Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Simultaneous detection of *Salmonella enterica*, *Escherichia coli* and *Listeria monocytogenes* in food using a light scattering sensor

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Keywords
BARDOT, biosensor, detection, *Escherichia coli* O157:H7, light scattering, *Listeria*, PCR, *Salmonella*, SELA, STEC.

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2018/2218: received 23 November 2018, revised 4 February 2019 and accepted 11 February 2019

doi:10.1111/jam.14225

Abstract

Aim: To investigate the use of a light scattering sensor, BActerial Rapid Detection using Optical scattering Technology (BARDOT) coupled with a multipathogen selective medium, *Salmonella, Escherichia* and *Listeria* (SEL), for concurrent detection of the three major foodborne pathogens in a single assay.
Methods and Results: BARDOT was used to detect and distinguish the three major pathogens, *Salmonella enterica*, Shiga toxin-producing *Escherichia coli* (STEC) and *Listeria monocytogenes* from food based on colony scatter signature patterns on SEL agar (SELA). Multiple strains of three test pathogens were grown on SELA, and BARDOT was used to generate colony scatter image libraries for inclusive (SEL Library) and exclusive (non-SEL Library) bacterial group. These pathogens were further differentiated using the SEL scatter image library. Raw chicken and hotdog samples were artificially inoculated with pathogens (100 CFU per 25 g each), and enriched in SEL broth at 37°C for 18 h and colonies were grown on SELA for 11–22 h before screening with BARDOT. The BARDOT sensor successfully detected and differentiated *Salmonella*, STEC and *Listeria* on SELA with high classification accuracy 92–98%, 91–98% and 83–98% positive predictive values (PPV) respectively; whereas the nontarget strains showed only 0–13% PPV. BARDOT-identified colonies were further confirmed by multiplex PCR targeting *inlB* gene of *L. monocytogenes*, *stx2* of STEC and *sefA* of *S. enterica* serovar Enteritidis.
Conclusions: The results show that BARDOT coupled with SELA can efficiently screen for the presence of three major pathogens simultaneously in a test sample within 29–40 h.
Significance and Impact of the Study: This innovative SELA-BARDOT detection platform can reduce turnaround time and economic burden on food industries by offering a label-free, noninvasive on-plate multipathogen screening technology for reducing microbial food safety and public health concerns.

Introduction

Microbial food safety is a global concern and reducing the foodborne disease burden is a major challenge for regulatory agencies and food industries (Nyachuba 2010). *Salmonella enterica*, *Listeria monocytogenes* and Shiga toxin-producing *Escherichia coli* (STEC) are the three major foodborne pathogens and are implicated in several outbreaks, which are commonly associated with ready-to-eat food, poultry, meats, fruits and vegetables, seafood, milk and dairy products (Zweifel and Stephan 2012; Walsh *et al.* 2014; Callejón *et al.* 2015). Rapid and

sensitive detection and identification of foodborne pathogens are critical for quality assurance and pathogen tracking within the food production and supply chain. Biosensor platforms are increasingly being tested as the new generation detection tools and are viewed as important critical development in food safety applications for routine surveillance in food processing facilities and for use in food defence emergencies (Velusamy *et al.* 2010; Gehring and Tu 2011; Ohk and Bhunia 2013; Bhunia 2014; Cho *et al.* 2014; Yoo and Lee 2016). Biosensors, in general, are sensitive and provide results much faster than the conventional methods. However, the assay accuracy depends on the specificity of biorecognition molecules used for capturing and reporting of an analyte and the quality of the sample matrix (Bhunia 2014).

We have developed a light scattering sensor called BActerial Rapid Detection using Optical scatter Technology (BARDOT) at Purdue University (Bae *et al.* 2007; Banada *et al.* 2007, 2009; Bhunia *et al.* 2012). BARDOT is an automated colony-screening system that utilizes a 635-nm red diode laser. When the laser beam impinges through the centre of individual colonies it generates scatter signature that is unique for a given bacterial species (Banada *et al.* 2009). These phenotypic characteristics of colonies are obtained from a standard plating method to provide a real-time, nondestructive and high-throughput method without any labelling reagents or probes (Banada *et al.* 2009; Bhunia *et al.* 2012; Singh *et al.* 2014). BARDOT has been used for the detection and identification of *Vibrio* species (Huff *et al.* 2012), *L. monocytogenes* (Banada *et al.* 2007, 2009), *S. enterica* (Singh *et al.* 2014; Abdelhaseib *et al.* 2016), Shiga toxin-producing *E. coli* (Tang *et al.* 2014), *Campylobacter* sp. (He *et al.* 2015), *Bacillus* species (Singh *et al.* 2015) and *Staphylococcus aureus* (Alsulami *et al.* 2018) from various naturally contaminated or spiked food samples. BARDOT was also used for detection and differentiation of several bacterial species belonging to the Enterobacteriaceae family (Singh and Bhunia 2016) and has been used to validate the application of pathogen enrichment device system for sample processing and preparation (Hahm *et al.* 2015). However, BARDOT has not been investigated for its ability to detect multiple foodborne pathogens simultaneously on a single selective/differential agar plate.

In the United States, the conventional culture-based method employs a standard United States Department of Agriculture- or the Food and Drug Administration-approved protocols for isolation and identification of *Listeria*, *Salmonella* and *E. coli* O157:H7, which require around 5–10 days to confirm the presence or absence in the test samples. These culture-based methods are still considered a gold standard and are used as a reference method to validate and compare the results of novel biosensors and foodborne pathogen detection methods before accreditation of new methods (Bhunia 2014). However, these methods are arduous and require individual selective media for initial isolation and subsequent phenotypic (biochemical) or molecular characterization for each pathogen identification. These conditions pose a constraint and burden on quality control and on testing laboratories associated with the food industry, where personnel has to use different selective media and confirmatory test for each pathogen with a turnaround time of 5–10 days. Therefore, the availability of a single selective enrichment medium for multiple pathogens coupled with an on-plate colony-screening technology is desirable, which will not only reduce the burden time but also reduce the microbiological testing cost per sample.

The current industry trend emphasizes the need for the detection of multiple pathogens on a single-assay platform to reduce the cost of testing per pathogen. Furthermore, a multipathogen detection strategy could mitigate the industry and regulatory needs in the mandatory testing of food products for many pathogens prior to sale. We investigated if BARDOT could be used for detection of the three major foodborne pathogens, *S. enterica*, STEC including *E. coli* O157:H7 and *L. monocytogenes* present in the same sample at any combinations. BARDOT-based pathogen detection relies on the use of a suitable pathogen-specific selective agar medium (Bhunia 2014). Therefore, for concurrent multipathogen detection, it is desirable to use a single selective agar medium that would favour the growth of all three pathogens equally while suppressing the growth of undesirable background bacteria, thus reducing the overcrowding of the plate and subsequent lessening of interference or false-positive results. We employed *Salmonella*, *Escherichia*, and *Listeria* (SEL), a semiselective multipathogen enrichment medium (Kim and Bhunia 2008) to determine if this could be used as an enrichment broth as well as a selective agar medium to generate unique scatter patterns of colonies for the three target pathogens.

Materials and methods

Bacterial cultures and growth media

Salmonella, *Listeria*, *E. coli* and other bacterial cultures used in this study are listed in Table 1. Bacterial cultures were revived from frozen (−80°C) glycerol (20%) stocks by inoculating a loop full of culture in brain heart infusion broth (BHI) or tryptic soy broth supplemented with 0·6% yeast extract and incubated at 37°C overnight. SEL agar (SELA) plates were prepared as described before (Kim and Bhunia 2008) with the addition of 1·5% agar. After autoclaving, antimicrobial supplements were added

to the tempered medium (45°C). The antimicrobial supplement contains acriflavine (0·01 g l^{-1}), cycloheximide (0·05 g l^{-1}), fosfomycin (0·05 g l^{-1}) and nalidixic acid (0·002 g l^{-1}) (Kim and Bhunia 2008). Buffered Listeria enrichment broth (BLEB) was prepared with antimicrobial supplement (0·01 g l^{-1} acriflavine, 0·05 g l^{-1} cycloheximide, and 0·04 g l^{-1} nalidixic acid). Other media used include BHI agar and TSA-YE (Neogen, Lansing, MI), xylose lysine tergitol 4 (XLT4, BD) for *Salmonella*, sorbitol MacConkey (BD, Franklin Lakes, NJ) for *E. coli*, and modified Oxford (MOX; Neogen) agar for *Listeria*. For creation of colony scatter signature libraries, bacterial cultures were decimally diluted, plated on agar plates, incubated at 37°C until the colony diameter reached 1·0 ± 0·2 mm and scanned using BARDOT.

Optimal incubation time to capture colony scatter patterns on SELA

Before developing the colony scatter image library, it was imperative to find the optimal incubation time to capture the scatter signature (Bae *et al.* 2008). To achieve this, representative cultures of *S. enterica*, *E. coli* and *Listeria* were incubated for a variable time period on SELA to select the optimal incubation time to capture the most distinguishing scatter signature. Scatter patterns of *E. coli*

Table 1 List of cultures used in this study and validation of scatter image libraries

			%PPV ± SEM match with the library		% PPV ± SEM match with SEL library		
Bacteria	No. of strains (*n*)	Source	SEL	Non-SEL	*Salmonella*	*E. coli*	*Listeria*
SEL group							
Salmonella enterica							
S. Enteritidis	03	CDC, Ottawa, NVSL	95 ± 5·4	5·0 ± 5·4	92·5 ± 3·3	4·5 ± 3·4	3 ± 2·7
S. Typhimurium	03	ADDL	97 ± 0·6	3·0 ± 0·6	92 ± 3·1	6·5 ± 3·3	1·5 ± 0·1
S. Newport	03	ISDH	93·6 ± 0·4	6·3 ± 0·4	90 ± 5·1	0·5 ± 0·3	9·5 ± 5·1
S. Heidelberg	02	ISDH	94 ± 2	6·0 ± 2·0	98 ± 1	0	2 ± 1
S. Javiana	03	ISDH	96·5 ± 2·9	3·5 ± 2·9	94 ± 3·2	5 ± 2·4	1 ± 0·8
STEC							
O157:H7	05	FDA, CDC, ATCC	97 ± 2·4	3 ± 2·4	6·8 ± 4·2	93·2 ± 4·2	0
O26:H11	02	P. Fratamico; BLCC	89 ± 6·9	11 ± 6·9	0	94 ± 5·9	6 ± 5·9
O45:H2	02	P. Fratamico	90 ± 3	10 ± 3	3·5 ± 2·5	95 ± 3·3	1·5 ± 0·7
O103:H11	02	P. Fratamico	92 ± 8	8 ± 8	0·5 ± 0·4	91·5 ± 4·8	8 ± 4·8
O111:NM	02	P. Fratamico	93·5 ± 4·5	6·5 ± 4·5	2·0 ± 0	94 ± 2	4 ± 1·5
O121:H19	02	P. Fratamico	93 ± 6·9	7 ± 6·9	3·5 ± 2·5	96·5 ± 2·5	0
O145:NM	02	P. Fratamico	87 ± 3·7	13 ± 3·7	2·0 ± 1·0	98 ± 1	0
Listeria sp.							
L. monocytogenes	05	CDC, FDA	96·4 ± 1·7	3·6 ± 1·7	1·0	2 ± 1·3	98 ± 1·3
L. ivanovii	03	ATCC	84·6 ± 7·9	15·3 ± 7·9	3 ± 1·6	2·0	95 ± 1·6
L. innocua	03	CDC	90·6 ± 7·3	9·4 ± 7·3	8 ± 1	3 ± 1·8	89 ± 2·4
L. seeligeri	03	ATCC; FDA	100	0	11 ± 0·8	5 ± 0·4	84 ± 2·8
L. welshimeri	03	ATCC; FDA	91·6 ± 6·4	8·3 ± 6·4	9 ± 7·3	7·5 ± 0·9	83·5 ± 7·5
L. marthii	01	ATCC	88	12	2	5	93
L. grayi	01	ATCC	100	0	9	3·5	83·5
Non-SEL group							
P. aeruginosa	01	ATCC	0	100	1	80	19
C. freundii	01	PRI	13	87	42	58	0
K. pneumoniae	01	NRRL	2	98	7	63	30
A. baumanii	01	PRI	0	100	0	38	62
S. marcescens	01	NRRL	4	96	16	72	12
H. alvei	01	BLCC	0	100	20	75	5
E. aerogenes	01	ATCC	0	100	21	63	16
S. aureus	01	ATCC	0	100	4	26	70

FDA, Food and Drug Administration, Silver spring, MD; CDC, Centers for Disease Control and Prevention, Atlanta, GA; ATCC, American Type Culture Collection, Manassas, VA; NRRL, Northern Regional Research Laboratory, Peoria, IL; PRI, Presque Isle Cultures, Erie, PA; P. Fratamico; USDA-ARS, ERRC, Philadelphia, PA; BLCC, Bhunia lab culture collection, Purdue University, West Lafayette, IN, ADDL, Animal Disease Diagnostic laboratory, Purdue University, West Lafayette, IN; PPV, positive predictive value (classification accuracy); SEM, standard error of mean; SELA, *Salmonella*, *Escherichia*, *Listeria* agar medium.

O157:H7 EDL 933 were captured at 9, 11–12 and 16 h; *S. enterica* serovar Enteritidis PT21 at 10, 12–13, 16 h; and *L. monocytogenes* F4244 at 18, 21–33, 25 h. Colony diameter was also measured at these time points to correlate the differentiating feature of the colony with their sizes to select an optimal incubation time.

Scatter image library generation and image analysis

To build a light-scatter image library, all freshly grown bacterial cultures (Table 1) were serially diluted in 20 mmol l^{-1} phosphate-buffered saline, pH 7·0 (PBS) plated onto SELA to obtain 50–100 colonies per plate, and incubated at 37°C until the colony diameter reached to 1 ± 0·2 mm. The colonies were then screened by BARDOT (Banada *et al.* 2009; Singh *et al.* 2014) where the scatter images were acquired by illuminating a 635-nm red diode laser through the centre of each colony (Tang *et al.* 2014). For building a scatter image library, at least images from 50 colonies were acquired from each Petri dish from two independent set of experiments. We created two sets of scatter image library: (i) SEL (inclusive group) and non-SEL (exclusive group) scatter image libraries which contained a total of 5406 scatter images consisting of 4197 scatter images for SEL and 1209 scatter images for non-SEL bacterial species (Table 1). These scatter image libraries were used to screen and differentiate inclusive bacterial panel consisting of *Salmonella*, *E. coli* and *Listeria* from the exclusive bacterial panel. (ii) Individual genus-specific library for *Salmonella* (top 5 serovars, 14 strains), *E. coli* (O157 and non-O157 STEC, 17 strains) and *Listeria*, primarily the *Listeria sensu stricto* group plus *L. grayi* (7 species, 20 strains), which contained a total of 4197 scatter images consisting of 1264 scatter patterns for *S. enterica*, 1108 scatter patterns for *E. coli* and 1825 scatter patterns for *Listeria* species. The data analysis with the scatter image libraries generated a cross-validation matrix, which provided classification accuracy in terms of positive predictive values (PPVs), that is, the probability of finding an identified colony as true positive to the specific group in the scatter image library set (Tang *et al.* 2014).

Bacterial colony scatter images acquired by BARDOT were analysed with image analysis software as described before (Ahmed *et al.* 2013) where two key features of the scattered patterns were used in the analysis: (a) Zernike feature, the rotation-invariant feature (circularly symmetrical patterns) representing a circular and radial feature in the image. (b) Haralick feature, the texture features representing granularity of the scatter image. The mixture of these two features together resulted in hundreds of signature attributes from a single 2D image, which could be used as an orthogonal basis for the fingerprint library. Also, to validate a specific pathogen identification, non-SEL scatter image libraries along with the separate scatter pattern libraries of *Salmonella*, *E. coli* and *Listeria* were used. To test the robustness of the scatter image library, we also tested the scatter pattern of each strain which was not included in the library against SEL, non-SEL, *Salmonella*, *E. coli* and *Listeria*-specific libraries and presented results in the form of percent PPV (Table 1).

Mixed culture analysis

For mixed culture analysis, a 100-μl aliquot of each freshly grown (BHI) culture of *S. enterica* serovar Enteritidis PT21, and *E. coli* O157:H7 EDL933 and 500 μl of *L. monocytogenes* F4244 were mixed and serially diluted and plated on SELA to achieve 50–100 colonies per plate in duplicate. Each plate was scanned at 12 and 22 h to acquire the scatter pattern of colonies. Scatter pattern of colonies was first matched with SEL and non-SEL bacterial libraries and then matched with genus-specific scatter image library as discussed above. BARDOT-identified colonies using the genus-specific (*Salmonella*, *Escherichia* and *Listeria*) scatter image library were further confirmed by multiplex PCR (mPCR) targeting specific genes of *Salmonella*, *E. coli* and *Listeria* as above.

Multipathogen detection from inoculated food products

A total of five hotdog packages and five raw chicken breast samples (450 g) were purchased from a local grocery store and were used for multipathogen detection using SELA. Twenty-five grams of each meat sample was weighed in a stomacher bag (Seward, Cincinnati, OH) and inoculated with about 100 CFU of *S.* Enteritidis, *L. monocytogenes* and *E. coli* O157:H7 separately or a mixture of all three pathogens and held for 20 min at room temperature in a biosafety cabinet. A 225 ml of SEL broth (Kim and Bhunia 2008) was added in each bag, homogenized for 2 min using a stomacher machine (Model 400 Bags; Seward Ltd, West Sussex, UK) and the bags were incubated at 37°C for 18 h for bacterial enrichment. An aliquot of each sample was serially diluted in PBS and surface plated on SELA. Plates were scanned using BARDOT after 11, 13 and 21 h of incubation at 37°C or when the colony diameter reached to about 1 mm, the scatter patterns of colonies on SELA were then compared with the scatter image libraries of SEL and non-SEL bacteria. Colonies obtained from SELA and the USDA method were further verified by mPCR using pathogen-specific gene primers.

PCR confirmation

Multiplex PCR assays were employed to validate the identity of the BARDOT-identified colonies. DNA was

extracted from freshly grown (4 h) culture by boiling (Ngamwongsatit *et al.* 2008). Ultrapure sterile water was used as a negative control. mPCR using the following pathogen-specific gene primers (Table S1) were used for confirmation of each pathogen: Fimbrial protein-encoding gene, *sefA*-specific primers for *S.* Enteritidis (Woodward and Kirwan 1996), invasion protein-encoding gene, *inlB*-specific primers for *L. monocytogenes* (Ericsson *et al.* 2000) and Shiga toxin-encoding gene, *stx2*-specific primers for *E. coli* O157:H7 (Cebula *et al.* 1995). Each PCR

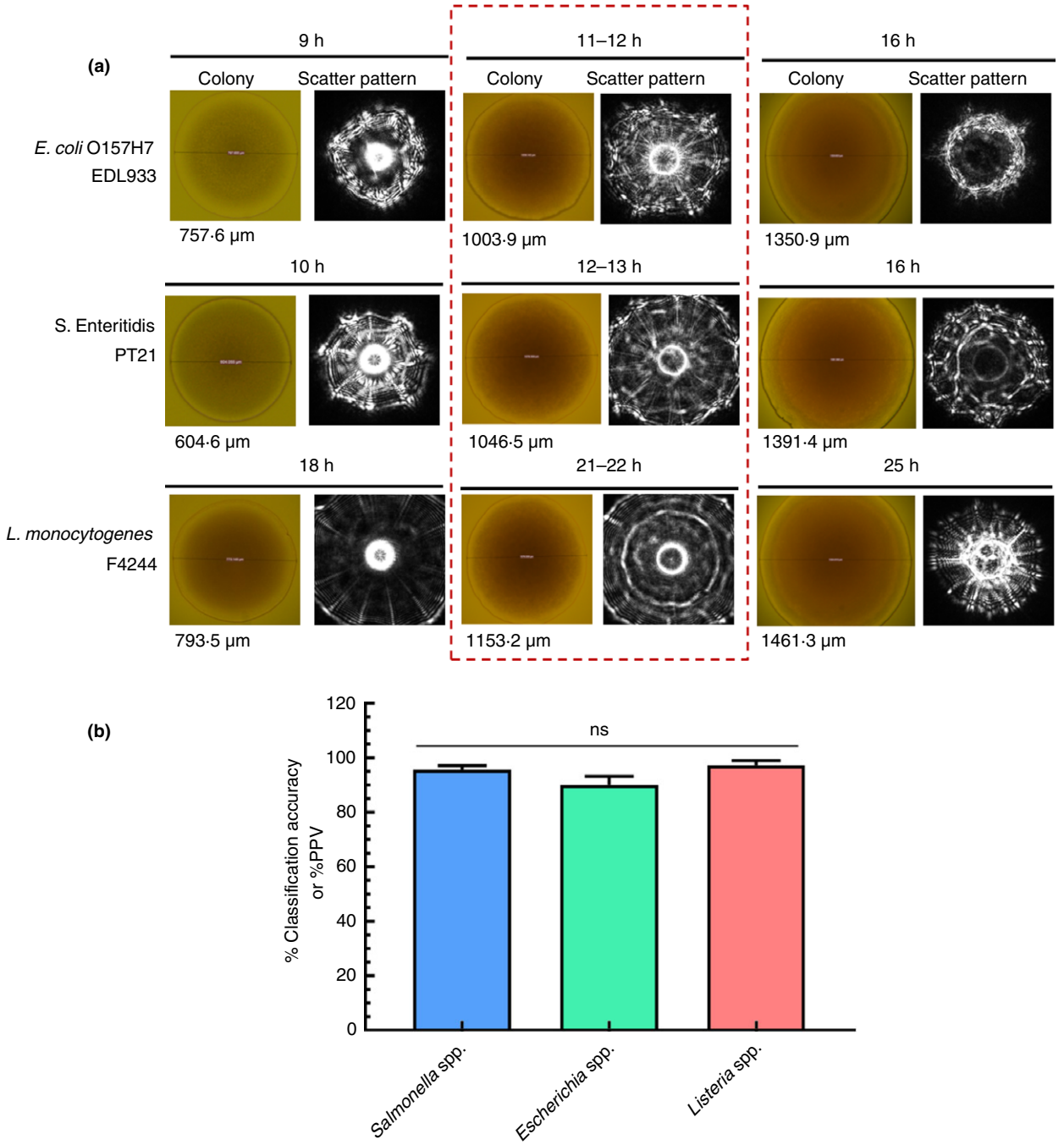

Figure 1 Time-dependent changes in colony size and scatter pattern of representative inclusive strains on SEL agar (SELA) plate. (a) Scatter pattern and colony size of *Escherichia coli* O157:H7, *Salmonella* Enteritidis PT21 and *Listeria monocytogenes* F4244 on SELA to find the optimal incubation time. (b) Comparison of scatter pattern of all strains belonging to *Salmonella* sp., *E. coli* serotypes and *Listeria* sp. on SELA in terms of classification accuracy (PPV). See also Table 1. Numerical values in a micrometre scale represent colony diameter at a specified time point. [Colour figure can be viewed at wileyonlinelibrary.com]

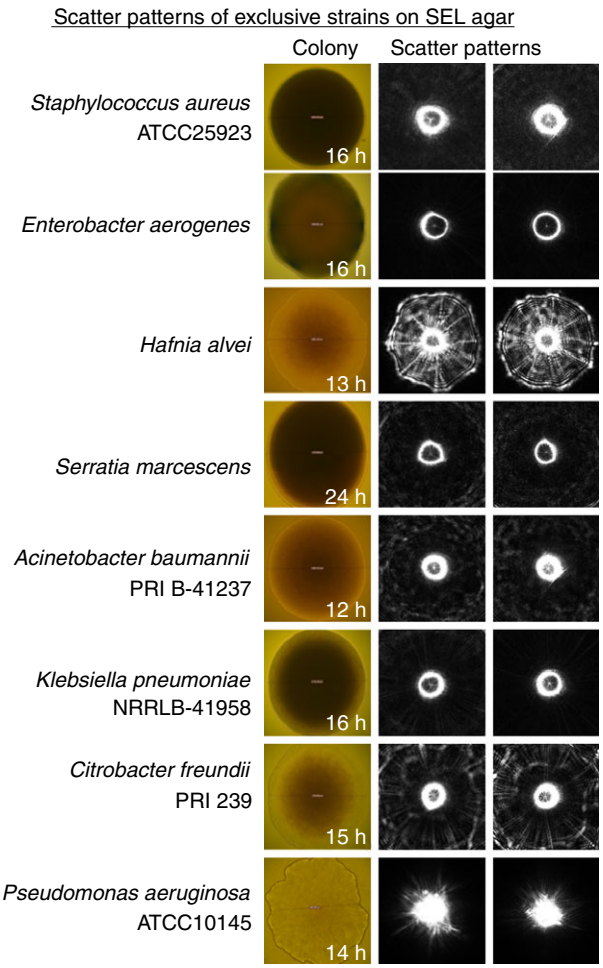

Figure 2 Scatter pattern of colonies of bacteria other than *Salmonella*, *Escherichia coli* and *Listeria* (exclusive or non-SEL) strains on SELA. Scatter patterns were acquired when the size reached 1·0 ± 0·2 mm. Inset numerical values represent the incubation time when colony scatter patterns were captured. [Colour figure can be viewed at wileyonlinelibrary.com]

mixture (25 μl) contained 100 ng of each DNA template, 15 pmol of each primer and 1 U of *Taq* polymerase, 200 µmol of each dNTP, 10 mmol l^{-1} Tris-HCl, 50 mmol l^{-1} KCl and 1·5 nmol l^{-1} $MgCl_2$. Thermal cycling (Applied Biosystems, Foster City, CA) condition included, DNA denaturation at 94°C for 3 min, 40 amplification cycles consisting of 1 min of denaturation at 94°C, 1·5 min of annealing at 60°C and 1·5 min of elongation at 72°C. Amplified DNA products were detected in agarose gel (1%, w/v) containing 3 μl of 10 mg ml^{-1} of ethidium bromide stock solution.

Statistical analysis

GraphPad Prism (ver. 7.04) was used to perform statistical analysis. Multiple comparisons were performed with the PPVs for species and serovars of *Salmonella*, *Escherichia* and *Listeria* scatter image libraries.

Results

Time-resolved scatter patterns of *Salmonella*, STEC and *Listeria* colonies

To find the optimal incubation time period, we examined colony growth and the changes in scatter patterns of *S. enterica*, *E. coli* and *Listeria* species over time on SELA. Time-resolved measurement of colony diameter of the three targeted pathogens showed incremental growth in colony size on SELA plate during 10–13 h of incubation for both *Salmonella* and *E. coli*, and consequent development in the signature scatter patterns (Fig. 1a). After 9–10 h of incubation, the colony size for *Salmonella* and STEC was about 604 and 757 μm, respectively, which was well below our desired 1 mm diameter. At this early time point, scatter patterns were not well-defined and also did not reveal any differentiating features; however, after 11–13 h incubation, the colony diameter reached 1003·9 ± 20·0 μm and showed characteristic and well-defined scatter patterns. At this time point, scatter patterns were highly distinguishable between *Salmonella* and *E. coli* strains (Fig. 1a). In case of *Listeria* species, *L. monocytogenes* took longer, that is, 21–22 h to reach the colony diameter of 1153·2 ± 10·0 μm and showed the characteristic spokes and concentric rings (Fig. 1a). Growth analysis plots of *E. coli*, *S.* Enteritidis and *L. monocytogenes* colony size *vs* incubation time on SELA also indicate that the colony diameter of 1 mm was reached in the logarithmic phase on the agar plate (Fig. S1). As *Listeria* is a slow grower, its growth profile was different from *E. coli* and *Salmonella* (Fig. S1). We also analysed the scatter image of *Salmonella*, *E. coli*, and *Listeria* using image classifier and compared the PPV difference in the scatter feature of *Salmonella*, *E. coli* and *Listeria*. The PPV values for *Salmonella*, *E. coli* and *Listeria* was very high 95·7, 90·1 and 97·4%, respectively, and were statistically insignificant ($P < 0·5$) suggesting high individual PPV for differentiating target pathogens using the image classifier (Fig. 1b). Taken together, these data suggest that optimal incubation time for *E. coli*, *Salmonella* and *Listeria* species on SELA is 11–12, 12–13 and 21–-22 h, respectively, and the classification accuracy for each pathogen identification was >90%.

Validation and comparison of SELA scatter image library

To test the robustness of scatter image libraries of SEL, non-SEL and genus-specific *Salmonella*, *E. coli* and *Listeria* developed in this study, we used scatter images, which were

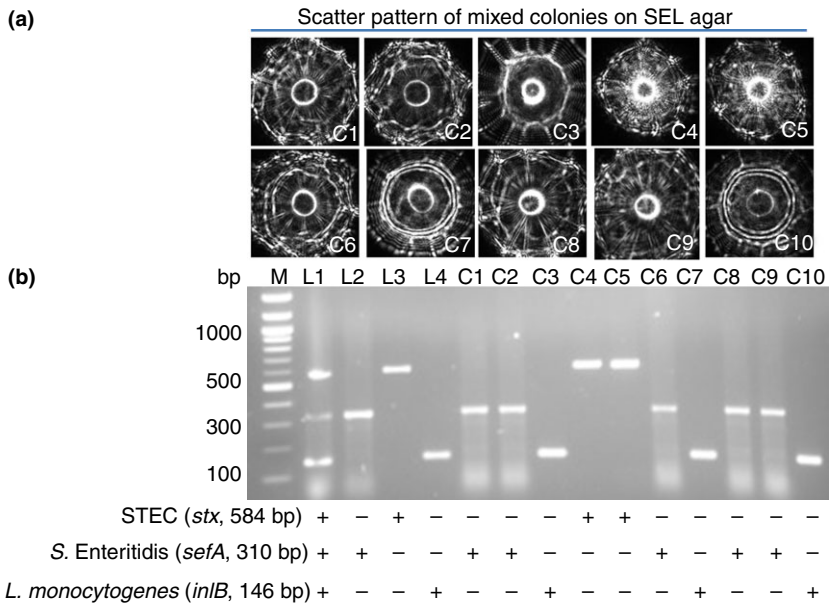

Figure 3 Mixed culture analysis using BARDOT on SEL agar (SELA) plate. (a) Scatter patterns of mixed colonies of *S.* Enteritidis PT21, *Escherichia coli* O157:H7 EDL 933, *Listeria monocytogenes* F4244 on SELA. (b) BARDOT-identified colonies were confirmed with mPCR using *E. coli* O157:H7, *Salmonella* and *L. monocytogenes*-specific primers. [Colour figure can be viewed at wileyonlinelibrary.com]

not used to train the libraries. Employing SEL library, the classification accuracy (%PPV) for *Salmonella* serovars was determined to be 93–97%, *E. coli* 87–97%, *Listeria* species 84–100% (Table 1), while for non-SEL (exclusive set) library, accuracy was low (0–15%) (Table 1, Fig. 2). These data demonstrate the robustness of the scatter image library, which was able to differentiate SEL (inclusive) bacteria from non-SEL (exclusive) bacteria with high classification accuracy and detect multipathogens on SELA.

Individual pathogen-specific libraries also showed high PPV scores. *Salmonella*-specific library (Fig. S2) yielded 90–98% PPV with *Salmonella* serovars, 0–6·5% with *E. coli* and 1–9·5% with *Listeria* (Table 1). Likewise, *E. coli*-specific library (Fig. S3) yielded 91–94% PPV with *E. coli*, 0–6·8% with *Salmonella* and 0–8% with *Listeria*. While *Listeria*-specific library (Fig. S4) yielded 83–98% PPV with *Listeria*, 0–11% with *Salmonella* and 2–7·5% with *E. coli*. Collectively, these data demonstrate that SEL, non-SEL and genus-specific *Salmonella*, *E. coli* and *Listeria* scatter image libraries are highly robust and are able to differentiate SEL (inclusive) bacteria from non-SEL (exclusive) and differentiate multipathogens (*Salmonella*, *E. coli* and *Listeria*) with high classification accuracy (Table 1).

Mixed culture analysis on SELA

Mixed culture analysis of *Salmonella* Enteritidis PT21, *E. coli* O157:H7 EDL 933 and *L. monocytogenes* F4244 on SELA revealed substantial differences in their scatter patterns. Scatter pattern of *L. monocytogenes* revealed a more circular feature compared to *S.* Enteritidis and *E. coli* O157, whereas scatter pattern of *E. coli* O157 was more granular between the central and proximal end of the colony (Fig. 3a). Matching of scatter pattern with the library showed more than 90% PPV match with the respective genus in the SEL library (data not shown). Five *S.* Enteritidis, three *L. monocytogenes* and two *E. coli* O157 colonies were randomly picked from the BARDOT-positive plates for the mPCR analysis targeting *S.* Enteritidis-specific *sefA* (310 bp), STEC-specific *stx* (584 bp) and *L. monocytogenes*-specific *inlB* (184 bp) genes (Table S1). All BARDOT-identified colonies were correctly amplified with mPCR resulting in 100% match with BARDOT results (Fig. 3b). Control samples consisting of *S.* Enteritidis, *E. coli* O157:H7 and *L. monocytogenes* were also amplified in uniplex and multiplex to confirm the working chemistry of mPCR (Fig. 3b).

Multipathogen detection from food samples using BARDOT on SELA

Bacterial colonies on SELA plate from inoculated meat samples (chicken drumstick and hotdogs) were screened with BARDOT at 11, 13 and 21 h (Fig. 4a) and colony scatter patterns were matched with the SEL library (Fig. 4b). Classification accuracy was determined to be >90% PPV for *Salmonella*, >91% PPV for *E. coli* and >83% PPV for *Listeria* when compared with the data presented in Table 1. Furthermore, visual comparison of scatter patterns of *Salmonella*, *E. coli* and *Listeria* colonies from spiked food samples with the images from the library revealed a remarkable homogeneity in the scatter patterns, while the scatter pattern of background commensal bacterial colonies from the chicken drumstick and

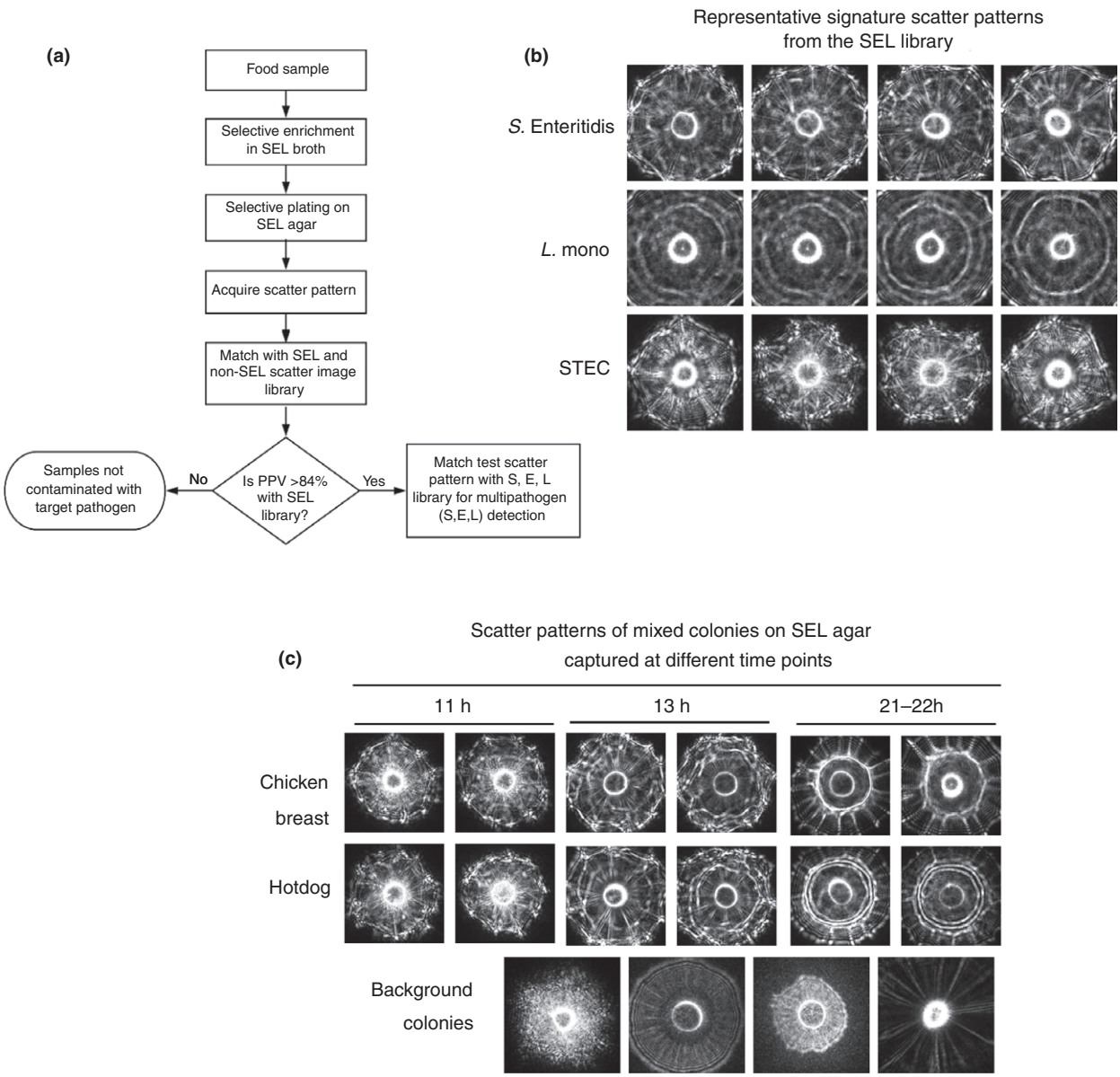

Figure 4 Application of BARDOT for detection of *Salmonella*, *Escherichia coli* and *Listeria* from inoculated raw chicken drumstick and ready-to-eat (hotdog) samples. (a) Flow-diagram for food sample testing. (b) Representative scatter patterns of *Salmonella enterica* serovar Enteritidis PT21 (*S. enterica*), *E. coli* O157:H7 EDL 933 (STEC) and *L. monocytogenes* F4244 (*L. mono*) colonies on SELA used in the training of SEL scatter image library. (c) Scatter patterns of colonies obtained from inoculated food samples at three different time points, 11 h (for *E. coli*), 13 h (for *Salmonella*) and 22 h for (for *Listeria*). Scatter patterns of background bacterial colonies are being also captured at these time points.

hotdog food matrix was substantially different from the three target pathogens (Fig. 4b,c).

A total of 115 BARDOT-positive colonies from two food samples were selected after matching with the SEL library. Of which 60 colonies were randomly picked and confirmed by mPCR. For the inoculated chicken sample, mPCR confirmed 13 of 15 colonies (86·7%) for both *S.* Enteritidis and *E. coli* O157:H7, while 15 of 15 colonies (100%) for *L. monocytogenes*. In hotdog samples, mPCR confirmed 14 of 15 colonies (93%) for *S.* Enteritidis, 13 of 15 colonies (87·6%) for *E. coli* O157:H7 and 15 of 15 colonies (100%) for *L. monocytogenes* (Table 2, Fig. 4). These results indicate that the BARDOT is capable of differentiating three major foodborne pathogens, *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes,* if present in the same sample and grown on the SEL agar after an enrichment in the SEL broth. However, depending on the target pathogen, the detection time including the enrichment

Table 2 Detection of *Salmonella*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* using BARDOT

	Colonies picked based on BARDOT identification			MPCR confirmation			Identification rate%			
Food samples	SE	EC	LM	SE	EC	LM	SE	EC	LM	Average identification rate%
Chicken breast (five samples)	15	15	15	13	13	15	86·7	86·7	100	91
Hotdog (five samples)	15	15	15	14	13	15	93	86·7	100	93·2
Control *S.* Enteritidis (3)	5	NA	NA	5	0	0	100	NA	NA	100
Control *E. coli* O157:H7 (3)	NA	5	NA	0	4*	0	NA	80	NA	100
Control *L. monocytogenes* (3)	NA	NA	5	0	0	5	NA	NA	100	100

*In one strain, *stx* gene was not amplified. NA, not applicable; SE, *Salmonella* Enteritidis; EC, *E. coli* O157:H7; LM, *L. monocytogenes*.

time may vary; a total of about 29 h turnaround time is required for *Salmonella* and *E. coli* O157:H7, and 40 h for *L. monocytogenes*. The above results indicate that due to dual level selective enrichment in both enrichment broth and on the plate, background commensal bacteria had little or no effect on the specific identification of target pathogens from food including raw poultry parts samples.

Discussion

Simultaneous multipathogen detection capability on a single-assay platform is highly desirable and economically advantageous since it can reduce the bench space required for handling a large number of samples, as well as supplies, reagents and labour, thus lowering the potential overall cost of testing each pathogen (Bhunia 2014; Huntington *et al.* 2018). For multipathogen detection, several methods are proposed such as mPCR (Bhagwat 2003; Mukhopadhyay and Mukhopadhyay 2007; Settanni and Corsetti 2007; Kawasaki *et al.* 2010), array-based immunosorbent assays (Chen and Durst 2006) and antibody-based array biosensor techniques (Taylor *et al.* 2006; Tu *et al.* 2011; Gehring *et al.* 2012, 2013, 2015; Ohk and Bhunia 2013). Therefore, our goal was to investigate if BARDOT could be used to detect and distinguish the three major foodborne pathogens, *S. enterica*, STEC including *E. coli* O157:H7 and *Listeria* species from food since these three are considered the leading causes of foodborne diseases in the United States (Scallan *et al.* 2011).

Rapid and sensitive detection and identification of pathogens are critical for quality assurance and pathogen tracing within the food production system. Preventive measures based on testing and screening the final products before distribution for human consumption would significantly improve food safety and public health; and consequently, reduce the incidence of foodborne outbreaks. Pathogen detection employs traditional detection methods based on biochemical, serological or DNA confirmation which includes sample enrichment in a liquid broth, plating on agar media to rely on phenotype-based identification, followed by molecular testing that requires DNA isolation and PCR amplification. All these methods take a minimum of 5–10 days to obtain confirmatory results and are labour intensive.

The investment of biosensor tools in the microbiological analysis and pathogen isolation could shorten assay time because of their improved sensitivity and prospect for direct application to food (culture independent) or enriched (culture dependent) samples (Bhunia 2008; Velusamy *et al.* 2010; Dwivedi and Jaykus 2011; Gehring and Tu 2011; Wang and Salazar 2015). Culture-dependent enrichment offers detection of live target pathogen at a very low concentration (0·2–2 CFU per 25 g). Additionally, selective enrichment of target (inclusive) pathogen offers selective inhibition of background (exclusive) bacteria up to certain extent, which makes the downstream assay more sensitive, reliable, and thus markedly reduces the chance for obtaining false-positive results. While biosensor-based screening technologies offer rapid and inexpensive methods for bacterial pathogen detection and identification (Jasson *et al.* 2010; Gehring and Tu 2011; Bhunia 2014; Wang and Salazar 2015).

We employed BARDOT, an optical scattering sensor that was developed at Purdue University in an interdisciplinary effort to advance the method for rapid, label-free, nondestructive, real-time detection of bacterial colonies including pathogens (Nebeker *et al.* 2001; Banada *et al.* 2007, 2009; Huff *et al.* 2012; Singh *et al.* 2014; Tang *et al.* 2014). However, all previous BARDOT applications relied on uniplex detection of foodborne pathogen and sometimes used the nonselective or differential agar medium for scatter pattern-based classification. The major drawbacks of BARDOT-based detection method on the nonselective agar medium are the generation of overlapping patterns of target organisms with the scatter patterns of colonies from the background commensals, which may

lead to a higher false-positive result. However, the accuracy of the assay can be improved by using a combination of selective enrichment broth and selective agar medium to reduce background commensal before employing BARDOT for detection. Therefore, we used SEL medium (Kim and Bhunia 2008) for both liquid enrichment and agar plate isolation. SEL was formulated by modifying the recipe of BLEB containing tryptic soy broth, yeast extract, sodium pyruvate and sodium phosphate, which support the growth of both healthy and injured bacteria (Buduamoako *et al.* 1992; Busch and Donnelly 1992) and antimicrobial agents; acriflavin, cycloheximide, fosfomycin and nalidixic acid. Previous results showed that SEL broth considered a semiselective enrichment broth for the concurrent growth of *S. enterica*, *E. coli* O157:H7 and *L. monocytogenes* present at various cell numbers in contaminated food samples with superiority to individual selective enrichment broths (Kim and Bhunia 2008). We also did not observe the growth of many background commensal bacteria on SELA when food samples were tested. It has been reported earlier that the growth of *Bacillus* sp., *Lactobacillus* sp., *Enterococcus faecalis*, *Proteus vulgaris*, *Lactococcus lactis* and *Yersinia enterocolitica* were suppressed on SEL (Kim and Bhunia 2008), while *Acinetobacter*, *Klebsiella*, *Citrobacter*, *Serratia, Staphylococcus, Pseudomonas* and *Enterobacter* sp. grew on SELA (this study, Fig. 2). Interestingly, these latter groups of bacterial species produced nearly opaque colonies on SELA which prevented the laser beam penetration through the colonies and scatter pattern generation thus aided consequent differentiation from target pathogens (*Salmonella*, STEC and *Listeria*).

Other advantages of BARDOT is that it can distinguish and differentiate colony profiles of petite colonies (0·8–1·0 mm diameter) which is even difficult for naked eyes to distinguish. BARDOT takes about 3–4 s per colony, that is, about 7 min for 100 colonies to generate scatter signatures, and requires about 15 min for image analysis. Furthermore, it is a nondestructive method and preserves the integrity of samples, unlike DNA-based molecular methods. The low-power (1 mW mm^{-2}) red diode laser (635 nm) used in BARDOT does not affect bacterial viability; therefore, the live cultures obtained from test samples can be used for other experimentation such as to monitor the effect of sanitizers for controlling the pathogens in the food processing/production plants, antibiotic sensitivity testing, pathogenicity analysis or other molecular characterization (Bhunia 2014; Singh *et al.* 2016).

In conclusion, the present work highlights the application of a laser light scattering technology coupled with multipathogen SEL agar medium to facilitate rapid detection of three pathogens (*Salmonella, E. coli* O157:H7, and *Listeria*). The SEL, non-SEL, and genus-specific colony scatter image libraries revealed high classification accuracies (84–100% PPV). Mixed culture and artificially inoculated food sample results underscored the scatter pattern-based discriminatory potential of BARDOT detection platform and demonstrated that this method can detect and identify *Salmonella* sp. and *E. coli* O157:H7 in about 29 h and *L. monocytogenes* in 40 h starting with the food sample. This novel label-free on-plate colony-screening technology is an economically favourable approach for food industry or regulatory agencies for the screening of the final products for multiple foodborne pathogens. This would also aid in ensuring food safety and reduce the incidence of human foodborne outbreaks while minimizing the overall cost by decreasing labour, assay time and reagents for individual pathogen testing.

Acknowledgements

This material is based upon work supported by the US Department of Agriculture, Agricultural Research Service, under Agreement No. 59-8072-6-001, and the Center for Food Safety Engineering at Purdue University, and the Ministry of Higher Education, Egypt. Any opinions, findings, conclusion or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the US Department of Agriculture.

Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Comparison of growth plots of *E. coli*, *S.* Enteritidis and *L. monocytogenes* on SELA.

Figure S2 Scatter patterns of multiple strains of top five human-outbreak associated *Salmonella* serovars (Enteritidis, Typhimurium, Heidelberg, Javiana and Newport) on SELA, which was used to train the SEL scatter image library.

Figure S3 Scatter patterns of multiple strains of *Escherichia coli* serovars, O157, O26, O45, O103, O111, O121, O145 on SELA, which was used to train the SEL scatter image library.

Figure S4 Scatter patterns of multiple strains of *Listeria* species (monocytogenes, grayi, rocourtiae, marthii, seeligeri, welshimeri, innocua and ivanovii) on SELA, which was used to train the SEL scatter image library.

Table S1 The primer sequences and the putative product sizes for each amplicon