus* (top) and *E. coli* O157 H7 (bottom) at ~245 µm; (h) profile comparison of *S. aureus* and *E. coli* O157 H7 at ~245 µm; (i) transmittance comparison of *S. aureus* and *E. coli* O157 H7 at ~245 µm.

**Improvements in rapid detection by scattering small (<1 mm) bacterial colonies**

The primary objective of this component is to make design changes that will allow the robust measurement of smaller colonies, and thus reduce the time to identification. Our long term goal
would be to measure a 100 µm colony. The current colony size is approximately 1 mm and is robust. To reduce colony size there are 3 fundamental modifications that must be made:

1. Reduce the laser diameter to accommodate smaller colonies
2. Increase the resolution of the imaging camera so that colony location can be identified
3. Decrease the imaging distance to the scatter camera to accommodate the significantly larger scatter patterns of small colonies.

We have built a prototype now with a 5 megapixel camera capable of identifying the smaller colonies but there are still a number of issues that must be dealt with. Additional funding from our NIH grant was used for most of the hardware prototyping. First, the software must be modified to accept the higher resolution image, and all of the calculations must be altered due to the significantly larger amount of data (doubling the resolution results in a four-fold increase in pixel number). The second issue is linking a smaller laser profile and this can be done by using a different lens, however, we cannot currently have a system that can measure both small and large colonies as this would require an automated lens system that we have designed but could only implement with future funding. Finally, the analytical engine must be changed as we are now dealing with significantly more data (slower speed) and larger data storage. For example, 50 colonies with a 5 megapixel camera now require over 300 megabytes per plate as opposed to around 50 megabytes with the previous camera. Regardless, we have now confidence that we can measure colonies around 300 µm which means a time reduction of 4-6 h over the previous 15-24 h of the first generation instruments. Our goal will be to approach 100 µm during the next round of funding which will mean much faster colony identification and make this system as effective as any current technology since most tools require sample preparation of a few hours regardless of the tools used.

Identification of serovars of Salmonella

The present investigation reports the application potential of laser optical sensor for detection and identification of Salmonella serovars, which relies on a non-invasive biophysical principle. Xylose lysine desoxycholate (XLD) agar was found to generate more discriminatory optical scatter patterns for Salmonella colonies in comparison to Brain heart infusion (BHI) agar medium based on DOTBAR analysis (Fig 3). Total 26 different Salmonella serovars comprising of 64 strains were used to build the scattering image library on XLD agar medium, which was further used to identify contaminating Salmonella isolates. Based on the recent Salmonella outbreaks as reported by CDC, eight Salmonella serovars (Salmonella Agona, Salmonella Enteritidis PT 21, Salmonella Hadar, Salmonella Heidelberg, Salmonella Montevideo, Salmonella Newport, Salmonella Typhi and Salmonella Typhimurium var. Copenhagen) were selected for detailed study. Scatter image and ribopattern based dendrogram revealed similar grouping of eight Salmonella serovars, which underscores and validate the discriminatory potential of the present technique. Spiked experiment with Salmonella Typhimurium var. Copenhagen in peanut butter did not reveal significant variation in scattering pattern over a period of 28 days. Laser optical biosensor revealed its application potential as screening tool for Salmonella contaminations in pork, turkey and chicken food samples, which was later confirmed by Salmonella serovar specific primers and ribotyping. The present laser optical sensor provides a non-destructive and simple tool for large scale screening of food contamination, which can be further confirmed, if necessary by molecular tools.
Identification of Shiga-toxin producing Escherichia coli (STEC)

Shiga-toxin producing Escherichia coli (STEC) strains are recognized as serious food-borne pathogens. STEC comprises of a diverse group of O-antigenic organisms, among which O157, O26, O111, O103, O121, O45, and O145 are most frequently implicated in cases of human diseases. Therefore, a sensitive and specific differentiation method for these serovars is in demand. Current culture-based isolation methodology followed by biochemical characterization is relatively time-consuming and may fail to correctly identify non-O157 STEC. Here we evaluated a novel light scattering technology for its ability to instantly differentiate colonies of various E. coli serovars on agar plates without compromising the colony integrity. A total of 20 E. coli strains of 7 different serotypes were grown on agar plates including Brain Heart Infusion (BHI) agar, Sorbitol MacConkey (SMAC) agar, Rainbow® Agar O157, BBL™ CHROMagar O157, and R&F® E. coli O157:H7 medium at 37°C until colony size reached to 1.1 ± 0.1 mm in diameter. The scatter patterns of the colonies were collected using BARDOT and serovar specific images were grouped using image analysis software. Optical microscopy was used to record colony morphology. Colonies of E. coli serovars produced distinct scattering patterns on each medium. Of all the media tested, both SMAC and Rainbow® Agar provided the best differential results allowing multi-class classification of all serotypes with average accuracy of 90% in 11-12 h, even though the colony morphology or colors were indistinguishable at that early stage of the growth.
Identification of Shiga-toxin producing Escherichia coli (STEC) using non-exhaustive learning (novelty detection) – study performed in collaboration with M. Murat Dundar (IUPUI)
The forward light scattering technology relies on statistical machine learning to perform recognition and classification. However, the currently used methods utilize traditional supervised techniques that assume completeness of training libraries. In other words, it is assumed that the classifier has an access to all the possible forms of the pathogens of interest. This restrictive assumption is known to be often false for real-life conditions, as we recently learned from the E. coli outbreak in Germany. Therefore, using additional funding from NIH (grant AI085531-01A1) we pursue a study demonstrating that detection and classification of unknown pathogens is indeed possible using the light-scatter based system.

![Fig 5. (A and B) Agarose gels showing results of the multiplex PCR assays targeting the stx2, stx1, and eaeA genes from different STEC strains.](image)

In our recent publications\(^a\) we demonstrated that classification of Salmonella phenotypes not only can be successfully performed with access to exhaustive training libraries, but can also be implemented in a nonexhaustive regimen, i.e., without assuming that access to the complete training set is always available. Although this study demonstrated clearly the feasibility of our approach, it also promoted speculation that a more complex distribution of phenotypes may lead to classification problems that ultimately would not be solvable with the help of parametric methods, i.e., techniques assuming some level of knowledge about the nature of the distributions.

We successfully completed a preliminary study showing an application of nonexhaustive learning technique to the problem of detecting non-O157 STEC. We showed promising preliminary results for models assuming both exhaustive and nonexhaustive training libraries, and we concluded that the complex nature of non-O157 STEC phenotype distribution indeed may require a new generation of statistical machine learning methods to cope with the extraordinary diversity of E. coli. Table 1 summarizes the results. It can be seen that even for the two most difficult cases (O103:H11, O103:H12) our system was able to detect presence of previously unseen classes, and correctly separate them from noise and background (with probability \(\approx 0.79\)).

Table 1. AUC values illustrating performance of the emerging pathogen detection system for 4-class and 7-class models. The O157 serotype was assumed to be always known. The models used in the study did not assume access to complete information regarding the classes. It was expected that any new instance submitted for classification might in fact belong to a new class for which the current system had no examples. This type of classification reflected a real-life situation in which a laboratory would not have information available about an emerging pathogen that might be encountered in tested samples at the outset of an outbreak.

Prototype development of mini-BARDOT

With the successful implementation of the BARDOT system, there is a great need of miniaturizing the system to a shoe-box size such that the system can be easily carried with the first-responders to the outbreak scenes rather than shipping the samples to the main laboratory. To enable this, a portable bacterial identification system called mini-BARDOT is currently being developed (Fig 6) with additional support from NIH grant # 1R56AI089511-01 (“A distributed clinical & biodefense national network for rapid organism identification”).

The overall footprint of mini-BARDOT is just 6”x 12”x 10.5” inches and only weighs 9 pounds. Since the main focus of the design was portability and accessibility, all the hardware connections to the computer have been upgraded to USB such that a simple laptop can be used to operate the system. In the next phase, integrating the output image with the regular BARDOT system will be implemented. Since the data captured via mini-BARDOT from remote location can be wirelessly transmitted to the main database system, it is crucial to test the image compatibility and classification results using the main database system against the mini-BARDOT images. These field tests are presently being performed.

![Image](image.png)

**Fig. 6.** Schematic diagram of the portable bacterial identification system using elastic light scattering patterns. The system consists of a linear stage, a rotational stage, and two camera (imaging and scattering), laser diode of 635 nm, and electroluminescent light for back-illumination. Approximate footprint 6”x 12”x 10.5”
Technology Transfer

1. BARDOT is currently being licensed by a startup company, Advanced Bioimaging Systems, LLC., West Lafayette, Indiana for commercial use by the microbiologists and food scientists for evaluation of food samples for possible contamination with foodborne pathogens.

International Cooperation/Collaboration

1. Three visiting scientists; one from Federal University at Pelatos, Brazil, from Jiangnan University, China and from France worked on BARDOT and evaluated its application on detection and identification of *Bacillus* and *Listeria* at Purdue University.

2. Arun Bhunia also visited Denmark Technical University (DTU) at Copenhagen and discussed BARDOT related research activities with the scientists at that institution.

3. West Virginia University: A BARDOT instrument has been installed in the microbiology department and initial data are encouraging.

4. University of California, Merced: Professor Dan Hirleman has now received a BARDOT instrument and is establishing programs to collaborate.

5. Battelle Laboratories, Columbus Ohio: An instrument was delivered to Battelle collaborators and initial studies have just begun.

Popular press articles


Publications and Presentations


6. Sun, X., Bae, E., Bai, N., Tang, Y., and Bhunia, A.K. Rapid detection and identification of *Bacillus* species based on label-free light scattering sensor (to be submitted to BMC Microbiology)


**Book chapter**


**Proceedings**


**Presentations**


11. Bae, E, 2011. Applying elastic light scattering in bacterial identification-From laboratory to a national surveillance network, Korea Institute of Science and Technology (KIST), Seoul, Korea, Jul 11, 2011

Thesis/Dissertation


Members of BARDOT team

1. Arun Bhunia: PI
2. J. Paul Robinson: Co-PI
3. Euiwon Bae: Co-PI
4. Bartek Rajwa: Co-PI
5. Atul K. Singh: Postdoc
6. Mary-Ann Roshni Amalaradjou: Postdoc
7. Valery Patsekin: Technical Associate
8. Yanjie Tang: Grad student: Strategies to scatterotype Shiga-toxin producing Escherichia coli (STEC) using scatterometer
9. Nan Bai: Grad student: Engineering of BARDOT to detect bacterial micro-colonies
10. Huisung Kim: Grad student: Engineering of BARDOT to detect bacterial micro-colonies
11. Dawei Ying: Grad student: Robust image analysis system for closely related bacterial serovars

Plans for collaboration with USDA-ARS

A second generation BARDOT unit was delivered to Dr. Shu-I Tu and Dr. George Paoli about 3 years back. Now, Advanced Bioimaging Systems, LLC is in the process of delivering the newest model of BARDOT to USDA in early January (1212).