RESEARCH ARTICLE

# Laser Optical Sensor, a Label-Free On-Plate *Salmonella enterica* Colony Detection Tool

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**ABSTRACT**   We investigated the application capabilities of a laser optical sensor, BARDOT (bacterial rapid detection using optical scatter technology) to generate differentiating scatter patterns for the 20 most frequently reported serovars of *Salmonella enterica*. Initially, the study tested the classification ability of BARDOT by using six *Salmonella* serovars grown on brain heart infusion, brilliant green, xylose lysine deoxycholate, and xylose lysine tergitol 4 (XLT4) agar plates. Highly accurate discrimination (95.9%) was obtained by using scatter signatures collected from colonies grown on XLT4. Further verification used a total of 36 serovars (the top 20 plus 16) comprising 123 strains with classification precision levels of 88 to 100%. The similarities between the optical phenotypes of strains analyzed by BARDOT were in general agreement with the genotypes analyzed by pulsed-field gel electrophoresis (PFGE). BARDOT was evaluated for the real-time detection and identification of *Salmonella* colonies grown from inoculated ($1.2 \times 10^2$ CFU/30 g) peanut butter, chicken breast, and spinach or from naturally contaminated meat. After a sequential enrichment in buffered peptone water and modified Rappaport Vassiliadis broth for 4 h each, followed by growth on XLT4 (~16 h), BARDOT detected *S.* Typhimurium with 84% accuracy in 24 h, returning results comparable to those of the USDA Food Safety and Inspection Service method, which requires ~72 h. BARDOT also detected *Salmonella* (90 to 100% accuracy) in the presence of background microbiota from naturally contaminated meat, verified by 16S rRNA sequencing and PFGE. Prolonged residence (28 days) of *Salmonella* in peanut butter did not affect the bacterial ability to form colonies with consistent optical phenotypes. This study shows BARDOT's potential for nondestructive and high-throughput detection of *Salmonella* in food samples.

**IMPORTANCE**   High-throughput screening of food products for pathogens would have a significant impact on the reduction of food-borne hazards. A laser optical sensor was developed to screen pathogen colonies on an agar plate instantly without damaging the colonies; this method aids in early pathogen detection by the classical microbiological culture-based method. Here we demonstrate that this sensor was able to detect the 36 *Salmonella* serovars tested, including the top 20 serovars, and to identify isolates of the top 8 *Salmonella* serovars. Furthermore, it can detect *Salmonella* in food samples in the presence of background microbiota in 24 h, whereas the standard USDA Food Safety and Inspection Service method requires about 72 h.

*S*almonella is a major food-borne human pathogen that causes salmonellosis and is a serious public health concern worldwide. There are about 2,610 serovars of *Salmonella*. In the decade from 1999 to 2009, the number of laboratory-confirmed *Salmonella* isolates obtained from human sources increased from 32,828 cases to 40,828 cases, and 72.8% of these were of the 20 most frequently reported serotypes according to the National Enteric Disease Surveillance program (1). Most of the *Salmonella* serovars belong to *Salmonella enterica* subsp. *enterica* (2, 3) and are associated with food-borne outbreaks and human infections (4). The U.S. Food and Drug Administration has mandated a zero toler-

ance for it in foods (Compliance Policy Guide [CPG] section 527.300, Microbial Contaminants and Alkaline Phosphatase Activity in Dairy Products [http://www.fda.gov/downloads/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/UCM238480.pdf], and CPG section 555.300, Foods, Except Dairy Products—Adulteration with Salmonella [http://www.fda.gov/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/ucm074553.htm]). *S. enterica* subsp. *enterica* is generally associated with gastroenteritis, septicemia, and reactive arthritis (5). The Centers for Disease Control and Prevention (CDC) estimates that the annual number of salmonellosis cases is approxi-

mately 1 million, resulting in 378 deaths (6). Outbreaks are associated with a wide variety of foods, including ground beef, ground turkey, sprouts, cantaloupes, jalapeño peppers, peanut butter, pistachios, powdered infant formula, salami, eggs, and sushi (7–9). Some well-publicized recent outbreaks include *S. enterica* serovar Typhimurium in peanut butter causing 714 illnesses (10), *Salmonella* Saintpaul in imported jalapeño peppers infecting 1,407 people (11), *Salmonella* Enteritidis in eggs causing about 1,500 illnesses (12), and more recently, *Salmonella* Bareilly in ground tuna affecting 160 people (8) and *Salmonella* Heidelberg in chicken affecting 134 people (13). In each of these outbreaks, hundreds of thousands of pounds of products were recalled, resulting in considerable financial losses and even bankruptcies, as well as potential criminal liability, for the companies involved. Therefore, there is a continued demand for improved technology and new assay methods for screening of foods for pathogens before they are released for human consumption (14).

Current *Salmonella* detection and identification involve a combination of culture-based methods coupled with metabolic fingerprinting, immunoassays, and nucleic acid-based methods (15–18). However, owing to the genetic similarity among serovars (19), antibodies or nucleic acid probes show cross-reactions, causing difficulties in the identification of *Salmonella* serovars (20). Various alternative biosensor-based methods (21–24) have been proposed, including fiber-optic sensors (25), surface plasmon resonance sensors, impedance sensors (22), magnetoelastic biosensors (26), and nanoparticle-based DNA sensors (27). However, these methods can detect only a selected serovar and do not allow pathogen recovery. Some tests may require advanced training of the operators. Thus, it is important to develop alternative rapid, user-friendly screening techniques that would allow real-time detection first and then identification of *Salmonella* serovars without requiring access to nucleic acid or antibody probes for each. Recently, matrix-assisted laser desorption ionization (MALDI)–time of flight mass spectrometry was reported as a phenotypic prescreening tool for the identification of *Salmonella* isolates before a conventional agglutination-based serotyping scheme could be performed (3). This method could be used for direct detection and identification of *Salmonella* in food samples, but each colony must be lifted from agar plates and placed on a MALDI sample tray as a thin film for analysis. In contrast, the laser light-scattering sensor designated BARDOT (bacterial rapid detection using optical scatter technology), developed at Purdue University (28, 29), could be used directly with colony cultures on agar plates to deliver high-throughput analysis and real-time detection and identification of *S. enterica* colonies.

BARDOT is a label-free nondestructive method in which a 635-nm laser beam passes through the center of a colony and generates a unique scatter signature (fingerprint) that can be used for detection and identification employing a scatter signature classification library (30, 31). This optical methodology preserves colony integrity and ensures the availability of colonies for subsequent testing. The technique has been demonstrated to differentiate *Escherichia*, *Listeria*, *Salmonella*, *Staphylococcus*, and *Vibrio* at the genus level with 90 to 99% accuracy (30). It was also successful in differentiating *Listeria* species (29, 32) and accurately detected *Listeria monocytogenes* on agar plates containing bacteria captured and preconcentrated with paramagnetic beads (33). More recently, BARDOT has been employed to detect several pathogenic species of *Vibrio*, including *Vibrio parahaemolyticus*, *V. vulnificus*, and *Vibrio cholerae*, in oyster and water samples (34). However, BARDOT's ability to identify bacteria at the serovar level has not been fully explored.

The overall objective of this study was to investigate BARDOT's abilities to differentiate the top 20 *S. enterica* subsp. *enterica* serovars, which are responsible for 72.8% of all *Salmonella* isolates reported to the CDC (1), from other bacterial species and to detect and identify them in food. An initial study involved finding the most suitable selective agar medium for the measurement of elastic light scatter signatures and the generation of a signature library of *S. enterica* serovars. Next, the ability of BARDOT to detect *Salmonella* in spinach, in chicken breast with a high natural microbial background level, and in a low-moisture model food system (peanut butter) was examined. The data show that this system can accurately detect most of the *Salmonella* serovars tested in the set of the 20 most prevalent serovars (positive predictive value [PPV] or classification precision level reaching 86%) and all serovars in the top 8 group (PPVs ranging from 68 to 93%), providing a valuable method for rapid screening of *Salmonella* for the benefit of the food-processing industry and regulatory agencies.

## RESULTS

**Optimal agar medium for colony scatter pattern generation.** One of the goals of this study was to find a suitable agar medium that would provide the best differentiation of *Salmonella* (Table 1) colonies on the basis of scatter patterns generated with the BARDOT instrument (Fig. 1). First, we examined scatter signatures formed by colonies of six randomly picked *Salmonella* serovars (Enteritidis, Hadar, Heidelberg, Montevideo, Newport, and Typhimurium) grown on nonselective brain heart infusion (BHI) agar and on selective media, including brilliant green (BG), xylose lysine deoxycholate (XLD), and xylose lysine containing tergitol 4, a sodium salt of 7-ethyl-2-methyl-4-undecanol hydrogen sulfate (XLT4) (Fig. 2A). The classification success levels, expressed as PPVs (or classification precision levels), for all six serovars on BHI (82.5%) and BG (86.2%) were high but significantly ($P < 0.05$) lower than those obtained with XLD (91.2%) and XLT4 (95.9%), which are used routinely for *Salmonella* isolation (35) (Fig. 2A). We chose XLT4 for this study, since it is more selective than XLD owing to the presence of tergitol 4, which inhibits some non-*Salmonella* bacteria. XLT4 has been recommended for the isolation of both nontyphoid and typhoid salmonellae (36). Moreover, the *Salmonella* colony scatter patterns obtained with XLT4 were more amenable to optical classification than those of colonies grown on XLD, as indicated by our tests with more than 30 serovars (data not shown).

Since bacterial colony scatter patterns are medium dependent (34, 37), one may ask whether the same type of medium obtained from different vendors would have any effect on detection and classification. XLT4 was procured from three suppliers (BD, Acumedia, and HiMedia), and scatter signatures of colonies of *S.* Typhimurium grown on plates with these media were examined. In general, the colonies cultured on the media obtained from BD and Acumedia exhibited very similar optical scatter patterns. The bacteria cultured on XLT4 from HiMedia produced patterns that were visually different but still within the range of acceptable variation (Fig. 2B). Additionally, *Salmonella* cell recovery was also slightly lower in this brand of XLT4 medium (Fig. 2C) than in the others. This suggests that the HiMedia formulation may exhibit a

**TABLE 1** Accuracy of BARDOT-based detection of *Salmonella* serovars on XLT4 agar plates tested with *Salmonella* and non-*Salmonella* libraries

| Organism | No. of strains tested[a] | Source(s)[b] | Avg % PPV ± SD[c] | |
|---|---|---|---|---|
| | | | Top 20 *Salmonella* serovar library | Non-*Salmonella* library |
| Top 20 *Salmonella* serovars (in order of incidence) | | | | |
| Enteritidis | 22 | ISDH, ATCC, BLCC | 98.8 ± 1.4 | 1.1 ± 1.4 |
| Typhimurium | 28 | ISDH, ATCC, BLCC | 94.8 ± 5.5 | 5.1 ± 6.0 |
| Newport | 4 | ISDH, ADDL | 99.6 ± 0.5 | 0.3 ± 0.5 |
| Javiana | 4 | ISDH | 94.0 ± 5.6 | 6.0 ± 5.6 |
| Heidelberg | 6 | ISDH, ATCC, BLCC | 100 ± 0.0 | 0.0 |
| Montevideo | 5 | ISDH, ATCC, BLCC | 100 ± 0.0 | 0.0 |
| I 4,[5],12:i:− | 4 | ISDH | 93.5 ± 9.9 | 6.5 ± 0.0 |
| Oranienburg | 3 | ISDH | 99.75 ± 0.5 | 0.2 ± 0.5 |
| Saintpaul | 4 | ISDH | 98.6 ± 2.3 | 1.3 ± 2.3 |
| Muenchen | 3 | ISDH | 99.75 ± 0.5 | 0.2 ± 0.5 |
| Braenderup | 3 | ISDH | 99.0 ± 2.0 | 1.0 ± 2.0 |
| Infantis | 5 | ISDH, UA | 98.2 ± 4.0 | 1.8 ± 4.0 |
| Thompson | 5 | ISDH, ATCC, BLCC, UM | 99.2 ± 1.5 | 0.7 ± 1.5 |
| Mississippi | 4 | ISDH | 92.3 ± 7.0 | 7.6 ± 7.0 |
| Paratyphi B | 4 | ISDH, PRI | 100 ± 0.0 | 0.0 |
| Typhi | 4 | ISDH, PRI | 90.5 ± 13.8 | 9.5 ± 13.8 |
| Agona | 3 | ISDH | 100 ± 0.0 | 0.0 |
| Schwarzengrund | 3 | ISDH | 64.5 ± 39.7 | 35.5 ± 39.7 |
| Bareilly | 4 | ISDH | 100 ± 0.0 | 0.0 |
| Hadar | 5 | ISDH | 99.5 ± 1.0 | 0.5 ± 1.0 |
| Miscellaneous *Salmonella* serovars | | | | |
| Anatum | 1 | EITC | 100 | 0 |
| Berta | 1 | BLCC | 16 | 84 |
| Brandenburg | 1 | BLCC | 15 | 85 |
| Choleraesuis | 2 | EITC | 70 | 30 |
| Gallinarum | 1 | UM | 100 | 0 |
| Havana | 1 | BLCC | 99 | 1 |
| Indiana | 1 | BLCC | 88 | 12 |
| Kentucky | 1 | UA | 90 | 10 |
| Litchfield | 1 | UA | 100 | 0 |
| Poona | 1 | PU | 90 | 10 |
| Pullorum | 1 | BLCC | 88 | 12 |
| Rubislaw | 1 | BLCC | 94 | 6 |
| Senftenberg | 2 | EITC, UA | 100 | 0 |
| Stanley | 1 | UM | 93 | 7 |
| Tennessee | 1 | UA | 100 | 0 |
| Thomasville | 1 | BLCC | 100 | 0 |
| Non-*Salmonella* bacteria | | | | |
| *C. freundii* | 2 | NRRL, ATCC | 13 | 87 |
| *E. coli* O157:H7 | 1 | USDA ARS | 10 | 90 |
| *E. coli* O26 | 1 | USDA ARS | 12 | 88 |
| *E. coli* O103 | 1 | USDA ARS | 15 | 85 |
| *H. alvei* | 1 | BLCC | 0 | 100 |
| *K. pneumoniae* | 2 | NRRL, PRI | 4 | 96 |
| *S. marcescens* | 2 | NRRL, BLCC | 10 | 90 |
| *S. flexneri* | 2 | PRI, CBS | 7 | 93 |

[a] The 161 strains used included 36 *Salmonella* serovars and 12 strains of non-*Salmonella* bacteria that grew on XLT4. Other non-*Salmonella* cultures that were tested on XLT4 agar but did not grow included *Acinetobacter baumannii* (n = 1), *Pseudomonas aeruginosa* (n = 2), *Providencia rettgeri* (n = 1), *Proteus mirabilis* (n = 2), *Proteus vulgaris* (n = 1), and *Yersinia enterocolitica* (n = 1).

[b] ATCC, American Type Culture Collection, Manassas, VA; BLCC, Bhunia Lab Culture Collection, Purdue University, West Lafayette, IN; ADDL, Animal Disease Diagnostic Laboratory, Purdue University, West Lafayette, IN; UA, Robert Story and Michael Slavik, University of Arkansas, Fayetteville, AR; UM, Department of Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada; EITC, Economic Innovation Technical Centre, Winnipeg, Manitoba, Canada; PRI, Presque Isle Cultures, Erie, PA; NRRL, Northern Regional Research Laboratory, Peoria, IL; PU, Bruce Applegate, Purdue University, West Lafayette, IN; CBS, Carolina Biological Supply Company, Burlington, NC.

[c] The top 20 *Salmonella* serovar library contained scatter patterns from the top 20 *Salmonella* serovars, and the non-*Salmonella* library contained scatter patterns of non-*Salmonella* bacteria that grew on an XLT4 agar plate. Standard deviations indicate variations in percent PPV. A PPV of ≥80% was considered to indicate true *Salmonella* bacteria. Only one or two strains were used for miscellaneous *Salmonella* serovars and non-*Salmonella* bacteria, and no standard deviation was calculated for these.

slight inhibitory effect on the growth of *S*. Typhimurium. For consistency, XLT4 medium from BD was used in all subsequent experiments.

Even though XLT4 has been used as a selective recovery medium for *Salmonella* in the official *Salmonella* isolation procedure, it also supports the growth of other microbiota (Table 1). Thus, it
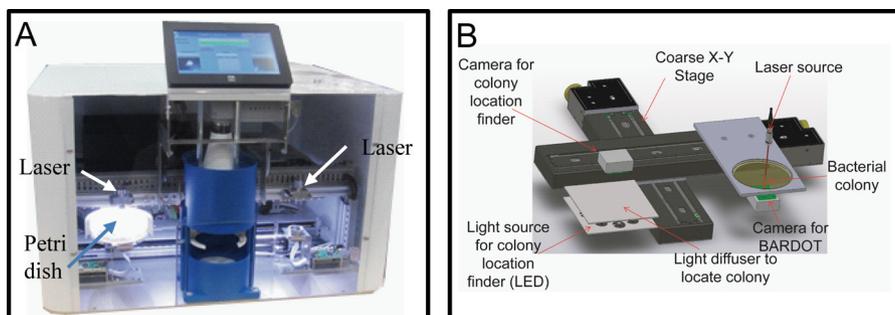
**FIG 1** Setup of the laser optical sensor designated BARDOT for bacterial colony detection and identification. (A) BARDOT instrument with dual scanning modules. (B) Schematic diagram of component design and setup.

was important to verify whether *Salmonella* scatter patterns can be differentiated from patterns of non-*Salmonella* bacteria that grew on XLT4, including *Citrobacter freundii*; *Hafnia alvei*; *Escherichia coli* serovars O157, O26, and O103; *Klebsiella pneumoniae*; *Shigella flexneri*; and *Serratia marcescens*. Collected data demonstrated that the scatter patterns of colonies of these non-*Salmonella* bacteria were visually distinct from that of the *Salmonella* serovars tested (Fig. 3A).

We also tested whether scatter patterns of *Salmonella* colonies would show similarity to those of non-*Salmonella* colonies at any time during the course of growth on XLT4. Therefore, colony scatter signatures of *S.* Enteritidis grown for 11 to 20 h were compared with scatter patterns of colonies of two model contaminants, *C. freundii* and *K. pneumoniae*. Although the scatter signatures evolve with time (owing to changes in colony size and organization) (31), at any given time point, all three stayed different from one another in appearance (Fig. 3B). The evidence is strong that BARDOT-based detection of *Salmonella* on XLT4 is feasible even in the presence of background microbiota (Fig. 3A). We discuss this issue further in later sections.

**Scatter image library for detection and identification of *Salmonella* on XLT4.** Initially, the XLT4-based scatter image library contained a total of 7,600 scatter images of colonies from 160 isolates, representing the top 20 human-derived *Salmonella* serovars (3 to 5 strains of each), an additional 16 *Salmonella* serovars (1 or 2 strains of each), and other non-*Salmonella* bacteria (6 genera, 12 strains) (Table 1). Scatter patterns (Fig. 4) and pulsed-field gel electrophoresis (PFGE) fingerprints (see Fig. S1 in the supplemental material) of the 20 most prevalent *Salmonella* serovars consisting of multiple strains are presented. The "non-*Salmonella*" library consisted of scatter patterns of other bacteria that were tested and grew on XLT4, including *C. freundii*; *H. alvei*; *E. coli* serovars O157, O26, and O103; *K. pneumoniae*; *S. flexneri*; and *S. marcescens* (Fig. 3A).

Next, we verified whether *Salmonella* colony scatter patterns could be accurately detected when tested against the top 20 *Salmonella* and "non-*Salmonella*" libraries. All but one serovar (Schwarzengrund) from the top 20 list showed 90.5 to 100% PPVs (Table 1). In the case of Schwarzengrund, only 64.5% of the colonies tested were detected as *Salmonella*. We also tested whether the library consisting of the most prevalent serovars can be used to detect an additional 16 *Salmonella* serovars from our collection. It was found that owing to the high dissimilarity of *Salmonella* patterns and non-*Salmonella* fingerprints, all but three of the serovars tested were detected with PPVs of 88 to 100%. The serovars that

were difficult to detect included Berta (PPV of 16%), Brandenburg (15%), and Choleraesuis (70%) (Table 1). Close examination of the scatter patterns produced by these serovars revealed smooth-textured patterns without characteristic distinguishable rings or spokes (Fig. 4; see Fig. S2 in the supplemental material). On the basis of the cross-validation experiment of each serovar with the scatter pattern libraries, it was determined that a PPV of >80% should be used for specific detection of *Salmonella*.

Subsequently, we verified whether the established library could be used for the identification of individual serovars. The procedure involved a *k*-fold cross-validation procedure in which the original collection of scatter patterns was randomly partitioned into *k* subsamples and one was left out in each iteration of training. The PPVs found via 10-fold cross-validation for the top eight *Salmonella* serovars ranged from 70 to 93% (Fig. 5A). However, the classification success decreased to 58 to 90% (Fig. 5B) and 38 to 83% (Fig. 5C), respectively, when the top 10 and top 20 serovars were classified. This decrease in classification accuracy is an expected effect of the increased number of classes.

We also examined the classification feasibility for the top two serovars (Enteritidis and Typhimurium) that were mixed and plated on XLT4. Mixed colonies of both were screened by BARDOT; the PPVs for Enteritidis and Typhimurium were 90% ± 2.4% and 85% ± 4.2%, respectively, and each colony identified by BARDOT was verified by serovar-specific colony PCR (Table 2).

**Colony scatter phenotype is related to molecular fingerprints of individual *Salmonella* serovars.** Subsequently, we examined the relatedness between colony scatter phenotypes and the genotypes (PFGE fingerprint patterns obtained with the enzymes XbaI and BlnI) for each serovar of the top eight *Salmonella* serovars (Fig. 6; see Fig. S3 in the supplemental material). Examination of dendrograms for both colony scatter patterns and PFGE results revealed highly similar hierarchical clusters among serovars. For example, in the PFGE-based dendrogram, three strains of *S.* Typhimurium (13ENT1288, 13ENT0899, and 13ENT1227) formed one cluster that was separated from strain 13ENT1140 (Fig. 6A). An identical grouping was also seen in the BARDOT-based dendrogram (Fig. 6B). Likewise, four strains (13ENT1033, 13ENT1058; 13ENT0972, and 13ENT1009) of serovar I 4,[5],12:i:− produced two distinct PFGE clusters similar to the BARDOT-based clusters (Fig. 6). A comparative display of the scatter patterns (represented as grayscale bitmaps) of some selected serovars (Infantis, Thompson, Hadar, and Schwarzengrund) and their respective PFGE patterns are shown in Fig. S2B in the supplemental material. Collectively, the data demonstrate general agreement
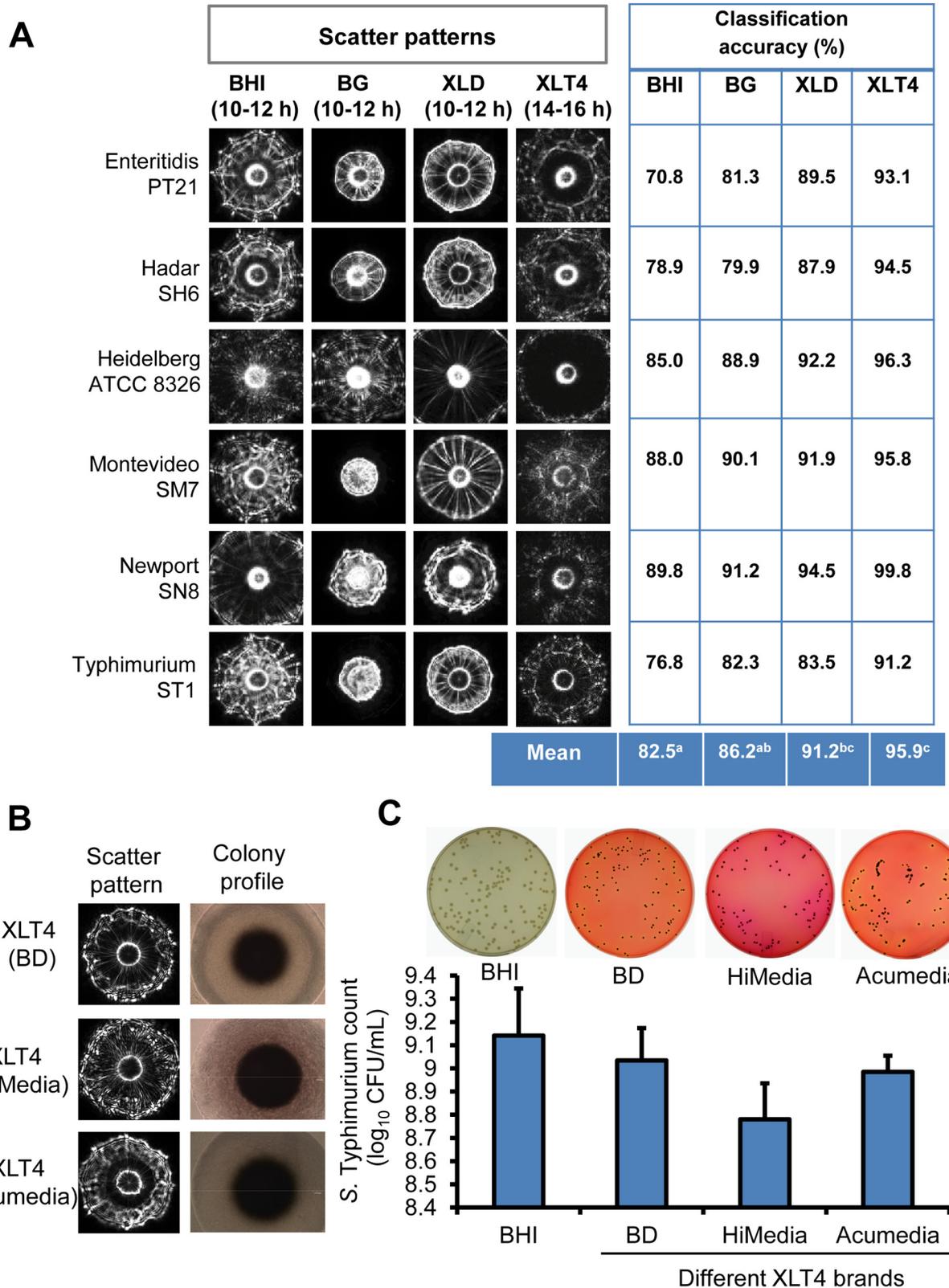
FIG 2  (A) Comparison of scatter patterns of selected *Salmonella* serovars on nonselective BHI agar and selective agar media, including BG, xylose XLD, and XLT4. Values with a, b, and c superscripts are significantly different ($P < 0.05$). In addition, the effects of different commercial brands of XLT4 agar on *S.* Typhimurium colony scatter patterns (B) and counts ($\log_{10}$ CFU/ml) (C) are presented.
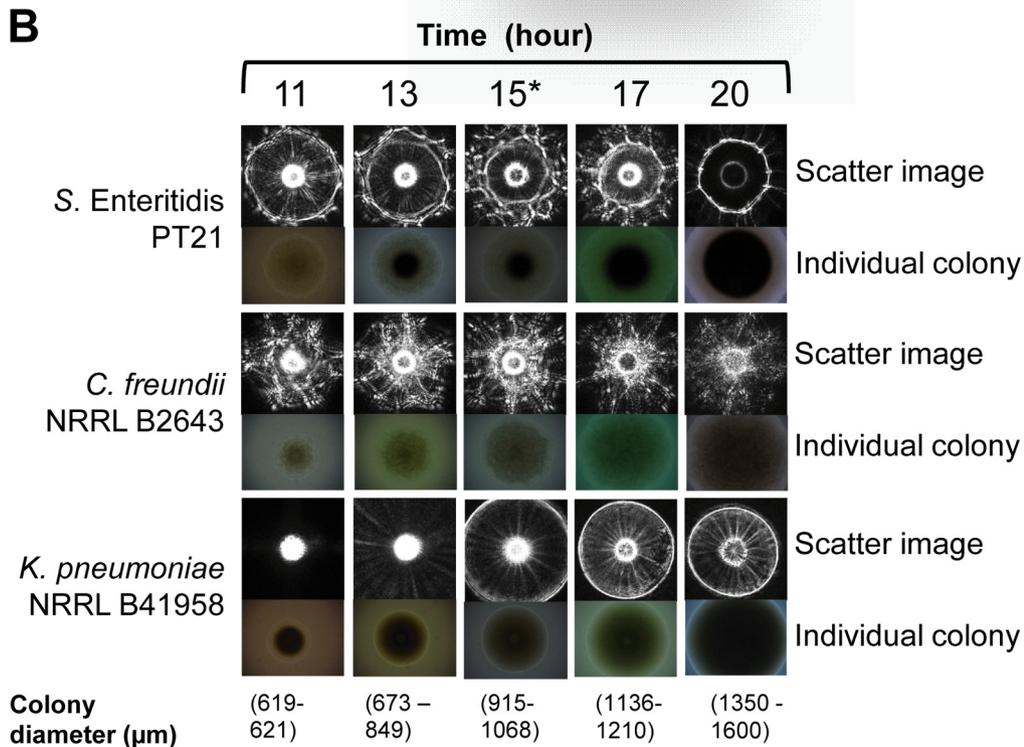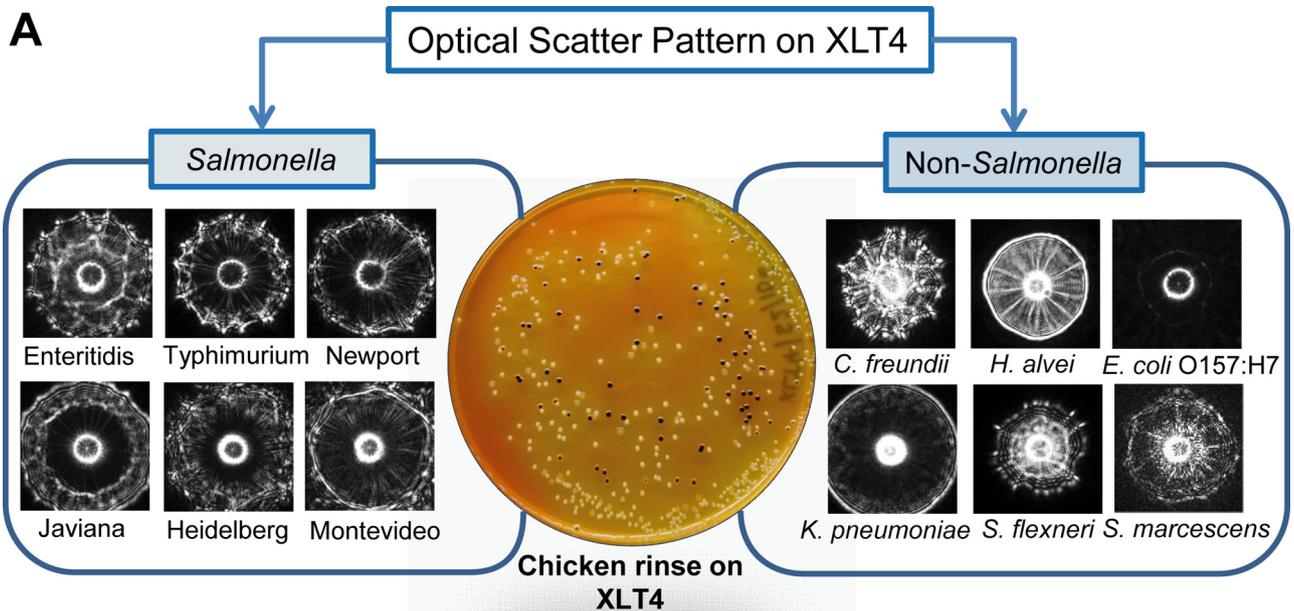
**FIG 3** Optical scatter pattern-based differentiation of *Salmonella* from non-*Salmonella* bacteria on an XLT4 agar plate. (A) Scatter pattern acquired from colonies (1.1-mm ± 0.2-mm diameter) after incubation for 14 to 18 h at 37°C. The *Salmonella* serovars include Enteritidis, Typhimurium, Newport, Javiana, Heidelberg, and Montevideo, and the non-*Salmonella* cultures include *C. freundii*; *H. alvei*, *E. coli* O157:H7, *K. pneumoniae*, *S. flexneri*, and *S. marcescens*. (B) Colony scatter pattern signatures of *Salmonella* and non-*Salmonella* cultures (*C. freundii* and *K. pneumoniae*) during growth from 11 to 20 h at 37°C. The corresponding colony diameters are also provided. The asterisk denotes the approximate time point of *Salmonella* scatter pattern acquisition.

between PFGE and BARDOT scatter signatures for each strain within a serovar (Fig. 6B).

**Detection of *Salmonella* in inoculated peanut butter, spinach, and chicken samples.** Experiments were performed to determine if BARDOT is able to detect and identify *Salmonella* present in peanut butter after an extended period of storage (28 days).

*Salmonella* bacteria were recovered from all test samples with or without an enrichment step. The scatter patterns of *S.* Typhimurium colonies on XLT4 were visually indistinguishable, regardless of the sampling time (day 0 or 28) (Fig. 7A). Serovar-specific PCR verified the colonies to be *S.* Typhimurium (Fig. 7B).

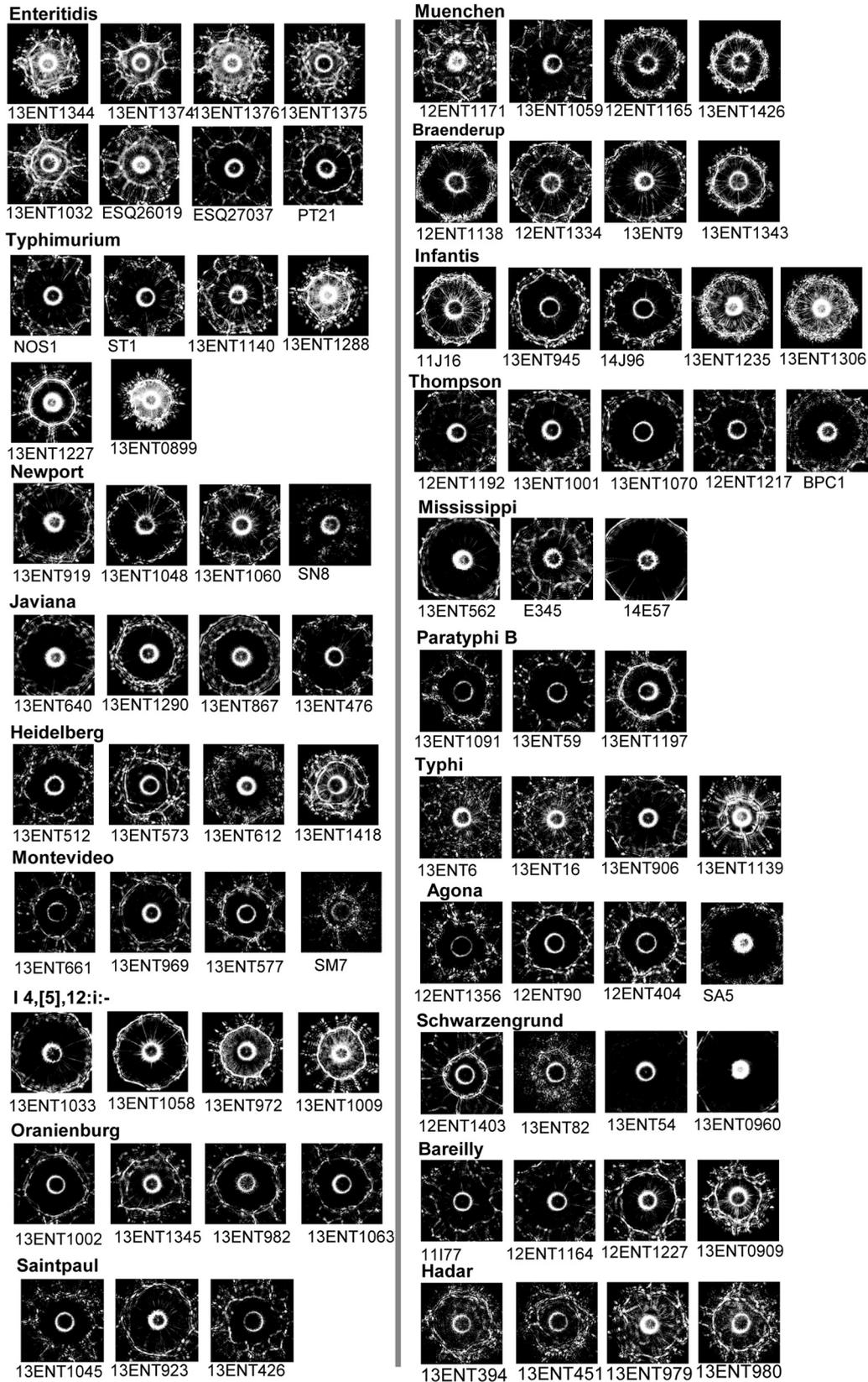We also optimized the procedure for the detection of *Salmo-*

**FIG 4** Scatter patterns of the top 20 human origin *Salmonella* serovars grown on XLT4 agar plates at 37°C for 16 h. Scatter patterns were acquired when the colony diameter reached 1.1 ± 0.2 mm.
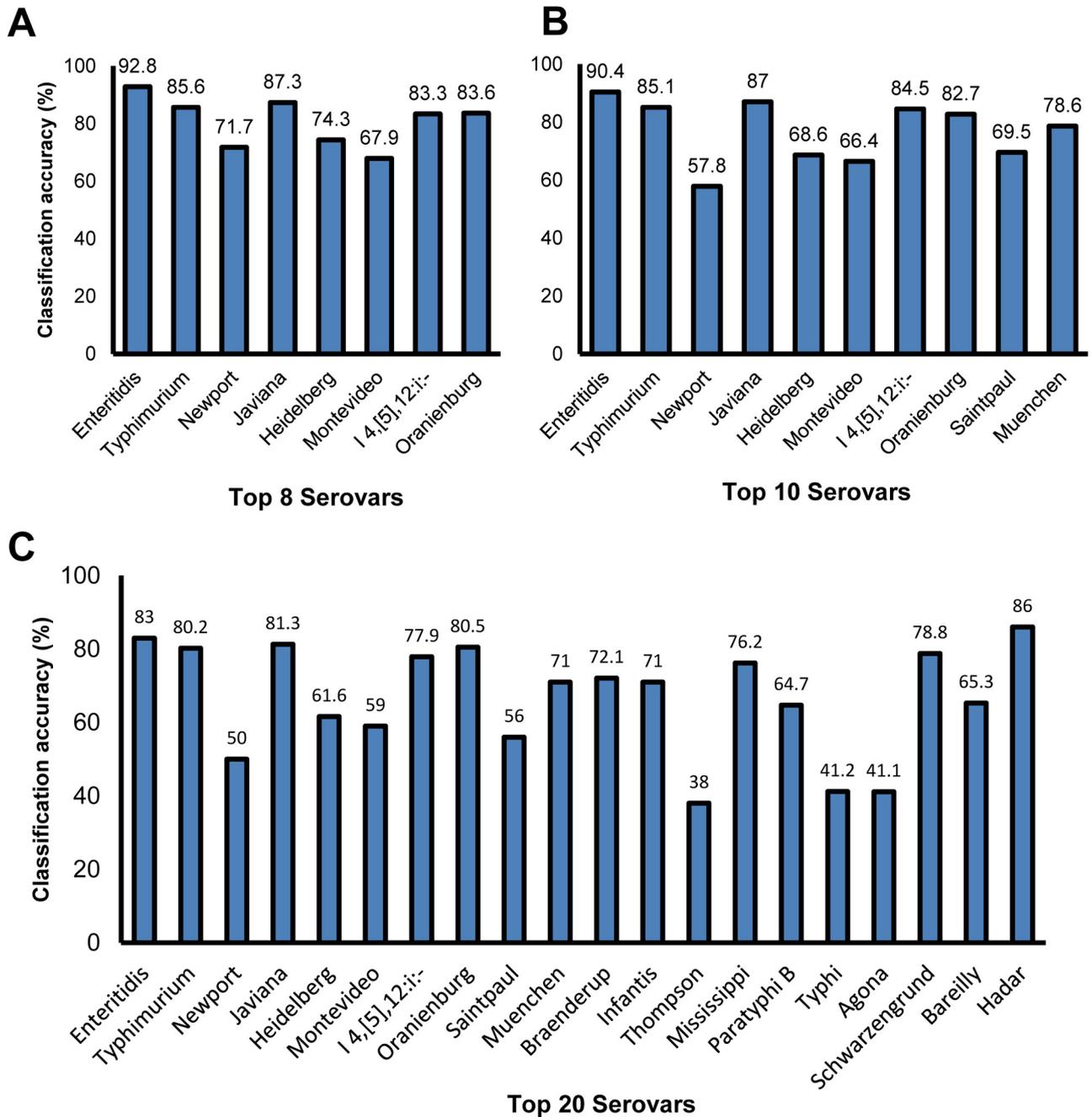
**FIG 5** Classification precision levels (PPVs) of each *Salmonella* serovar classified against the scatter pattern library consisting of the top 8 serovars (A), the top 10 serovars (B), and the top 20 serovars (C).

*nella* from peanut butter samples that received low levels of inoculum ($1.2 \times 10^2 \pm 0.1 \times 10^2$ CFU/30 g). As a preliminary trial experiment, employing sequential enrichment in buffered peptone water (BPW) and modified Rappaport Vassiliadis (mRV) broth for 4 h each, followed by plating on XLT4 (16 h), we were able to detect *S.* Typhimurium with a PPV of 84% in 24 h (see Table 3; see Table S1 in the supplemental material) by using the top eight *Salmonella* serovar library. When the samples were enriched in only one enrichment broth, i.e., BPW for 4 h, followed by plating on XLT4, BARDOT was able to identify *S.* Typhimurium

with a PPV of 82%. However, we observed the formation of a thin film of peanut butter on the surface of the agar plates that occasionally interfered with the optical detection system. This problem was eliminated when the samples were enriched sequentially in BPW (4 h) and mRV broth (4 h) or in an enrichment broth for 24 h to ensure that the sample matrix could be diluted out before plating on the agar surface (a technique recommended by the USDA Food Safety and Inspection Service [FSIS] procedure that employs 24-h enrichment) (38).

Likewise, a shortened enrichment protocol was also used to

**TABLE 2** Detection of *S.* Typhimurium and *S.* Enteritidis in a mixed culture on XLT4 agar plate by BARDOT

| Serovar identified by BARDOT on mixed-culture plate[a] | No. of colonies identified by BARDOT | Avg PCR confirmation of colonies ± SD[b] | Avg identification efficiency (%) ± SD[c] |
|---|---|---|---|
| *S.* Enteritidis | 50 | 45.0 ± 1.4 | 90.0 ± 2.4 |
| *S.* Typhimurium | 50 | 42.5 ± 2.1 | 85.0 ± 4.2 |

[a] Colonies of *S.* Enteritidis and *S.* Typhimurium identified by BARDOT were picked from replicate XLT4 plates for PCR confirmation. Colonies of *S.* Enteritidis and *S.* Typhimurium on a mixed-culture plate were identified as *S.* Enteritidis or *S.* Typhimurium after matching of colony scatter patterns with the top eight *Salmonella* serovar library. *S.* Enteritidis PT21 and *S.* Typhimurium var. Copenhagen were used as a mixed inoculum.
[b] Colonies of *S.* Enteritidis and *S.* Typhimurium were confirmed by PCR with *S.* Enteritidis- and *S.* Typhimurium-specific primers as described in Materials and Methods.
[c] Identification efficiency was calculated as the number of PCR-positive colonies that were randomly picked from a plate after identification with the library divided by the total number of BARDOT-positive colonies. Standard deviations were calculated from results obtained with 50 colonies analyzed in two separate experiments.

detect artificially inoculated *S.* Typhimurium in fresh produce (spinach). BARDOT was able to detect and identify *S.* Typhimurium in the presence of a high natural background microbial load of $7.9 \times 10^5 \pm 1.1 \times 10^5$ CFU/ml after 4 h of enrichment in BPW (Fig. 8). Selected BARDOT-positive colonies were further confirmed by 16S rRNA gene sequencing. Furthermore, selected colonies of background microbiota that produced scatter patterns showing very high similarity to patterns from the library consisting of non-*Salmonella* bacteria were found to be either *E. coli* or *Enterobacter* species, as confirmed by 16S rRNA gene sequencing. It is worth mentioning that the background bacterial counts in spinach were $6.15 \times 10^6 \pm 1.4 \times 10^6$ CFU/ml when samples were enriched in mRV broth at 37°C for 4 h. When the enrichment temperature was raised to 42°C for 4 h, the bacterial count was only $1.6 \times 10^5$ CFU/ml, indicating that enrichment at 42°C is desirable, though none of these enrichment conditions apparently affected *Salmonella* detection by BARDOT.

A shortened enrichment protocol was also used for detection of *S.* Enteritidis in artificially inoculated chicken breast samples. Ninety-one percent of the scatter patterns of *S.* Enteritidis colonies grown from chicken samples were correctly detected by using a library consisting of the top eight *Salmonella* serovars. The scatter images of the colonies were distinct from the background microbial scatter patterns (data not shown). BARDOT-based analysis needed a total of 24 h, and the results were in agreement with the standard USDA FSIS procedure that was run in parallel and that took about 72 h to complete (Table 3). Results were verified by PCR with *S.* Typhimurium serovar-specific primers tested with five randomly picked colonies from XLT4 for each sample (Table 3). These data indicate that BARDOT could be used for rapid screening of food samples for *Salmonella* in 24 h or less and does not depend on the examination of physical characteristics (color, shape) of the colonies on an agar plate, which may require up to 48 h to develop.

**Detection and identification of *Salmonella* from naturally contaminated raw meat and poultry.** Isolates from whole chicken carcasses (CRTB1, CRTB7, CRTB29, CRMB68, and CRMB13) and pork (APK1) gave PPVs of 90 to 100% when compared with the top 20 *Salmonella* library (Fig. 8 and Table 4). The identities of all of the isolates (*Salmonella* and non-*Salmonella*) were confirmed at the genus level after sequencing of the 16S rRNA gene (1,360 to 1,410 bp). Isolate ATK1 gave a PPV of only 67%; however, 16S rRNA gene sequencing revealed it to be a *Salmonella* species. ATK1 may represent a minority of serovars that produce colonies difficult to recognize (Table 4) and may warrant further testing of samples. All isolates from chicken carcasses (CRMY9, CRMY50, and CRTY15) that gave PPVs of 82 to 94% when com-

pared with the non-*Salmonella* library were classified as non-*Salmonella* isolates and found to be *E. coli* by 16S rRNA gene sequencing. Eighty-two percent of the spinach isolate (SPB1) colonies were classified as non-*Salmonella* isolates. These colonies were subsequently identified as *Enterobacter* species, indicating BARDOT's ability to discriminate between *Salmonella* and non-*Salmonella* bacteria when they are growing on the same plate (Table 4; Fig. 8).

To determine the serovar of each *Salmonella* isolate, the scatter patterns were classified by using the top eight *Salmonella* serovar library. The PPVs of all of the isolates were well below our acceptability range (68 to 93%; Fig. 5A), indicating that none of the isolates belonged to the top eight serovars. Further verification by both serotyping and PFGE analysis of the isolates indicated that all chicken isolates (CRTB1, CRTB7, CRTB29, and CRMB68) were *S. enterica* serovar Mbandaka, while the pork isolate (APK1) was identified as serovar Schwarzengrund and the turkey isolate (ATK1) was identified as serovar 4,5:r:− (Table 4; Fig. 8), and these serovars are not part of the top eight serovar library.

## DISCUSSION

The multidisciplinary approach to bacterial detection and identification has led in recent years to the development of various innovative technologies that have had a significant impact on food safety and public health (39, 40). The laser optical sensor described in this report was developed as a rapid (real-time), label-free, nondestructive, on-plate detection and identification tool for use with bacterial pathogens (14, 29, 30, 34). Here we employed BARDOT for rapid detection of the top 20 *S. enterica* serovars, which are responsible for 72.8% of all *Salmonella*-associated outbreaks (1).

The BARDOT system uses a low-power (1 mW/mm²) red diode laser (635 nm) to produce a scatter pattern over an exposure time of 1 to 2 s. During this process, an analyzed colony absorbs 1 to 2 mJ/mm²; thus, no measurable cellular damage that could affect subsequent confirmation by molecular methods is expected. The BARDOT-exposed cells were routinely cultured for additional experiments, demonstrating their viability. Nevertheless, subtle effects of laser light on bacterial physiology cannot be ruled out without conducting further studies. Typically, lasers with bactericidal activity must deliver high levels of power (7.3 mW/30 s) in order to inactivate bacteria, as demonstrated with *Streptococcus sanguis* (41). A high-power (30 to 1,500 mJ/mm²) blue laser (470 nm) has been shown to kill methicillin-resistant *Staphylococcus aureus* (42).

In the course of this research, we first examined multiple media (BHI, BG, XLD, and XLT4) to find the most discriminatory for-
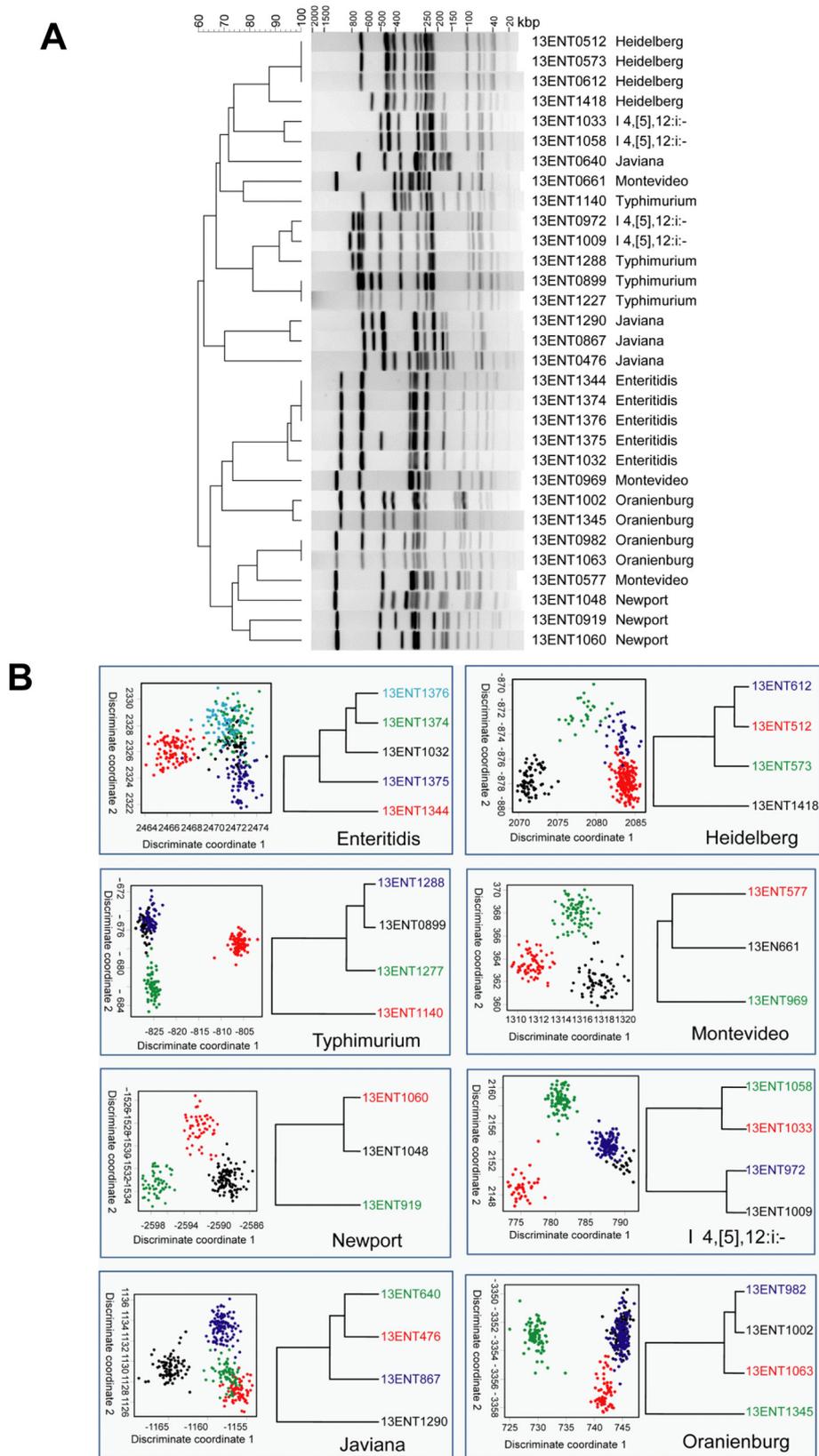
FIG 6 Relationship of genotypic fingerprint patterns of eight *S. enterica* serovars with scatter patterns based on the colony phenotype. A dendrogram based on PFGE-based (XbaI) genotypic fingerprint of selected serovars (A) shows clustering similar to scatter pattern-based clustering (B). Phenotypic classification of *Salmonella* serovars was determined on the basis of minimum spanning tree visualization of serovar similarities and Kendall distances between every pair of colony signatures in the training set grown on XLT4 plates.
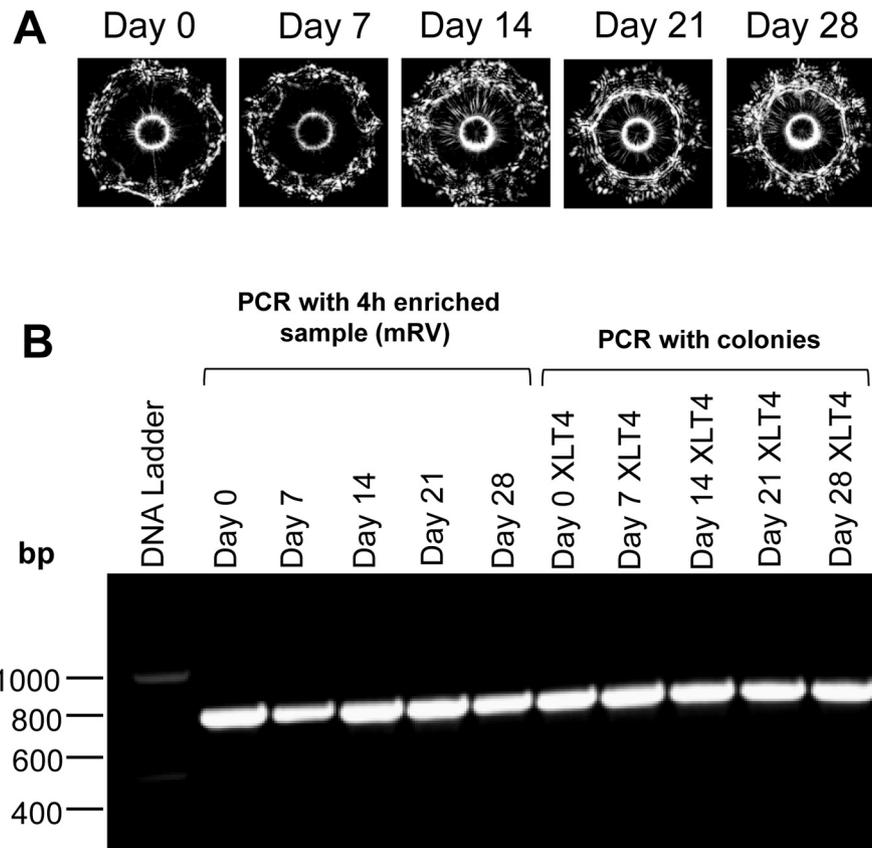
**FIG 7** Scatter patterns of colonies of *S.* Typhimurium var. Copenhagen on XLT4 that were inoculated and stored in peanut butter for up to 28 days. (A) Scatter pattern of *S.* Typhimurium colonies. (B) PCR confirmation (755 bp) of selected colonies targeting a metabolic gene.

mulation for the *Salmonella* serovars tested (30, 34, 37). The scatter signatures of bacterial colonies are affected by the medium type, as they are directly related to the colony morphotype shaped by the accumulation of metabolic by-products, exopolysaccharides, and cellular distribution and arrangement within a colony (30). Medium formulation, agar concentration (37), oxygenation, and humidity are known factors that also affect bacterial growth and the colony phenotype (43, 44). XLT4 was selected for further studies since it allowed for the best result in differentiating the six representative *Salmonella* serovars (95.9% PPV, Fig. 2). Moreover, XLT4 is also the USDA-recommended medium for *Salmonella* isolation owing to its high selectivity (36, 45). We also found that the use of different commercial brands of XLT4 medium led to variations in the scatter patterns. This result may have been an effect of the differences in medium formulation (protein and sugar contents). However, the variability happened to be insignificant in terms of *Salmonella* detection with a scatter pattern library. For consistency, we used XLT4 from a single source (BD, Sparks, MD) for the entire study.

BARDOT-based pathogen detection and identification require a robust scatter image library. Since *Salmonella* serovars are diverse (there are >2,600), we focused our study primarily on the 20 most important *Salmonella* serovars (1). The *Salmonella* library contained 50 to 100 scatter patterns per strain. Each of the 20 serovars was represented by three to five strains (a total of 123 strains) to account for strain-related phenotypic variability. We compared scatter pattern-based fingerprints with genotypic

(PFGE) fingerprints. The data showed that phenotypic similarities closely followed genotypic similarities. This confirmed the observation by Ben-Jacob et al. (46) that the phenotypic and morphotypic properties of microorganisms are correlated with the phylogenetic relationships. In addition, we also built a laser scatter library of non-*Salmonella* bacteria (*Citrobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Serratia*, and *Shigella*) that can grow on XLT4 during food sample testing (Fig. 3; Table 1). The time-lapse analysis of scatter patterns formed by *S.* Enteritidis and the two representative non-*Salmonella* organisms (*C. freundii* and *K. pneumoniae*) did not show similarity between *Salmonella* and non-*Salmonella* patterns during the course of colony growth, indicating a potential for the robust detection of *Salmonella* in the presence of background colonies on XLT4 within the specified detection time window (Fig. 3B).

Since *Salmonella* is considered to be a food adulterant, we tested the suitability of BARDOT for the detection of the presence of *Salmonella* in food. Out of 20 *Salmonella* serovars, 19 were detected with PPVs (precision levels) of over 90% (Table 1) when matched against the library containing scatter images of both *Salmonella* and non-*Salmonella* bacteria. Only colonies of serovar Schwarzengrund were detected with a lower precision level of 64.5% because of strain variation within the serovar; two of three strains tested had similar scatter patterns and PFGE subtypes (see Fig. S2 in the supplemental material). Of the 16 miscellaneous serovars tested, all but 3 were detected with PPVs of ≥88% when compared to the top 20 *Salmonella* serovar library. The scatter
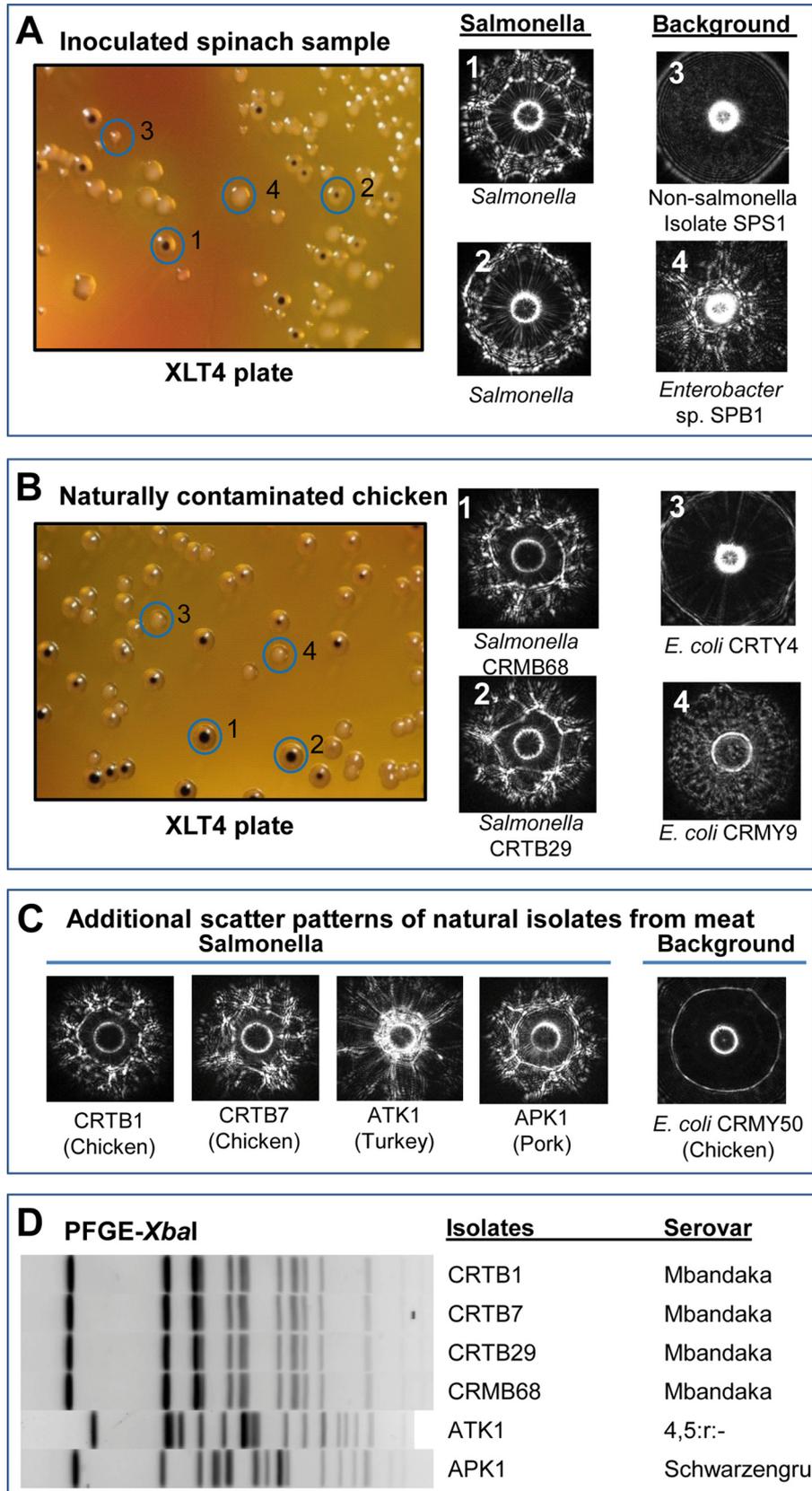
**FIG 8** Detection and identification of *Salmonella* in inoculated spinach (A) and uninoculated chicken (B) samples in the presence of background microbiota. Panel C shows the scatter signatures of *Salmonella* isolates and background isolates from different meat samples, and panel D represents PFGE analysis patterns that were matched with the PulseNet national database. Identities of isolates were also determined by 16S rRNA gene sequencing and serotyping (see also Table 4).

**TABLE 3** Detection of *S.* Typhimurium in peanut butter samples by BARDOT-based and USDA FSIS-based procedures

| | Enrichment time (h) | | Growth on XLT4 | | |
|---|---|---|---|---|---|
| | BPW, | mRV broth, | No. of | BARDOT match with top 8 | |
| Method and sample[a] | 37°C | 42°C | CFU/ml | *Salmonella* serovar library (%)[b] | PCR |
| BARDOT | | | | | |
|   Expt 1 | | | | | |
|     Uninoculated | 4 | 0 | 0 | NA[c] | NA |
|     Inoculated with *S.* Typhimurium | 4 | 0 | $4.7 \times 10^2$ | 82 | + |
|     Control *S.* Typhimurium | 4 | 0 | $3.4 \times 10^2$ | 84 | + |
|   Expt 2 | | | | | |
|     Uninoculated | 4 | 4 | 0 | NA | NA |
|     Inoculated with *S.* Typhimurium | 4 | 4 | $4.7 \times 10^2$ | 84 | + |
|     Control *S.* Typhimurium | 4 | 4 | $4.0 \times 10^2$ | 85 | + |
| USDA FSIS | | | | | |
|   Expt 1 | | | | | |
|     Uninoculated | 24 | 0 | 0 | NA | NA |
|     Inoculated with *S.* Typhimurium | 24 | 0 | $1.1 \times 10^8$ | 82 | + |
|     Control *S.* Typhimurium | 24 | 0 | $3.7 \times 10^8$ | 83 | + |
|   Expt 2 | | | | | |
|     Uninoculated | 24 | 24 | 0 | NA | NA |
|     Inoculated with *S.* Typhimurium | 24 | 24 | $1.5 \times 10^9$ | 84 | + |
|     Control *S.* Typhimurium | 24 | 24 | $1.2 \times 10^9$ | 85 | + |

[a] Peanut butter samples were inoculated with $1.2 \times 10^2 \pm 0.1 \times 10^2$ CFU of *S.* Typhimurium var. Copenhagen and enriched in BPW and mRV broth for different time periods. Enriched samples were plated after 10-fold serial dilutions on XLT4 agar. Control *S.* Typhimurium represents *S.* Typhimurium var. Copenhagen enriched at the same inoculation level in BPW and RV broth without peanut butter to observe the effect of enrichment steps on the light-scattering pattern of a *Salmonella* serovar.
[b] BARDOT match represents percent similarity to *S.* Typhimurium var. Copenhagen included in the *Salmonella* library database consisting of the top eight human-derived serovars showing a PPV range of 68 to 93% for identification (Fig. 5A); PCR positive (+) indicates positive amplification of an *S.* Typhimurium serovar-specific primer set from a randomly picked colony after BARDOT matching.
[c] NA, not applicable.

patterns of these three serovars (Berta, Brandenburg, and Choleraesuis) do not show characteristic distinguishable features (spokes or rings) that are typically seen in other *Salmonella* serovars (Fig. 4; see Fig. S2A in the supplemental material), and all were confirmed to be *Salmonella* by 16S rRNA sequencing. Nevertheless, these data indicate that most *Salmonella* colonies produce light scatter patterns similar enough to be used in a binary classification setting (*Salmonella* versus non-*Salmonella*), even though only a selected few serovars are represented in the training set. This suggests the feasibility of using the top 20 *Salmonella* serovar library for rapid screening of XLT4 plates for the presence of other *Salmonella* serovars.

BARDOT also successfully detected *Salmonella* in inoculated peanut butter, spinach, and raw chicken within 24 h, and the results were in agreement with the standard USDA FSIS method completed in about 72 h (Table 3). In the peanut butter inoculation study, *Salmonella* bacteria were recovered from all of the test samples and the scatter patterns were visually indistinguishable when the bacteria were taken on day 0 or on day 28 (Fig. 7A). Such consistency indicates that long-term storage, even in a stressful environment, apparently does not perturb the scatter pattern. The likely explanation may point to enrichment in BPW and mRV broth or on solid agar (XLT4), which allowed bacterial resuscitation from unfavorable conditions (9). Similar results were obtained in our previous studies in which *Listeria* (30) and *Vibrio* (34) bacteria were preexposed to high temperature, low pH, and high osmotic stress, as well as a viable-but-nonculturable state for *Vibrio*, prior to plating. This study indicates that regardless of the period of time that microorganisms have been present in a food product, BARDOT, like molecular methods, should still be able to detect them accurately.

We did not find any background or non-*Salmonella* colonies on the plates in either inoculated or uninoculated peanut butter samples; however, both spinach and chicken samples contained background microbiota that produced colonies on XLT4 (Fig. 8). The scatter patterns of these background colonies were distinct from those of *Salmonella*, indicating that BARDOT can be used for detection of *Salmonella* in food even in the presence of background microbiota. The background colonies were identified by 16S rRNA sequencing as *E. coli* in chicken and *Enterobacter* species in spinach samples. BARDOT was also able to detect *Salmonella* in naturally contaminated food samples, and a majority of them were confirmed by 16S rRNA gene sequencing or PFGE (Table 4; Fig. 8). These findings strongly suggest that BARDOT is capable of rapid screening for the presence of *Salmonella* on XLT4 agar plates.

In practice, BARDOT can detect a single target colony in the presence of multiple nontarget colonies on the same agar plate (33, 34). Therefore, the prolonged enrichment commonly practiced for most culture-based methods may not be necessary. However, interference from the sample matrix must be dealt with by optimizing sample preparation and enrichment procedures for different types of samples (meats, fruits and vegetables, flours, dairy products, spices, etc.). Paramagnetic bead-based capture of bacteria from complex matrices can be adopted to increase cell numbers and also to avoid interference from food matrices before plating for BARDOT-based detection (33, 47).

With an interest in identifying *Salmonella* at the serovar level, three data sets were used to train the pattern classifier. The data set with the top 8 serovars that includes Enteritidis, Typhimurium, Newport, Javiana, Heidelberg, Montevideo, I 4,[5],12:i:−, and Oranienburg, which are responsible for 58% of food-borne out-

**TABLE 4** BARDOT-based detection and identification of *Salmonella* in naturally contaminated food

| Isolate | % PPV with BARDOT Library | | BARDOT-based detection[a] | 16S rRNA gene sequencing and verification (NCBI accession no.) | Identification by serotyping | Subtype determined by PFGE |
|---|---|---|---|---|---|---|
| | Top 20 *Salmonella* library | Non-*Salmonella* library | | | | |
| CRTB1 (Chicken) | 94 | 6 | *Salmonella* | *Salmonella* sp. (KF574804) | Mbandaka | Mbandaka |
| CRTB7 (Chicken) | 97 | 3 | *Salmonella* | *Salmonella* sp. (KF574801) | Mbandaka | Mbandaka |
| CRTB29 (Chicken) | 90 | 10 | *Salmonella* | *Salmonella* sp. (KF574801) | Mbandaka | Mbandaka |
| CRMB68 (Chicken) | 100 | 0 | *Salmonella* | *Salmonella* sp. (KF574809) | Mbandaka | Mbandaka |
| CRMB13 (Chicken) | 95 | 5 | *Salmonella* | *Salmonella* sp. (KF574810) | NT[b] | NT |
| CRMY4 (Chicken) | 57 | 43 | Non-*Salmonella* | *E. coli* (KF574805) | NT | NT |
| CRMY9 (Chicken) | 6 | 94 | Non-*Salmonella* | *E. coli* (KF574806) | NT | NT |
| CRMY50 (Chicken) | 7 | 93 | Non-*Salmonella* | *E. coli* (KF574802) | NT | NT |
| CRTY15 (Chicken) | 6 | 94 | Non-*Salmonella* | *E. coli* (KF574803) | NT | NT |
| APK1 (Pork) | 97 | 3 | *Salmonella* | *Salmonella* sp. (KF574808) | Schwarzengrund | Schwarzengrund |
| ATK1 (Turkey) | 67 | 33 | Undefined | *Salmonella* sp. (KF574807) | 4,5:r:− | 4,5:r:− |
| SPB1 (Spinach) | 18 | 82 | Non-*Salmonella* | *Enterobacter* sp. (KF574799) | NT | NT |

[a] BARDOT was also used for serotype identification with the top eight *Salmonella* serovar library, but the analysis did not yield any serotype identification, indicating that none of these isolates belong to the top eight serovars (see text for explanation).

[b] NT, not tested.

breaks, showed classification precision levels of 67.9 to 92.8%, while the top 10 and top 20 serovars resulted in PPVs ranging from 57.8 to 90.4% and 38.0 to 86%, respectively (Fig. 5). The low classification precision level of some serovars could be attributed to the similarity of scatter pattern features within or between the strains of different serovars. Since the top eight *Salmonella* serovar library gave the best results, we used it for the identification of *S.* Enteritidis and *S.* Typhimurium, obtaining PPVs of 90 and 85%, respectively, when both serovars were grown as a mixed culture on XLT4 plates.

Furthermore, we attempted to identify the serovars of *Salmonella* isolates from naturally contaminated food by using the top eight *Salmonella* serovar library; the percent classification values were well below our acceptability range (67.9 to 92.8%), indicating that possibly none of the isolates belonged to the top eight serovars (Fig. 5; Table 4). Further examination by serotyping and PFGE indeed confirmed all of the chicken isolates to be *S. enterica* serovar Mbandaka, the pork isolate (APK1) to be Schwarzengrund, and the turkey isolate (ATK1) to be 4,5:r:− (Fig. 8). None of these serovars is part of the top eight *Salmonella* serovar library, which may explain why we were unable to identify the natural food isolates at the serovar level. Furthermore, Mbandaka is not even included in the top 20 serovars and Schwarzengrund had the lowest PPV (64.5%) when compared with the top 20 *Salmonella* serovar library (Table 1). Taken together, these data indicate that identification of *S. enterica* at the serovar level would have limited success owing to overall low classification accuracy with the top 20 *Salmonella* serovars during a cross-validation experiment (Fig. 5).

Thus, modified growth medium formulations and image analysis programs may be necessary to improve the classification of serovars.

In conclusion, the present work demonstrates that BARDOT is suitable for rapid (real-time) on-plate detection (screening) of *Salmonella* colonies (88 to 100% accuracy) obtained by the classical microbiological culture-based method; colonies are available for further testing, including molecular or serological tests. Employing a shortened (4 h each) preenrichment (in BPW) and selective enrichment (in mRV broth), followed by 16 h of growth on an XLT4 agar plate, BARDOT accurately detected *S. enterica* in the presence of high levels of background microbiota from inoculated spinach and chicken and in naturally contaminated food products in about 24 h. Long storage of bacteria in peanut butter (28 days) did not affect the scatter pattern of *S.* Typhimurium. BARDOT would be able to identify *S. enterica* at the serovar level, especially serovars Typhimurium and Enteritidis, but with limited success for the other serovars, owing to overlapping scatter patterns. A new generation of BARDOT (Advanced Bioimaging Systems, LLC, West Lafayette, IN) is being manufactured with an attached incubator that holds a large number of petri plates for high-throughput automated screening of plates with a minimum of user intervention. After an initial investment in the equipment, BARDOT can be operated with a minimum of consumables and supplies and should be an attractive tool for the food industry, regulatory agencies, and diagnostic laboratories, even in resource-constrained remote locations.

## MATERIALS AND METHODS

**Cultures and growth media.** Bacterial cultures (Table 1) were stored as 10% glycerol stocks at −80°C. All top 20 *Salmonella* serovars were supplied by the Indiana State Department of Health (ISDH) and verified by serotyping (48) and PFGE analysis (49) (see below). To develop a scatter image library, frozen stocks were streaked onto XLD agar plates and incubated at 37°C for 12 h to obtain pure colonies. An isolated single colony was selected and propagated in mRV broth (38) at 37°C for 16 h prior to plating. Cultures were serially diluted in 20 mM phosphate-buffered saline (PBS), pH 7.4, and appropriate diluents were surface plated on appropriate agar plates and incubated at 37°C for 12 to 16 h to achieve a colony diameter of 1.1 ± 0.2 mm, which is suitable for acquiring scattering patterns. Dehydrated media BG, BHI, mRV broth, and XLD were purchased from Acumedia (Neogen, Lansing, MI), and XLT4 was from BD (Sparks, MD), Acumedia (Neogen), and HiMedia (VWR). For most of our studies, XLT4 from BD was used. To prepare fresh agar plates, 19.0 ± 1.0 ml/plate (disposable petri dish) was poured and used within 2 to 3 days.

**Acquisition of colony scatter patterns.** A prototype of a commercial laser light-scattering sensor, BARDOT (Advanced Bioimaging Systems) (Fig. 1), originally developed in our laboratory (28, 29) was used to collect scatter signatures of colonies (see Materials and Methods in the supplemental material for details).

Our first experiment was performed to select an agar medium that would provide the most differential scatter patterns among *Salmonella* serovars. Fresh cultures of the *Salmonella* serovars listed in Fig. 2A were diluted in PBS, and appropriate diluents were surface plated on BHI, BG, XLD, and XLT4 agar to obtain 30 to 100 CFU/plate and incubated at 37°C for 12 to 16 h to achieve a colony diameter of 1.1 ± 0.2 mm. Scatter patterns were analyzed by using a custom-developed pattern recognition algorithm that relies on machine-learning algorithms and training with scatter images of reference bacteria, and data are presented as classification precision levels or PPVs (32, 50). We also examined the scatter signatures and colony counts of *Salmonella* on XLT4 medium procured from three suppliers (BD, Acumedia, and HiMedia).

Next, we collected the scatter signatures of the top 20 human-derived *Salmonella* serovars (3 to 5 strains of each serovar, a total 123 strains) and non-*Salmonella* bacteria that grew on XLT4 agar to build our scatter image libraries (Table 1). For each strain, scatter patterns of at least 50 to 100 colonies were collected and a total of about 7,600 scatter images were included in the library.

Time-lapse measurement of scatter pattern and microscopic images (Leica Microscope, Buffalo Grove, IL; ×10 magnification) of colonies of *S.* Enteritidis and representative non-*Salmonella* bacteria (*C. freundii* and *K. pneumoniae*) were collected at 11, 13, 15, 17, and 20 h of incubation at 37°C.

**Scatter patterns and phylogeny.** For every scatter pattern represented as a grayscale image, a vector of features was computed. The vector consisted of 78 features, including pseudo-Zernike moments and Haralick cooccurrence matrix-based features, to aid in our image analysis and development of a phylogenetic tree for each serovar (29, 32, 51). For a detailed description, see Materials and Methods in the supplemental material.

**PFGE.** All of the top 20 serovars (8) used in this study were analyzed by PFGE as previously described, and the fingerprint patterns were matched with the CDC PulseNet database (49). The BARDOT-generated colony phenotypic scatter fingerprints were compared with the PFGE fingerprints of the top eight *Salmonella* serovars (Enteritidis, Typhimurium, Newport, Javiana, Heidelberg, Montevideo, I 4,[5],12:i:−, and Oranienburg) representing 31 strains (for a detailed description, see Materials and Methods in the supplemental material).

**BARDOT analysis of mixed cultures of *S.* Typhimurium and *S.* enteritidis.** The ability of BARDOT to differentiate between *S.* Typhimurium and *S.* Enteritidis in a mixed culture on an XLT4 agar plate was investigated. An aliquot of 0.5 ml of $1.3 \times 10^9 \pm 0.1 \times 10^9$ CFU/ml from each culture was mixed, serially diluted, plated on XLT4, and incubated at 37°C for 16 h. The scatter patterns of colonies were compared with those of the top eight *Salmonella* serovar library. The identity of each colony was further verified by serovar-specific PCR (see below).

**Application of BARDOT as a screening tool to detect *Salmonella* in artificially and naturally contaminated food products. (i) Optimization of enrichment steps for BARDOT-based detection.** Since peanut and peanut butter products have been implicated in *Salmonella* outbreaks (10), we used peanut butter as a model food matrix for BARDOT evaluation. We tested both meat and nonmeat products (spinach and peanut products); hence, we used a modified USDA FSIS protocol for enrichment and plating (38). The amount of peanut butter and the volume of BPW were scaled down to 30 ± 2.5 g/sample and 270 ± 2.5 ml/sample, respectively. Each 30 ± 2.5-g portion of sample was placed in a Whirl-Pack bag, inoculated with $1.2 \times 10^2 \pm 0.1 \times 10^2$ CFU of *S.* Typhimurium var. Copenhagen, left in a laminar-flow hood for 15 min, mixed with 270 ± 2.5 ml BPW, and blended in a Stomacher 400 (Seward, United Kingdom) at 200 rpm for 5 min. Some preenriched samples were also transferred to mRV broth, and both enrichments were done for various time periods at 37°C (see Table S1 in the supplemental material). Samples were decimally diluted in PBS, surface plated on XLT4 agar plates, and incubated at 37°C for 12 to 16 h or until colonies reached a diameter of 1.1 ± 0.2 mm. Plates were analyzed by BARDOT. The identities of selected colonies were also confirmed by serovar-specific PCR (see below). In parallel, the samples were also analyzed by the standard USDA FSIS procedure for ready-to-eat food (38).

**(ii) Detection of *Salmonella* in artificially inoculated chicken and spinach samples.** The shortened enrichment protocol was also used for artificially inoculated chicken and spinach samples. In short, 25 ± 2.5 g each of raw chicken breast and fresh spinach procured from a local grocery store was artificially inoculated separately with *Salmonella* Enteritidis PT21 at $1.2 \times 10^2 \pm 0.4 \times 10^2$ cells and *S.* Typhimurium var. Copenhagen at $1.2 \times 10^6 \pm 0.4 \times 10^6$ cells, respectively. Samples were blended in 225 ± 4.5 ml of BPW in a stomacher bag and incubated at 37°C for 4 h. One milliliter of preenriched sample was inoculated into 10 ml of mRV broth and enriched at 42°C for 4 h before plating for BARDOT analysis.

**(iii) Detection and identification of *Salmonella* serovars in chicken carcass, pork, and turkey meat samples.** Chicken carcass, pork, and turkey meat samples (a total of 15) were processed by following the USDA FSIS microbiology laboratory guidelines (38). Enriched samples were diluted and plated on XLT4, and colonies were screened with BARDOT and matched with the top 20 *Salmonella* and non-*Salmonella* libraries. Isolate identities were further confirmed by 16S rRNA gene sequencing (see below) (52). To identify *Salmonella* at the serovar level, the scatter patterns were matched with the top 8 *Salmonella* serovar library since this library had classification accuracies of 68 to 93%, much better than the top 10 (58 to 90%) or top 20 (38 to 86%) *Salmonella* serovar library. The serovar of each natural isolate was further determined by a conventional serotyping scheme (48) and PFGE (49) at the ISDH laboratory (Indianapolis, IN).

**(iv) Analysis of peanut butter samples over an extended period of storage.** In order to verify whether the low-moisture, high-fat, and high-salt environment of peanut butter may exert stress on *Salmonella* cells, leading to a change in colony formation by surviving bacteria, we inoculated peanut butter samples (30 ± 2.5 g) with $1.2 \times 10^2 \pm 0.1 \times 10^2$ CFU of *S.* Typhimurium ST1 and stored them at room temperature for up to 28 days (53). Three 30 ± 2.5-g portions of the samples were removed on days 0, 7, 14, 21, and 28 of storage; preenriched in BPW for 4 h at 37°C; and enriched in mRV broth for 4 h at 42°C before surface plating on XLT4. Colonies on plates were analyzed with BARDOT after ~16 h of growth, and the results were verified by *S.* Typhimurium-specific PCR.

**PCR analysis and 16S rRNA sequencing.** *Salmonella* isolates were verified by PCR with *Salmonella* genus-specific primers derived from the invasion gene activator *hilA* (forward, 5′-CGGAACGTTATTTGCGCCA TGCTGAGGTAG-3′; reverse, 5′-GCATGGATCCCCGCCGGCGATTG TG-3′) (54). For identification of *Salmonella* at the serovar level, *S.*

Typhimurium-specific primers (STM4492-F, 5′-ACAGCTTGGCCTAC GCGAG-3′; STM9942-R, 5′-AGCAACCGTTCGGCCTGAC-3′) (55) and S. Enteritidis-specific primers (IE1L, 5′-AGTGCCATACTTTTAATG AC-3′; IE1R, 5′-ACTATGTCGATACGGTGGG-3′) (56) were used.

The 16S rRNA gene (~1,400 bp) was amplified as previously described (52), and each amplicon was sequenced at the Purdue University Genomics Facility.

**Data analysis.** Statistical analysis of the quantitative values was performed by one-way analysis of variance (SPSS ver. 21 software). Tukey's honestly significant difference multiple-comparison tests were used to demonstrate the significant differences in the classification accuracy values obtained with different media. Scatter patterns from two experiments were cumulatively used to make a scatter image library of *Salmonella* serotypes in the different media used in this study. Data with *P* values of <0.05 were considered significantly different, with a high individual score level.

**Nucleotide sequence accession numbers.** The sequences of the background *E. coli* or *Enterobacter* species that produced scatter patterns showing very high similarity to patterns from the library consisting of non-*Salmonella* bacteria were deposited in the NCBI database and assigned the accession numbers listed in Table 4. The 16S rRNA gene sequences of the following taxa were submitted to the NCBI GenBank database and assigned the accession numbers shown: S. Agona SA4, JQ228522; S. Enteritidis PT21, JQ228519; S. Hadar SH6, JQ228523; S. Heidelberg ATCC 8326, JQ228521; S. Montevideo SM7, JQ228524; S. Newport SN8, JQ228525; S. Typhi ST3, JQ228520; S. Typhimurium var. Copenhagen ST1, JQ228518.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org /lookup/suppl/doi:10.1128/mBio.01019-13/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Figure S1, TIF file, 1.2 MB.
Figure S2, TIF file, 1.1 MB.
Figure S3, TIF file, 1.2 MB.
Table S1, DOCX file, 0.1 MB.

## ACKNOWLEDGMENTS

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