Food Microbiology 47 (2015) 28–35

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Rapid identification and classification of *Campylobacter* spp. using laser optical scattering technology

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ARTICLE INFO

Article history:
Received 14 May 2014
Received in revised form
10 September 2014
Accepted 8 November 2014
Available online 15 November 2014

Keywords:
Campylobacter
Detection
Optical laser scattering technology
Light scatter image
Passive filtration

ABSTRACT

Campylobacter jejuni and *Campylobacter coli* are the two important species responsible for most of the *Campylobacter* infections in humans. Reliable isolation and detection of *Campylobacter* spp. from food samples are challenging due to the interferences from complex food substances and the fastidious growth requirements of this organism. In this study, a novel biosensor-based detection called BARDOT (BActerial Rapid Detection using Optical scattering Technology) was developed for high-throughput screening of *Campylobacter* colonies grown on an agar plate without disrupting the intact colonies. Image pattern characterization and principal component analysis (PCA) of 6909 bacterial colonies showed that the light scatter patterns of *C. jejuni* and *C. coli* were strikingly different from those of *Salmonella* spp., *Escherichia coli* O157:H7, and *Listeria monocytogenes*. Examination of a mixed culture of these microorganisms revealed 85% (34/40) accuracy in differentiating *Campylobacter* from the other three major foodborne pathogens based on the similarity to the scatter patterns in an established library. The application of BARDOT in real food has been addressed through the analysis of *Campylobacter* spiked ground chicken and naturally contaminated fresh chicken pieces. Combined with real-time PCR verification, BARDOT was able to identify *Campylobacter* isolates from retail chicken. Moreover, applying passive filtration to food samples facilitated the isolation of pure *Campylobacter* colonies and therefore overcame the interference of the food matrix on BARDOT analysis.

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1. Introduction

Campylobacter is a major pathogen associated with foodborne illness in the United States (CDC, 2011; Scallan et al., 2011). It can cause food poisoning and gastroenteritis in humans and animals. Among 23 identified species or subspecies, *Campylobacter jejuni* and *Campylobacter coli* account for the majority of cases of *Campylobacter* infections (Friedman et al., 2000; Man, 2011). It is well known that *Campylobacters* preferably inhabit or colonize the intestines of all avian species. High numbers of the bacterial cells have been found in colonized poultry and wild birds (Hue et al., 2010; Irwin et al., 2013). Due to the spread of *Campylobacter* and cross-contamination during food processing, consumption of undercooked chicken or turkey products, unpasteurized milk, and untreated water are considered to be the primary routes of transmission (Denis et al., 2011; Heuvelink et al., 2009; Sheppard et al., 2009).

Campylobacter is Gram-negative, spiral-shaped, and highly motile bacterium with a single flagellum at one or two ends of the cell, which mediates its rapid corkscrew-like motility (Silva et al., 2011). It is a fastidious microorganism which requires a nutrient rich medium (*e.g.*, Brucella) to support its growth. Due to the slow growth rate of *Campylobacter*, traditional culture-based methods for isolation and identification, which are laborious and time-consuming, can take up to 5 days. In recent years, PCR has become the preferred method for detecting *Campylobacter* spp. from a variety of sources because of the technical advantages in speed, specificity, and ability to multiplex (Al Amri et al., 2007; Debruyne et al., 2008; Katzav et al., 2008; Muller et al., 2011).

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http://dx.doi.org/10.1016/j.fm.2014.11.004
0740-0020/Published by Elsevier Ltd.

More recently, real-time PCR has become the method of choice due to its highly sensitive and quantitative detection (He and Chen, 2010; He et al., 2010; Leblanc-Maridor et al., 2011; Ronner and Lindmark, 2007; Sails et al., 2003). However, due to the interference from complex food substances and background microorganisms, effective methods to isolate and subsequently identify *Campylobacter* from food samples remain limited.

The BARDOT system (BActerial Rapid Detection using Optical scattering Technology; Advanced Bioimaging Systems, West Lafayette, IN), initially developed by Purdue University, is an optical laser sensor-based method for rapid identification of bacterial colonies grown on agar medium (Bae et al., 2007; Banada et al., 2007). It directly applies a 635 nm red diode laser beam (low power; 1 mW/mm^2) to the center of a colony and creates a circular symmetric scatter pattern without destroying the cells. The resulting forward light scattered image is automatically captured by a charge-coupled device (CCD) sensor. Distinctive colony scatter patterns among bacterial species are produced depending on the colony's physical characteristics (size, shape, and refractive index, etc.), cell surface chemical compositions, and the interactions with different agar media. These light scattering images are automatically processed using image feature extraction and analysis software associated with the BARDOT instrument. Classification of bacterial samples is based on their similarity to known scatter patterns in a library. The major advantages of using the BARDOT system for bacterial identification are rapidity, automation, and high-throughput processing, none of which are destructive to the morphology of intact colonies. Previously, the BARDOT has been successfully used to identify and differentiate 108 *Listeria* strains belonging to 6 different species (Banada et al., 2007). Additionally, it has been used to differentiate *Listeria, Staphylococcus, Salmonella, Vibrio,* and *Escherichia* at the genus (Banada et al., 2009; Huff et al., 2012; Singh et al., 2014), species (Banada et al., 2007; Huff et al., 2012), and even at the serovar (Singh et al., 2014) level.

Recently, a hyperspectral imaging technique based upon colony spatial and spectral (wavelength between 400 and 900 nm) information for bacteria identification was reported for Shiga Toxin-producing *Escherichia coli* strain determination (Windham et al., 2013). However, the effect of food matrix on colony spatial and spectral characteristics as well as the application to direct detection of pathogens in food samples has not been addressed.

The specific objective of this study was to explore the laser scatter imaging system for rapid screening and preliminary identification of *Campylobacter* spp. from other major foodborne pathogens. If identified as presumptive positives, suspect colonies were confirmed through multiplex qPCR or 16S rRNA sequencing. Application of BARDOT to the isolation and detection of *Campylobacter* from real food has been demonstrated in both spiked and naturally contaminated chicken samples. Interference of chicken matrix and background microflora on BARDOT analysis has been overcome by using a passive filtration technique.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Twenty-six *Campylobacter* strains were used in this study, consisting of 17 strains of *C. jejuni* and 9 strains of *C. coli* (Table 1). Eight strains of *C. jejuni* isolated from environmental and clinical samples were provided by Norman J. Stern (USDA-ARS) (Callicott et al., 2008). The remainder of the *Campylobacter* strains were provided by Robert E. Mandrell (USDA-ARS) and were also isolated from clinical or environmental sources. All *Campylobacter* colonies were grown on Brucella agar (Becton Dickinson, Sparks, MD) which was supplemented with cefoperazone, vancomycin, trimethoprim (each 20 μg/mL) and cycloheximide (50 μg/mL). Plates were incubated for 28–40 h at 42 °C in a microaerobic workstation (Don Whitely Scientific, Ltd., Shipley, UK) with an atmosphere consisting of 85% N_2, 10% CO_2, 5% O_2, and 82% relative humidity. Other microorganisms used for BARDOT library generation and validation are also included in Table 1. These organisms were grown under the same conditions as the *Campylobacter* spp.

Table 1
Bacterial strains used in this study.

Organism	No. of strain tested	Source
Campylobacter jejuni	17	USDA ARS[a], Naval Res. Medical Center,
Campylobacter coli	9	USDA ARS
Enterococcus cecorum	3	Chicken isolate[b]
Enterococcus spp./*faecium*	1	Chicken isolate
Enterococcus faecalis	2	ATCC[c], Unknown
Escherichia K12	1	ATCC
Escherichia coli O157:H7	8	ATCC, CDC[d], USDA FSIS[e], FDA[f], PA Dept. of Health
Listeria monocytogenes	12	ATCC, USDA ARS, Purdue Univ.,
Salmonella Anatum	1	ATCC
Salmonella Enteritidis	5	ATCC, CDC, Wyoming Dept. of Health, Univ. of Pennsylvania
Salmonella Heidelberg	1	USDA FSIS
Salmonella Infantis	1	ATCC
Salmonella Kentucky	2	USDA FSIS
Salmonella Montevideo	1	ATCC
Salmonella Newport	2	CDC
Salmonella Orienberg	1	ATCC
Salmonella St. Paul	1	CDC
Salmonella Typhimurium	4	ATCC, CDC, unknown
Staphylococcus aureus	1	ATCC
Staphylococcus epidermidis	1	ATCC
Staphylococcus intermedius	1	ATCC
Staphylococcus spp.	4	Chicken isolate
Staphylococcus xylosus	1	ATCC

[a] U.S. Department of Agriculture, Agricultural Research Service.
[b] Identified by 16S rRNA sequencing in this study.
[c] American Type Culture Collection.
[d] Centers for Disease Control and Prevention.
[e] U.S. Department of Agriculture, Food Safety and Inspection Service.
[f] Food and Drug Administration, U.S. Department of Health and Human Services.

2.2. BARDOT analysis

Screening and preliminary identification of the samples were done through the use of the Bacteria Rapid Detection using Optical Scattering Technology (BARDOT) system (Advanced Bioimaging Systems, West Lafayette, IN), followed by confirmation of the bacteria with a multiplex PCR assay or 16S rRNA sequencing. BARDOT instrument directly scans individual colonies on a Petri dish plate by using a 635 nm wavelength red diode laser. The light scatter image was automatically captured by a CCD sensor and then analyzed using associated DotBar software (Purdue University, West Lafayette, IN). The system only takes 6 s to scan and classify each colony after all the parameters were set up in the instrument and software. Bacterial identification is based on the characteristics of colony scattering images and comparison to the scatter patterns in a library consisting of known microorganisms. The mechanics of BARDOT and associated image analysis software has been previously described (Banada et al., 2007, 2009).

2.3. Construction of Campylobacter scatter image library

A library containing 26 *Campylobacter* strains, as well as other bacteria of interest, was generated prior to analysis of experimental samples. Briefly, appropriate dilutions of fresh bacterial cultures

were spread plated onto Brucella agar plates containing 20 mL of medium per plate to obtain well separated colonies. An incubation time between 28 and 40 h under the microaerobic conditions described above was determined to be suitable for *C. jejuni* and *C. coli* strains generating colonies within the diameter range of 0.927 ± 0.192 mm, an optimum colony size for BARDOT scanning with a 1 mm laser beam. For each strain, 50 to100 scatter images were captured and composed into the library. When sufficient scatter images for all of the test strains had been obtained and analyzed, a *Campylobacter* library was generated.

2.4. Image analysis and classification

Bacterial light scatter images acquired by BARDOT instrument were represented as greyscale bitmaps and normalized by signal intensity correction and background subtraction. All of the colony scatter images were processed using the DotBar software as described previously (Rajwa et al., 2010). Briefly, two sets of numerical features (pseudo-Zernike moments and Haralick texture descriptors) representing the characteristics of a bacterial colony were used for extracting image data. For each species in the library, over a thousand images were collected from different strains and then converted to large datasets. These datasets were computed and differentiated using 208 elements of the features extracted from each image. The degree of differentiation among these datasets was statistically calculated and presented by a principal component analysis plot and cross-validation results. Bacterial samples were classified against a selected library under the same experimental conditions and image analysis parameters used for constructing the library.

2.5. Mixed cultures

Fresh cultures of *C. jejuni* and *C. coli* were mixed together with overnight grown cultures of *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* in Brucella broth. Appropriate dilutions of the mixed culture were plated onto Brucella agar and incubated at 42 °C under microaerophilic conditions as described above. After 24 and 28 h incubations, colonies in the same set of plates were scanned and classified against the *Campylobacter* scatter image library. To keep tracking the changes of individual colonies in a plate, the agar plate was consistently placed at the same position inside a plate holder at the different times of scanning.

2.6. Preparation of chicken samples

Chicken samples were analyzed for both spiked and naturally occurring *Campylobacter*. Aliquots of 450 g of fresh chicken pieces were weighed into Whirl-Pak stomacher bags with filters (Nasco, Ft. Atkinson, WI), and 200 mL of 0.1% buffered peptone water (BPW) (Becton, Dickinson and Co., Sparks, MD) was added. The samples were manually massaged for 2 min and the filtered chicken rinse was collected. For a spiked sample, 0.5 mL of each diluted *C. coli* RM 2221 and *C. jejuni* 81–176 culture was added to achieve a final concentration of 7500 CFU for each species.

The chicken rinse was then concentrated by centrifugation (Avanti J-25; Beckman Coulter, Brea, CA) at 15,000 × g for 10 min. The pellet was resuspended in 4 mL of 0.1% BPW. Aliquots of 100 μL of 1:10 diluted and undiluted suspensions were spread plated onto Brucella agar and then incubated under the microaerobic conditions described above.

In addition, irradiated ground chicken spiked with *Campylobacter* was also tested for the application of BARDOT in food samples as well as the possible interference from chicken matrix. To filtered stomacher bags, 50 g aliquots of irradiated ground chicken were aseptically added and then spiked with *C. jejuni* 81–176 and *C. coli* RM 2221 to achieve a final concentration of 7500 CFU for each species. To the spiked ground chicken, 150 mL of 0.1% BPW was added and manually massaged. The slurry was filtered through cheesecloth before being subjected to the centrifugation and plating procedures described above.

2.7. DNA extraction

DNA extraction was performed in a 96-well plate format. To a PCR plate, 20 μL of PrepMan Ultra reagent (Applied Biosystems, Foster City, CA) was added to each well. Each suspect colony was selected and placed in a well. The entire plate was placed in a thermocycler (GeneAmp® PCR system 9700; Applied Biosystems) and heated at 99 °C for 10 min to lyse the cells, followed by a cooling step at 20 °C for 2 min. The plate was centrifuged at 6600× g for 10 min. Aliquots of 10 μL of the supernatant were transferred to a new plate for qPCR analysis.

2.8. Multiplex real-time PCR and 16S rRNA sequencing

A multiplex real-time PCR or 16S rRNA sequencing was used to confirm the colonies identified by BARDOT as *C. jejuni* or *C. coli*. The multiplex qPCR was performed on a 7500 Real-Time PCR system (Applied Biosystems, Carlsbad, CA) using optical microtiter plates (Applied Biosystems). *C. jejuni* or *C. coli* specific gene targets, primers and probes, as well as reaction conditions used for the multiplex qPCR were described by He et al. (He et al., 2010). The primers and procedures used for 16S rRNA sequencing identification of unknown bacteria were also reported previously (Irwin et al., 2008).

2.9. Passive filtration

Passive filtration of spiked ground chicken samples was employed to prevent chicken matrix from being directly plated. Ten grams of irradiated ground chicken was spiked with approx. 10, 100, and 1000 CFU/g of *C. jejuni* 81–176 or *C. coli* RM 2221. The inoculum was manually massaged into the ground chicken suspended in 50 mL of Bolton broth (Oxoid, Basingstoke, Hampshire, England) with Bolton's supplement (Oxoid) and laked horse blood (Remel, Lenexa, KS). The liquids were recovered to sterile 50 mL tubes and enriched at 42 °C under microaerobic conditions for 24 h. Ten-fold dilutions of the enriched samples were prepared using Bolton broth with Bolton's supplement and laked horse blood. Sterile cellulose acetate membrane filters (0.65 μm; Millipore, Billerica, MA) were placed in the center of Brucella agar plates. Four drops each containing 20 μL of diluted enriched sample were placed on the filter. The drops were soaked into the filter and agar for 15 min at room temperature before the filter was carefully removed to allow *Campylobacter* cells to move through the filter onto the agar medium. After incubation, the number of cells that passed through the filters was counted, and randomly selected plates were scanned with the BARDOT for presumptive identification. In addition, cell concentrations of the post-enrichments were established using a 6 × 6 drop plating format (Chen et al., 2003).

3. Results and discussion

3.1. Construction of Campylobacter scatter image library

The first essential step of BARDOT identification of *Campylobacter* spp. was to build a library of the light scatter images representing most *C. jejuni* and *C. coli* strains. In this study, a collection of 17 strains of *C. jejuni* and 9 strains of *C. coli* were used for the construction of the *Campylobacter* library. Upon completion of

scanning 50–100 colonies of each strain, a library containing a total of 2248 *Campylobacter* scatter images was generated using BARDOT and its associated image analysis software. Fig. 1 shows a representative sampling of light scatter patterns of *C. jejuni* and *C. coli* colonies collected from these strains. All of the *Campylobacter* scatter patterns in the library display a high degree of similarity.

Bacterial colony morphology is an important aspect for bacterial identification. Light scatter patterns of bacterial colonies mostly depend on colony morphology, such as shape, size, edge, elevation, texture, pigment, and opacity (Bae et al., 2011; Banada et al., 2009). These characteristics can be greatly affected by cultural media and other growth conditions. For consistency, all of the *Campylobacter* colonies subjected to BARDOT analysis were grown on Brucella agar and incubated at 42 °C under microaerobic conditions which were found to allow all of the tested strains to grow preferably. An incubation time between 28 and 40 h was determined to yield *C. jejuni* and *C. coli* colony sizes ranging from 0.927 ± 0.192 mm, which was adequate for forward laser scanning by the BARDOT instrument equipped with a 1 mm diameter laser beam. The time required for developing the suitable size of *Campylobacter* colonies is consistent with the incubation time (24–48 h) recommended by U.S. Food and Drug Administration's Bacterial Analytical Manual (http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm072616.htm).

3.2. Evaluation of Campylobacter scatter image library

To examine if the established library could be used for differentiating *Campylobacter* from other major foodborne pathogens, a total of 4661 scatter images of different strains of *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* (Table 1) were captured and compared to the *Campylobacter* scatter images in the library. From visual observation, the light scatter patterns of *C. jejuni* and *C. coli* colonies are significantly different from those of *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* (Fig. 2A). All of the *Campylobacter* scatter images display multiple smooth concentric rings surrounding the central scatter region whereas the other organisms have asymmetric spiked outward rings in their scatter patterns.

For automatic classification, principal component analysis (PCA) was employed to analyze the large datasets of numerical image features extracted from the library. Fig. 2B shows the distribution of five microorganisms generated from PCA of 6909 scatter images. In this plot, scatter patterns of different *C. jejuni* and *C. coli* strains are positioned in the same area to form a *Campylobacter* cluster, which is distinct from the cluster of *L. monocytogenes* and the third cluster containing overlapped *E. coli* O157:H7 and *Salmonella* spp.

Subsequently, performance of the library was evaluated using 5-fold cross validation with 10 repetitions. The result of cross validation on training dataset is presented as a form of confusion matrix in Table 2. The classification accuracy rates for *Campylobacter* spp., *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* were determined to be 99.1%, 90.1%, 94.8%, and 90%, respectively. However, the accuracy rates for differentiating the species between *C. jejuni* and *C. coli* were 79.2% and 84.0%, which was moderately lower than the classification rates at the genus level. This result is consistent with previous reports of which the appearance of *C. jejuni* colonies was highly similar to *C. coli* in scanning electron micrographs (Ng et al., 1985). Furthermore, this library was evaluated with 365 scatter images of C. *jejuni* and *C. coli* which are independent of the training set. Among these tested colonies, 303 of them (83.01%) were correctly classified. The rest of them were misidentified as *E. coli* O157:H7 (10.69%), *Salmonella* spp. (6.03%), or *L. monocytogenes* (0.27%).

3.3. BARDOT identification of Campylobacter in mixed cultures

To test the differentiation ability of the *Campylobacter* library, a mixed culture consisting of *C. jejuni*, *C. coli, S.* Typhimurium, *E. coli*

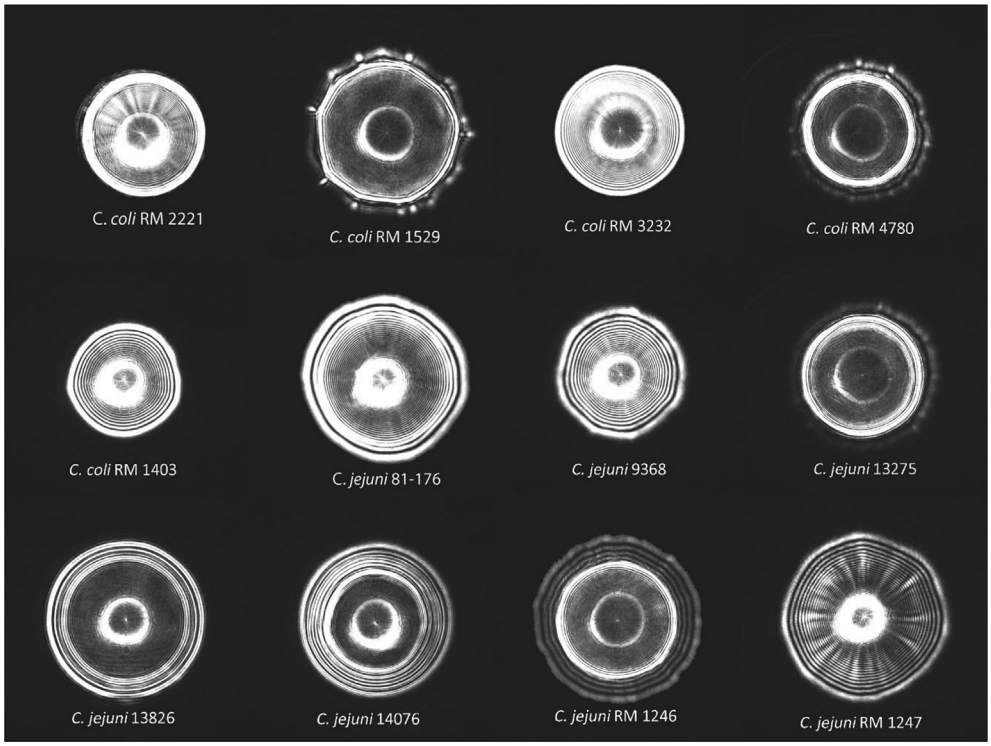

Fig. 1. Light scatter patterns of *C. jejuni* and *C. coli* colonies. A total of 2248 *Campylobacter* scatter images were collected from 17 strains of *C. jejuni* and 9 strains of *C. coli* grown on Brucella agar. Representative scatter patterns of various strains of *C. jejuni* and *C. coli* are shown in here.

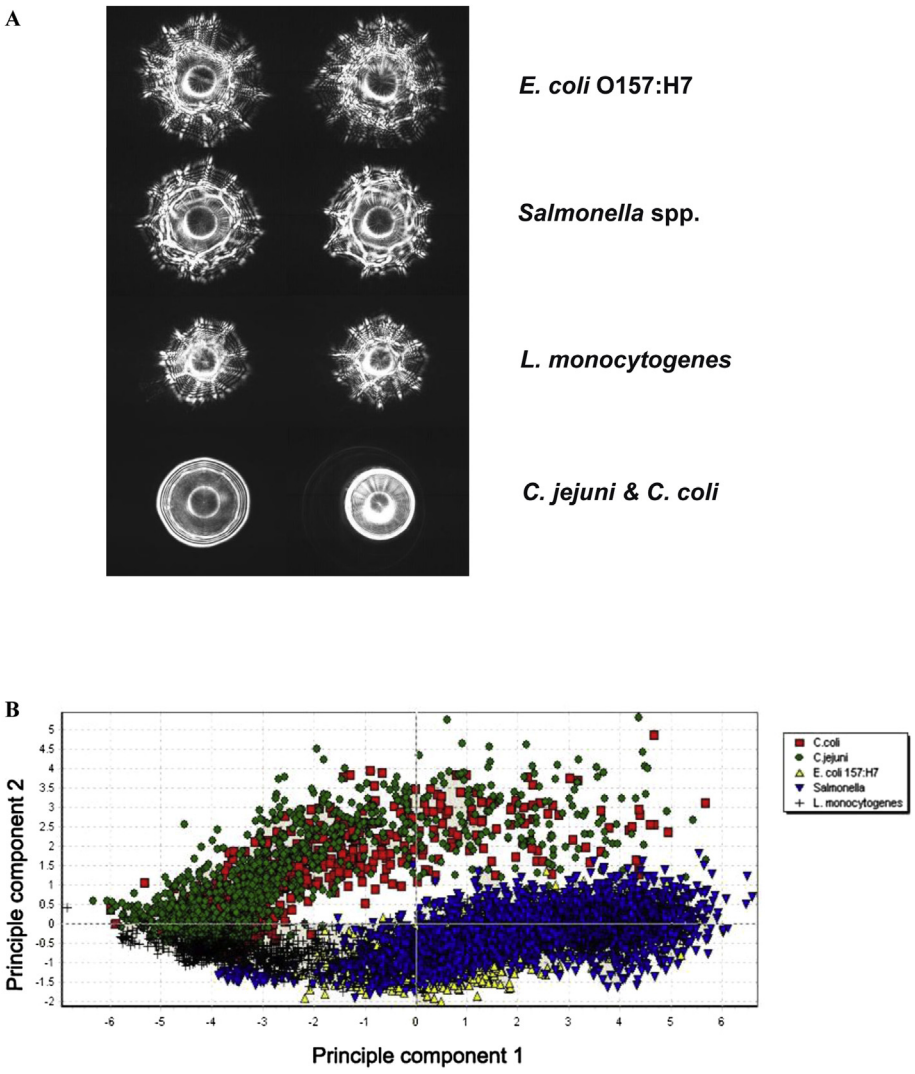

Fig. 2. (A) Comparison of light scatter patterns of *E. coli* O157:H7, *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. Each of the scatter images was acquired from a different strain of these pathogens. (B) Principal component analysis of bacterial scatter images in *Campylobacter* library. Over 6900 light scatter images representing a large strain collection of the five major foodborne pathogens were examined via image feature extraction and principal component analysis.

O157:H7, and *L. monocytogenes* was plated on Brucella agar, incubated under microaerobic conditions, and then subjected to BARDOT identification. Under the growth conditions favorable for *Campylobacter*, the colonies of *C. jejuni* and *C. coli* still grew smaller than others due to the nature of their slow growth. Compared to pure cultures, the growth of *Campylobacter* colonies was not affected by the presence of other bacteria.

After scanning 60 colonies in five plates of the mixed cultures, a total of 40 individual colonies were identified as *Campylobacter* by BARDOT analysis. Of these potential *Campylobacter* colonies, all of the 33 colonies (100%) scanned after 28 h incubation were confirmed as such using multiplex qPCR, whereas only 1 out of the 7 colonies (14.3%) scanned after 24 h incubation were confirmed as *Campylobacter*. This result suggested that a minimum of 28-h growth of *Campylobacter* colonies was required for accurate identification of *Campylobacter* spp. from the mixed bacterial culture using the BARDOT system. It should be noted that these colonies were scanned after 24 and 28 h incubations because prolonged incubation resulted in most of the *Salmonella*, *E. coli*, and *Listeria* colonies outgrowing the upper boundary of a colony size parameter (1.3 mm diameter) in the scanning instrument.

3.4. BARDOT analysis of Campylobacter spiked in chicken samples

The BARDOT system including the scatter image library of *Campylobacter* was further evaluated with both fresh chicken pieces and irradiated ground chicken samples inoculated with a mixed culture of *C. jejuni* 81–176 and *C. coli* RM 2221 at 7500 cells of each strain per sample. With directly plating each of the rinses, the matrix associated with the chicken coated the surfaces of the agar

Table 2
Classification confusion matrix of *Campylobacter* library.

	C. coli	*C. jejuni*	*E. coli* O157:H7	*Salmonella* spp.	*L. monocytogenes*
C. coli	**79.2**	20.3	0.3	0.1	0.2
C. jejuni	15.1	**84**	0.1	0.4	0.5
E. coli O157:H7	0.1	0	**90.1**	9.2	0.5
Salmonella spp.	0	0	4	**94.8**	1.1
L. monocytogenes	0.1	0.3	2.8	6.8	**90**

Note: Each row of the matrix corresponds to the ground truth information while each column corresponds to the actual classification result. The diagonal numbers represent the rates of correct classification and the off-diagonal numbers are the rates of misclassification.

medium which resulted in indistinct scatter images of *Campylobacter* colonies. Fig. 3 shows representative *C. jejuni* and *C. coli* scatter patterns captured from irradiated ground chicken (A) and fresh chicken pieces (B) after directly plating the chicken rinses. As expected, the matrix of ground chicken had far more interference than fresh chicken pieces on the quality of colony scatter images.

To reduce the effects of chicken matrix on BARDOT-based detection of *Campylobacter*, passive filtration was employed instead of directly plating ground chicken rinse on Brucella plates. This filtration method has been used to isolate *Campylobacter* from fecal suspensions, water samples (Jokinen et al., 2012), naturally contaminated broiler meat (Speegle et al., 2009), and retail chicken samples (Quinones et al., 2007). The high motility, corkscrew morphology, and small diameter of *Campylobacter* cells are key attributes to its ability to pass through the filter membrane.

By using a passive filtration method, both *C. jejuni* and *C. coli* spiked at different concentrations into ground chicken were able to be filtered to pure colonies on agar plates. Table 3 shows the numbers of cells passed through the membrane filter and recovered from 20 μL of diluted/undiluted chicken enrichments. A maximal passage of 3.8 log CFU/ml (3.92% recovery rate) of *Campylobacter* was obtained from the 24-hr enriched chicken samples. The chicken matrix collected on the filter appeared to not inhibit the *Campylobacter* cells from penetrating into the agar below. Similar results were reproduced in three independent experiments. In contrast, none of the *E. coli* O157:H7 B1409, *S.* Enteritidis 15159, and *L. monocytogenes* ATCC19111 cells at concentrations of 10^4–10^6 CFU/ml were able to pass through the membrane filters (data not shown). Thus, these rod-shaped, less motile (or non-motile) bacteria were restricted by the membrane.

Selective passage of *Campylobacter* but not the other pathogens through 0.65 μm pore size of the membrane filter not only facilitated the isolation of *Campylobacter* colonies from complex food samples but also overcame the interference of chicken matrix on the follow-up BARDOT detection. Fig. 3C shows the greatly improved *Campylobacter* scatter images acquired from the spiked ground chicken after passage through this filter. The quality of these scatter images acquired from ground chicken samples appears to be equivalent to those from pure cultures. BARDOT was able to accurately classify dozens of randomly selected colonies as *Campylobacter* spp. This technique is also efficient without adding extra steps or additional time to the assay. Moreover, the practicality of passive filtration has been recently demonstrated by the successful isolation of 10 *C. jejuni* or *C. coli* strains from 9 out of 17 retail meat packages after culture enrichment. It was confirmed by the PCR assay that no other background microorganisms passed through the membrane filter (data not shown).

Table 3
Numbers of *Campylobacter* colonies recovered from passive filtration.

Dilution of enrichment	Spiked level (CFU/g)					
	C. Jejuni			*C. coli*		
	17	170	1700	28	280	2800
No dilution	75[a]	137	TNTC[b]	102	TNTC	TNTC
1:10	35	113	75	5	88	73
1:100	6	11	11	1	6	84

[a] The number of *Campylobacter* colonies recovered from passive filtration of 20 μL of diluted or undiluted chicken enrichments spiked with *C. jejuni* or *C. coli*.
[b] TNTC: too numerous to count.

3.5. Identification of Campylobacter naturally occurred in fresh chicken samples

Fresh chicken pieces (wings, breasts, thighs, and legs) from local grocery stores were analyzed for the presence of naturally occurring *Campylobacter* spp. After directly plating the chicken rinses without an enrichment or passive filtration step, many colonies with different morphologies were generated from the background microorganisms in the food. However, the majority of colonies scanned by the BARDOT did not visually resemble the scatter patterns of known *Campylobacter* strains in the library, therefore were excluded for further analyses.

By using the image library merely consisting of the above five pathogens, BARDOT was unsuccessful in differentiating *Campylobacter* spp. from the background microorganisms with similar scatter patterns. Among the scatter patterns visually similar to *Campylobacter*, 24 colonies were randomly picked and subjected to 16S rRNA sequencing for strain identification. Of these, 8 were identified as *Enterococcus cecorum*, 7 each as *Staphylococcus* spp., 2 each as some “uncultured bacterium” and “N/A”, and 1 each as

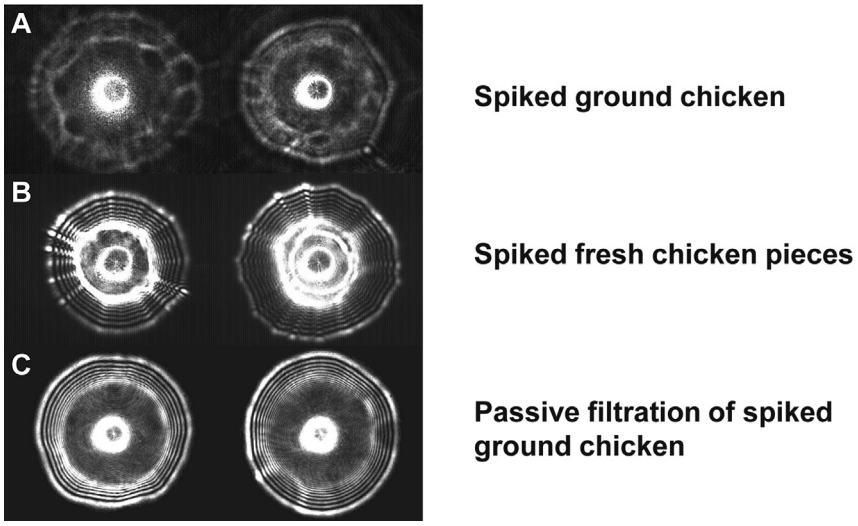

Fig. 3. Light scatter patterns of *C. jejuni* and *C. coli* colonies from chicken samples. Scatter images acquired from directly plating chicken rinses of ground chicken (A) or fresh chicken pieces (B) spiked with a mixture of *C. jejuni* and *C. coli* cultures. (C) Scatter images obtained from the spiked ground chicken rinses that were passed through 0.65 μm pore size of membrane filters onto agar plates.

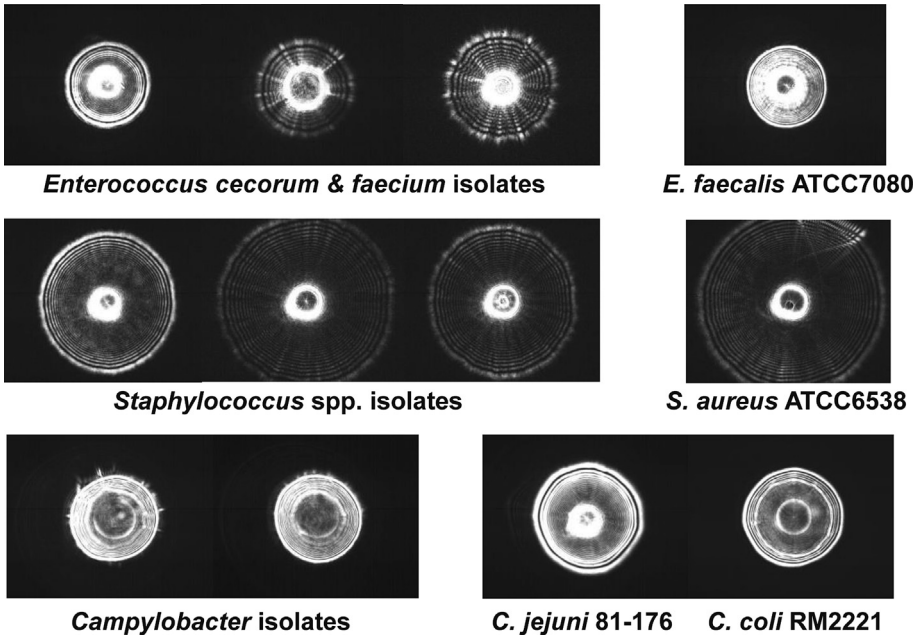

Fig. 4. Light scatter patterns of bacterial isolates from naturally contaminated chicken. These bacterial isolates purified from chicken rinses shared similar scatter patterns with *Campylobacter*. The genus and species information of these isolates was provided by 16S rRNA sequencing. Light scatter patterns of some known bacterial strains are included as references.

Enterococcus spp./*Enterococcus faecium*, *Pantoea* spp./*Buttiauxella* spp., a "swine manure bacterium", *Lactobacillus salivarius*, and *Hafnia alvei*. Apparently, there was a certain degree of similarity in the colony scatter patterns between these bacteria and *Campylobacter* spp. (Fig. 4).

Armed with this new information, the objective was to build a more comprehensive library. Using 4 *Staphylococcus* spp., 3 *E. cecorum*, and 1 *Enterococcus* spp./*E. faecium* isolates from the fresh chicken samples along with several ATCC strains of *Staphylococcus* spp. and *Enterococcus faecalis* (Table 1), an enhanced *Campylobacter* library was constructed in hopes of weeding out some of the false positives from chicken samples. Table 4 shows the results of cross validation of the enhanced library. Over 90% of accuracy was achieved in classifying the genera of *Campylobacter*, *Enterococcus*, and *Staphylococcus* although their light scattering patterns appears to be visually similar to each other. The same set of data from naturally contaminated chicken samples was then re-analyzed against this library and the results are shown in Table 5. Two of the colonies from 450 g sample of chicken wings were identified as potential *Campylobacter* by BARDOT and also confirmed as *C. jejuni* by the multiplex qPCR assay. The improved results from re-analysis indicated that the more comprehensive the library, the more accurate the identification of target organisms by the BARDOT system.

Apparently, there is a discrepancy between the predicted accuracy of training dataset and the actual performance of classifying unknown *Campylobacter* strains in food. In cross validation of the library, training dataset of thousands of images was randomly split for training and validating the training results. After an extensive (5-fold) cross validation exercise to tune the parameters and fit into the model, a high accuracy was predicted. In retail chicken samples, a high variation of scatter patterns was produced from a wide range of background microorganisms. With incomplete environment strains in the image library and unknown target information in the chicken samples, BARDOT had a relative high false positive rate in identifying *Campylobacter* isolates from real food. Therefore, it is necessary to use qPCR or 16S rRNA sequencing method to confirm the preliminary results of BARDOT screening of food samples.

Table 4
Classification confusion matrix of enhanced *Campylobacter* library.

	C. coli	*C. jejuni*	*Enterococcus*	*Staphylococcus*
C. coli	**81.4**	13.4	5	0.2
C. jejuni	8	**91**	0.6	0.4
Enterococcus	3.1	0.2	**94.4**	2.4
Staphylococcus	0	0.1	0.3	**99.6**

Note: Each row of the matrix corresponds to the ground truth information while each column corresponds to the actual classification result. The diagonal numbers represent the rates of correct classification and the off-diagonal numbers are the rates of misclassification.

4. Conclusions

We have demonstrated a rapid and high-throughput method for screening potential *Campylobacter* colonies using laser optical scattering technology. A colony scattering image library has been constructed and enhanced using known *Campylobacter* strains and various chicken isolates. Upon the completion of the scattering image library of target microorganisms, the BARDOT system was able to automatically scan and classify large numbers of bacterial colonies on Petri dish plates without the disruption of intact

Table 5
Identification of *Campylobacter* from fresh chicken pieces using enhanced library.

Sample	Enhanced *Campylobacter* library			Confirmed by PCR	
	Campylobacter	*Enterococcus*	*Staphylococcus*	*C. jejuni*	*C. coli*
Wings	2[a]	1	14	2	0
Breast + pkg liner	2	19	55	0	0
Breast 1	1	2	23	0	0
Breast 2	3	68	144	0	0
Legs	4	104	454	0	0

[a] The number of bacterial colonies identified.

samples for a follow-up qPCR or 16S rRNA sequencing verification. It tremendously saves time and effort for preliminary identification of target microbial pathogens in food, such as raw meat, which typically contains large numbers of background microbiota. Furthermore, applying passive filtration technique to *Campylobacter* in food eliminated the interference from ground chicken matrix, resulting in pure colonies and high quality of colony scatter images. Combined with passive filtration, BARDOT has been proven to be an effective method for the detection and classification of *Campylobacter* from real food samples.

Disclaimer

The USDA is an equal opportunity provider and employer. Mention of trade names is solely for the purpose of providing specific information and does not imply recommendation. Prof. Bhunia has recently gained partial ownership of Advanced Bioimaging Systems, West Lafayette, IN on March 14, 2014. His contribution to the study design and execution of the research was done prior to his ownership of this company.

Acknowledgments

This research was supported by a cooperative agreement between the Agriculture Research Service, U.S. Department of Agriculture (project 8072-42000-072-02G) and the Center for Food Safety Engineering at Purdue University. We thank Dr. Beatriz Quinones at the USDA-ARS-WRRC for supplying the cellulose ester membrane filters, and Drs. Nereus Gunther and George Paoli at the USDA-ARS-ERRC for providing some of the bacterial strains.

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