Evaluation of Rapid Molecular Detection Assays for *Salmonella* in Challenging Food Matrices at Low Inoculation Levels and Using Difficult-to-Detect Strains

GINA RYAN,† SHERRY ROOF, LAURIE POST, and MARTIN WIEDMANN

1Department of Food Science, College of Agriculture and Life Sciences, Cornell University, Ithaca, New York 14853, USA; and 2Mars Global Food Safety Center, Huairou, Beijing, People’s Republic of China

MS 15-098: Received 5 March 2015/Accepted 11 May 2015

ABSTRACT

Assays for detection of foodborne pathogens are generally initially evaluated for performance in validation studies carried out according to guidelines provided by validation schemes (e.g., AOAC International or the International Organization for Standardization). End users often perform additional validation studies to evaluate the performance of assays in specific matrices (e.g., specific foods or raw material streams of interest) and with specific pathogen strains. However, these types of end-user validations are typically not well defined. This study was conducted to evaluate a secondary end user validation of four AOAC-validated commercial rapid detection assays (an isothermal nucleic acid amplification, an immunoassay, and two PCR-based assays) for their ability to detect *Salmonella* in two challenging matrices (dry pet food and dark chocolate). Inclusivity was evaluated with 68 diverse *Salmonella* strains at low population levels representing the limit of detection (LOD) for each assay. One assay detected all strains at the LOD, two assays detected multiple strains only at 10 times the LOD, and the fourth assay failed to detect two strains (*Salmonella bongori* and *S. enterica subspp. houtenae*) even at 1,000 times the LOD; this assay was not further evaluated. The three remaining assays were subsequently evaluated for their ability to detect five selected *Salmonella* strains in food samples contaminated at fractional levels. Unpaired comparisons revealed no significant difference between the results for each given assay and the results obtained with the reference assay. However, analysis of paired culture-confirmed results revealed assay false-negative rates of 4 to 26% for dry pet food and 12 to 16% for dark chocolate. Overall, our data indicate that rapid assays may have high false-negative rates when performance is evaluated under challenging conditions, including low-moisture matrices, strains that are difficult to detect, injured cells, and low inoculum levels.

An increasing number of rapid assays are available for detection of key foodborne pathogens in a wide range of food matrices. These proprietary assays have several advantages over traditional culture-based reference assays, including shorter turn-around times, costs savings due to fewer resources required (e.g., labor and culture media), automation, and increased throughput. Initial validation of new (alternative) detection assays evaluates whether results obtained with the alternative are not significantly different from results obtained with the appropriate reference assay. Currently, several validation schemes are used for validation of commercially available alternative assays. These validation schemes are based on four system standards: (i) the European and international standard EN ISO 16140 from the International Organization for Standardization (ISO), including the French organization Association Française de Normalisation (AFNOR) and the European organization MicroVal (3, 24); (ii) AOAC standards, including the Official Methods of Analysis (OMA) and Performance Tested Methods (PTM) of the AOAC Research Institute (2); (iii) NordVal standards from the Nordic Committee on Food Analysis in the Nordic countries (33); and (iv) the Health Canada standards (21).

With the exception of the AOAC PTM scheme, all validation schemes mentioned above comprise three phases: (i) establishment of performance characteristics, usually by a single lab (method developer), (ii) validation of the performance characteristics by an independent laboratory in a precollaborative study, and (iii) evaluation of performance ruggedness, either by a single lab or in a multilab collaborative study. The AOAC PTM scheme includes an independent single-lab validation study but does not include a multilab collaborative study. For the precollaborative studies, the similarities between the various validation schemes (e.g., ISO 16140 and AOAC PTM) extend to the type of analyses included in the protocols (e.g., inclusivity and exclusivity and analysis of the degree of correspondence between the alternative and reference assays). The schemes differ somewhat in specific technical rules guiding experimental design and the statistical methods used in the validation study, e.g., specific analytical performance
criteria, experimental design (e.g., type and number of samples tested), calculations, and interpretation (2, 21, 24, 33). For inclusivity testing, the allowable level of pure bacterial broth culture can range from 10 to 100 times the limit of detection (LOD) (2, 21, 24, 33). Although at least 50 strains are typically required for inclusivity testing, this number may differ among validation schemes for different pathogens; for example, ISO 16140 requires 30 strains, whereas AOAC requires a minimum of 50 strains except for Salmonella, for which a minimum of 100 strains is required (2, 24). Guidance for selection of strains to be tested in inclusivity or comparative matrix experiments is limited; validation guidelines generally state only that strains included in the study should be representative of genetic and phenotypic diversity and associated with the food matrix (2).

The validation schemes also differ in the claims allowed for the matrices evaluated. For example, AFNOR includes an “all foods” claim, whereas AOAC standards limit claims for approval to those specific food matrices that a given assay was tested with, which means an all-foods claim is not applicable for AOAC standards (2). The ISO standard is currently undergoing revision to harmonize it with AOAC standards. The all-foods claim has now been replaced with a claim for a “broad range of foods” and will require method developers to identify the actual food categories and food types used in the validation study (24). The criteria used for selection of a specific matrix (i.e., diversity and range of food types or formulations) that is representative of a food category also differ for the various validation schemes. For example, standards based on EN ISO 16140 require testing of three separate food matrices (e.g., raw, heat processed, and cured for meat products) per category, for a total of 15 different food matrices; the number of matrices required for AOAC testing depends on the applicability of the method (3, 24, 33). For both ISO and AOAC validation schemes, naturally contaminated samples are preferred for comparative evaluation studies, but samples artificially contaminated at fractional positive levels are allowed (2, 24). The range of strains evaluated for detection in food matrices also differs among the validation schemes. AOAC requires testing with one strain per individual food matrix evaluated, but ISO 16140 schemes require testing with one strain per food category (e.g., meat products). Thus, under ISO 16140 schemes the same strain could be tested with all three food matrices for a given category (2, 24). Differences also exist for data analyses and reporting guidelines. For validation schemes based on AOAC OMA and ISO 16140, a detailed analysis of the assay performance may be based on results obtained from paired enrichment cultures (relative accuracy, relative sensitivity, and relative specificity) or unpaired enrichment cultures (probability of detection [POD]). However, for AOAC PTM precollaborative studies, only the assay specificity (i.e., use of paired samples to compare presumptive-positive results obtained with the alternative assay with positive results confirmed with the reference culture assay) is typically reported (2, 24).

A limitation of the current schemes is that they do not require an evaluation that stringently addresses the effect of strain and food matrix variation on assay performance, even though these factors can greatly impact microbial recovery and detection. For example, certain genetically distinct pathogen strains may not be detected or they may be detected with reduced sensitivity in a given assay; these strains with reduced detectability may easily be overlooked in an evaluation when the strain set is not appropriately selected. Food matrices within categories can differ greatly (e.g., with regard to water content, pH, and fat content), and this variation can have a considerable effect on microbial recovery and detection (e.g., when foods contain polyphenols and complex polysaccharides) (14). For example, different types of chocolate (e.g., milk and dark chocolate) may vary considerably in their phenolic and fat contents, which can influence the severity of pathogen cell injury, the recovery of injured cells during enrichment culture, and the inhibition of pathogen detection from enrichment cultures (36, 40). Recommendations to address a number of factors influencing test outcomes (e.g., aging of inoculated samples, presence of competitor strains, and inoculum stress such as cell injury) are included in the U.S. Food and Drug Administration (FDA) (42) guidelines for internal validation of alternative methods; however, similar standards are not uniform across the different validation schemes and consequently may leave the end user exposed to unknown risks when a validated assay is used under conditions that differ from those tested in the original validation study.

The aim of the current study was to stringently evaluate commercially available rapid molecular detection assays for their ability to detect diverse Salmonella strains under conditions simulating a worst-case scenario of difficult-to-detect strains, injured cells, low inoculation level, and a challenging matrix. This study also was designed to develop and publish a general approach that can be used for secondary user specific evaluation of commercial assays that have already been evaluated using a validation scheme such as ISO or AOAC.

**MATERIALS AND METHODS**

**Bacterial strains and media.** A total of 68 Salmonella strains were used in this study: (i) 63 strains of Salmonella enterica subsp. enterica serotypes, (ii) 4 strains (1 each) of S. enterica subspecies salamae, arizonae, diarizonae, and houtenae, and (iii) 1 strain of Salmonella bongori. The strain set was assembled from several collections, including the Food Safety Laboratory (Department of Food Science, Cornell University, Ithaca, NY) and the American Type Culture Collection (Manassas, VA) (see supplemental Table 1 for complete strain details; all supplemental material is available at https://foodsafety.foodscience.cornell.edu/research-and-publications-supplementary-materials-manuscripts/2015).

Isolates were selected to represent strains commonly associated with human disease (e.g., Salmonella Enteritidis), strains commonly associated with nonhuman sources (based on Salmonella serotypes reported to the Centers for Disease Control and Prevention) and food contamination, and strains commonly reported in different regions of the world (e.g., Salmonella Virchow, which is commonly found in southeast Asia) (9, 22). All strains were stored at −80°C in brain heart infusion (BHI) broth supplemented with 15% glycerol (BD, Franklin Lakes, NJ). All culture media were purchased from BD.
TABLE 1. Inclusivity and analytical sensitivity results for commercial rapid Salmonella detection assays evaluated

<table>
<thead>
<tr>
<th>Assay</th>
<th>Target</th>
<th>Predicted LOD (CFU/ml)</th>
<th>Salmonella strain or serotype</th>
<th>Lowest level detected a</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Nucleic acids</td>
<td>$10^3$</td>
<td>All 68 strains</td>
<td>LOD</td>
</tr>
<tr>
<td>B</td>
<td>Nucleic acids</td>
<td>$10^4$</td>
<td>Typhimurium</td>
<td>LOD (variable)</td>
</tr>
<tr>
<td>C</td>
<td>Nucleic acids</td>
<td>$10^4$</td>
<td>Alachua</td>
<td>LOD + 1 log unit</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>II salmonae</td>
<td>LOD + 1 log unit</td>
</tr>
<tr>
<td>D</td>
<td>Bacterial surface molecules</td>
<td>$10^3$</td>
<td>IIIa arizonae</td>
<td>LOD (variable)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IIIb diarizonae</td>
<td>LOD + 3 log units</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IV houtenae</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>V bongori</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Alachua</td>
<td>LOD + 1 log unit</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Minnesota</td>
<td>LOD + 1 log unit</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kentucky</td>
<td>LOD + 1 log unit</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Typhi</td>
<td>LOD + 1 log unit</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Adelaide</td>
<td>LOD + 1 log unit</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mississippi</td>
<td>LOD + 1 log unit</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>13,22:b:--</td>
<td>LOD + 1 log unit</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Virchow</td>
<td>LOD + 1 log unit</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Loubomo</td>
<td>LOD + 1 log unit</td>
</tr>
</tbody>
</table>

a LOD, limit of detection, i.e., the lowest level of target organism that the assay can consistently detect.
b Lowest level of a given strain(s) that was detected in both replicate samples. LOD + 1 or 3 log units indicates 1 or 3 log units above the LOD. Variable, strain was detected in only one of two replicate samples at the LOD; ND, not detected at any level up to the LOD + 3 log units.

c For assay C, three strains not belonging to S. enterica subsp. enterica were poorly detected; consequently assay C was eliminated from further study.

Rapid detection assays evaluated. Four enrichment-based commercial rapid detection assays with AOAC validation for dark chocolate and dry pet food matrices were selected for evaluation from a larger collection of assays, using a previously published approach (45). A set of 30 detection assays were initially evaluated using several criteria (e.g., reported performance characteristics, cost, and assay throughput) with a scoring system. Based on the overall quantitative score sums for each assay, four high-ranking assays were selected for further evaluation: an isothermal nucleic acid amplification assay, two PCR-based assays, and an immunoassay that detects bacterial surface molecules (see Table 1).

Inclusivity. Pure cultures of all 68 Salmonella strains were used for inclusivity testing. Bacterial suspensions for individual strains were prepared as follows. Frozen stock cultures were streaked onto BHI agar and incubated at 37°C for 18 h. Individual colonies were inoculated into BHI broth and grown at 37°C (with shaking) to an optical density at 600 nm of approximately 0.40 ± 0.02 (model Spectronic 20D+, Thermo Fisher Scientific, Waltham, MA), which yielded approximately $4 \times 10^9$ CFU/ml. The broth cultures were diluted to target levels in buffered peptone water (BPW) and tested with all four detection assays according to the respective assay guidelines. The LODs for each assay were verified by testing strains representing 10 common serotypes at three cell levels: (i) the reported or experimentally determined LOD for each assay, (ii) the LOD + 1 log unit (i.e., 1 log unit above the LOD), and (iii) the LOD + 3 log units (i.e., 3 log units above the LOD). The LOD for assay D was experimentally determined by testing 10 strains at levels representing a 3-log range ($10^3$ to $10^6$ CFU/ml). Full inclusivity testing with all 68 strains was performed with two biological replicates at the LOD and LOD + 1 log unit for each assay. The LOD was $10^3$ CFU/ml for assay A, $10^4$ CFU/ml for assays B and C, and $10^5$ CFU/ml for assay D (Table 1). If a given strain was not detected at the LOD + 1 log unit, the evaluation was repeated with LOD + 3 log units.

Viable microbial counts were determined for all test suspensions by plating appropriate dilutions onto BHI agar with a spiral plater (model Autoplate 4000, Advanced Instruments, Norwood, MD). Plates were incubated at 37 ± 2°C for 24 ± 1 h, and colonies were counted with a QCount (model 530, Advanced Instruments).

Salmonella reduction on dry pet food and dark chocolate. Preliminary experiments were performed to determine the die-off kinetics over 2 weeks at 25 ± 2°C for Salmonella populations inoculated on two low-moisture matrices: dry pet food and dark chocolate. For each strain evaluated, BPW suspensions were prepared from cells collected from BHI agar cultures (grown for 14 h at 37°C) and diluted to $5 \times 10^6$ CFU/ml (29). For dry pet food, individual kibble pieces (approximately 1 g) were loosely distributed across the bottom of sterile petri plates, surface inoculated with three 1-μl volumes of the respective bacterial suspension (representing a total inoculum of approximately $1.5 \times 10^7$ CFU), and dried at room temperature for 1 h. For dark chocolate, four 1-g aliquots were prepared from melted chocolate in sterile petri plates and held at room temperature for 1 h to harden. Individual pieces were surface inoculated with three 1-μl volumes of the respective bacterial suspension (representing a total inoculum of approximately $1.5 \times 10^7$ CFU). Samples inoculated with sterile BPW served as negative controls. All inoculated and control samples were stored at 25 ± 2°C for up to 2 weeks in sterile covered plastic containers kept in a dry heat incubator (model 414005-128, VWR, Radnor, PA). Counts of viable Salmonella colonies for each strain were determined for 10 replicate samples at several time points during the 2-week storage period: 1 h, 24 h, 7 days, and 14 days postinoculation. Data obtained from at least three independent trials were used to determine the bacterial reduction kinetics for each strain. At each time point, 1-g replicate samples were diluted 1:10 in BPW or nonfat dry milk (NFDM) in sterile 4-oz (120-ml) Whirl-Pak bags (VWR) for dry pet food and dark chocolate, respectively, and
homogenized by stomaching at 230 rpm for 2 min. Appropriate dilutions were prepared from sample homogenates in cold BPW, and 50-µl aliquots were plated on BHI agar plates (equilibrated to room temperature) in duplicate and incubated at 35 ± 2°C for 24 ± 1 h. *Salmonella* colonies were then enumerated. BHI agar inoculated with negative control samples were negative for microbial growth at all time points evaluated. For each strain, the log reduction of *Salmonella* on a given matrix was calculated as the mean log difference between the initial inoculum and the mean number of viable *Salmonella* colonies recovered from replicate samples at each time point.

**Matrix inoculation at fractional positive levels.** Comparative evaluations with the inoculated matrices (dry pet food and dark chocolate) were performed with separate sets of five *Salmonella* strains for each matrix. Strains were selected to represent serotypes of importance in public health (e.g., *Salmonella Typhimurium* and *Salmonella Mississippi*), serotypes relevant to a given food matrix (e.g., *Salmonella* Kentucky, which has been associated with the environment of relevant food processing facilities), strains that were detected only at the LOD + 1 log unit with a given assay in the initial inclusivity experiments (e.g., *Salmonella* Alachua, which was detected only at LOD + 1 log unit with assays B and D). Both matrices were inoculated with *Salmonella* Typhimurium and *Salmonella* Alachua; dry pet food was also inoculated with *Salmonella* serotypes Adelaide, Minnesota, and Kentucky, and dark chocolate was also inoculated with serotypes Mississippi, Virchow, and 13,22:b–.

Twenty replicate samples were contaminated with each strain at fractional positive levels (i.e., contamination level yielding 50% ± 25% positive results) (2). Uninoculated dry pet food and dark chocolate samples (five samples per matrix per experiment) were prepared by surface inoculating *Salmonella* species with the FDA Bacteriological Analytical Manual (BAM) assay 3 days before matrix inoculation. Bacterial strain suspensions in BPW were prepared from overnight plate cultures as described above and dilutted to approximately 10^3 CFU/ml before inoculation. To determine contamination levels yielding fractional positive results for each matrix, samples were inoculated at several levels based on estimated strain log-reduction rates to target a 1 CFU/25 g test portion after a 2-week storage period. For dry pet food, individual kibble pieces were loosely distributed in sterile petri plates and surface inoculated with each strain at three levels (5, 7, and 10 CFU/g). For dark chocolate, 1-g aliquots were prepared in sterile petri plates as previously described, and individual pieces were surface inoculated at 8, 12, 16, and 20 CFU/g. BPW-inoculated samples were used for negative controls. Contaminated samples were held at room temperature for 1 h to dry and stored at 25 ± 2°C for 2 weeks in sterile covered plastic containers in a controlled dry heat incubator.

**Sample preparation and testing.** For each strain, samples were inoculated at different levels and screened for viable *Salmonella* on day 10 postinoculation to predict which contamination level would yield fractional positive results in the full evaluation conducted on day 14 postinoculation. Screening was performed using a modified BAM approach (41). For each contamination level, 10 individual samples (1 g each) were screened on day 10. These samples were diluted 1:10 in 4-oz Whirl-Pak filter bags containing 9 ml of lactose broth (LB) medium for dry pet food or NFDM for dark chocolate. The preenrichment samples were held at room temperature for 1 h to soften the food matrix and then homogenized in a stomacher, and the pH was adjusted to 6.8 with sterile 1 M NaOH. Dark chocolate preenrichment samples were supplemented with 0.018 ml of a 1% brilliant green solution. All preenrichment samples were incubated for 24 h at 35 ± 2°C. A 1-ml aliquot from each enrichment culture was inoculated into 9.0 ml of tetraphionate (TT; Oxoid, Basingstoke, UK) broth and incubated for 24 h at 35 ± 2°C, and 50-µl aliquots were then subcultured onto xylose lysine deoxycholate (XLD; Neogen, Lansing, MI) agar and incubated at 35 ± 2°C for 24 h. Samples were considered positive when typical *Salmonella* colonies were recovered on XLD agar. The contamination level yielding four to six positive results from 10 replicate samples (i.e., subsequently predicted to yield fractional positive results on day 14) was selected for full testing with all assays.

The full comparative evaluation was initiated on day 14 after matrix inoculations. For each strain tested, 20 replicate samples were evaluated with each assay (assays A, B, and D and the BAM enrichment sets) according to the respective assay guidelines. Sample preenrichments were prepared by combining 1 g of contaminated sample and 24 g of unincoculated sample in a sterile 55-oz (1.65-liter) Whirl-Pak filter bag containing 225 ml of the appropriate enrichment medium. Controls tested with each assay were five uninoculated negative controls and two positive controls spiked with 100 CFU of *Salmonella* Typhimurium; positive control samples were prepared by surface inoculating *Salmonella* Typhimurium cells in BPW suspension onto a 1-g sample of kibble 1 h before inoculation of control preenrichments. The BAM method was used as the reference method. Each 25-g sample was diluted 1:10 in LB medium for dry pet food or NFDM for dark chocolate and allowed to stand for 1 h at room temperature (25 ± 2°C), and the pH was adjusted to 6.8; dark chocolate enrichments were supplemented with 0.45 ml of a 1% brilliant green solution. After incubation at 35 ± 2°C for 24 h, a 1.0-ml aliquot was transferred to 9 ml of TT broth and a 0.1-ml aliquot was transferred to Rappaport-Vassiliadis (RV; Oxoid) medium, and cultures were incubated (with shaking) at 35 ± 2°C and 42 ± 2°C, respectively, for 24 h. A 50-µl aliquot of each of the TT and RV broth cultures was plated onto XLD agar, Hektoen enteric (HE) agar, and bismuth sulfite (BS) agar and incubated at 35 ± 2°C for 24 h. A single presumptive *Salmonella* colony from the RV-XLD selective enrichment-plating medium combination (or from the TT-XLD combination when no colonies were present on RV-XLD agar plates) was subcultured onto BHI agar and incubated at 37 ± 2°C for 24 h. Presumptive *Salmonella* colonies were confirmed by PCR amplification of the *Salmonella*-specific invA gene (28).

**Paired culture confirmation.** All assay sample enrichments were confirmed by culture methods. Appropriate aliquot volumes were subcultured into selective media (RV and TT broths) within 1 h of the recommended incubation time for each assay and then plated on selective agar (e.g., XLD) and submitted for molecular confirmation by invA PCR.

**MPN analysis.** Contaminated food samples used to inoculate preenrichment cultures were enumerated on day 14 (the same day sample preenrichments were prepared) using a modified AOAC most-probable-number (MPN) assay at three consecutive twofold dilutions (10 tubes at 1:5, 20 tubes at 1:10, and 10 tubes at 1:20). The AOAC method stipulates 5 tubes at the lowest and highest dilutions (i.e., the 1:5 and the 1:20 dilutions) (2), but we used 10 tubes at these dilutions. This change increased the sensitivity of the assay. *Salmonella* MPNs were estimated using the MPN calculator provided in the BAM (41).

**Statistical analysis.** Analysis of unpaired sample results was conducted according to the AOAC POD statistical model (2).
McNemar’s exact analysis was performed on paired sample results. A one-way analysis of variance was used to compare the log reduction for *Salmonella* serotypes recovered from dry pet food and dark chocolate samples. The effect of serotype on log reduction was considered significant at $P < 0.05$. The statistical analyses for paired data, including calculating error rates and McNemar’s exact test, were performed using the exact 2x2 package in the R (version 3.0.2) program (37).

**RESULTS**

**Ability of assays to detect the test set of 68 *Salmonella* strains.** We initially evaluated the ability of the four commercial rapid detection assays to detect the 68 selected *Salmonella* strains (63 *S. enterica* subsp. *enterica* serotypes, four *S. enterica* non-*enterica* strains, and one *S. bongori* strain) at the LOD for each assay (Table 1). As a first step, the LOD for each assay was verified using pure cultures of 10 common serotypes; LODs were 10$^3$ CFU/ml (assay A), 10$^4$ CFU/ml (assays B and C), and 10$^5$ CFU/ml (assay D) (Table 1). Subsequent evaluation of all four assays with all 68 strains revealed that assay A detected all 68 strains at the LOD. For assay B, the *Salmonella* Alachua strain was detected at LOD + 1 log unit and the *Salmonella* Typhimurium strain was variably detected at the LOD. For assay D, nine strains were detected only at the LOD + 1 log unit, including two strains representing clinically important *Salmonella* serotypes (Typhi and Mississippi) (9). For assay C, five *Salmonella* strains not belonging to *S. enterica* subsp. *enterica* were poorly detected at the LOD, with two strains (*S. enterica* subsp. *houtenae* and *Salmonella bongori*) not detected at 1,000 times the LOD and one strain (*S. enterica* subsp. *diarizonae*) detected only at 1,000 times the LOD (Table 1).

**Salmonella reduction on dark chocolate and dry pet food.** Viable microbial counts were used to determine the log reduction of *Salmonella* strains in both dark chocolate and dry pet food during 2 weeks at room temperature. On dark chocolate, the mean (±standard deviation) log reduction of the five *Salmonella* strains differed significantly by serotype ($P < 0.001$) and ranged from 1.40 ± 0.22 CFU/ml (*Salmonella* Mississippi) to 1.83 ± 0.27 CFU/ml (*Salmonella* Typhimurium). On dry pet food, no difference in the log reduction (1.04 ± 0.07 CFU/ml; $P > 0.05$) was found among serotypes. These data indicate that inoculation protocols and specifically initial inoculation levels for different food matrices may have to be optimized and evaluated separately for different *Salmonella* serotypes.

**Salmonella detection in dry pet food.** Five strains from five *Salmonella* serotypes (Adelaide, Alachua, Kentucky, Minnesota, and Typhimurium) were used to evaluate the ability of assays A, B, and D to detect *Salmonella* in dry pet food contaminated at fractional positive levels (Tables 2 and 3). All of the uninoculated control samples tested negative for *Salmonella* by the culture-based method. The contamination level for individual strains ranged from 0.52 to 0.94 MPN/25 g (supplemental Table 2). The combined detection frequency for all five strains did not differ significantly among any of the three assays and the reference assay, based on the AOAC POD model (Table 2). The POD is defined as the positive outcome for a qualitative method for a given matrix at a given level (2). Overall, 51 of 100

### Table 2. Comparison of rapid assay results and FDA BAM reference assay results for detection of *Salmonella* in unpaired sample enrichment cultures

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Assay</th>
<th>No. of positive samples</th>
<th>POD</th>
<th>95% CI</th>
<th>No. of positive samples</th>
<th>POD</th>
<th>95% CI</th>
<th>dPOD</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry pet food</td>
<td>A 51</td>
<td>0.51</td>
<td>0.41, 0.61</td>
<td>57</td>
<td>0.57</td>
<td>0.47, 0.66</td>
<td>−0.06</td>
<td>−0.19, 0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 68</td>
<td>0.68</td>
<td>0.58, 0.76</td>
<td>67</td>
<td>0.67</td>
<td>0.57, 0.75</td>
<td>0.01</td>
<td>−0.12, 0.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D 46</td>
<td>0.46</td>
<td>0.37, 0.56</td>
<td>57</td>
<td>0.57</td>
<td>0.47, 0.66</td>
<td>−0.11</td>
<td>−0.24, 0.03</td>
<td></td>
</tr>
<tr>
<td>Dark chocolate</td>
<td>A 61</td>
<td>0.61</td>
<td>0.51, 0.70</td>
<td>60</td>
<td>0.60</td>
<td>0.50, 0.69</td>
<td>0.01</td>
<td>−0.12, 0.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 56</td>
<td>0.56</td>
<td>0.46, 0.65</td>
<td>60</td>
<td>0.60</td>
<td>0.50, 0.69</td>
<td>−0.04</td>
<td>−0.17, 0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D 55</td>
<td>0.55</td>
<td>0.45, 0.64</td>
<td>60</td>
<td>0.60</td>
<td>0.50, 0.69</td>
<td>−0.05</td>
<td>−0.18, 0.08</td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**
- Assays were evaluated with 20 replicate samples for each of the five *Salmonella* serotypes tested per food matrix; thus, a total of 100 samples were tested for each assay for each matrix.
- POD, probability of detection, fractional positive results for the test assay.
- BAM sample enrichments were subcultured into RV and TT selective media and then isolated on XLD, HE, and BS agar plates; selected *Salmonella* colonies were confirmed by invA PCR assay.
- POD, difference between the rapid assay and BAM assay POD values in unpaired samples (POD calculated as the number of confirmed positive results divided by the total number of samples).
- 95% CI, 95% confidence interval. Intervals for dPOD values that include zero indicate that the rapid assay and BAM assay results are not significantly different at the 5% level.
- Unpaired sample BAM and assay B evaluation with dry pet food was performed separately from that for assays A and D; therefore, the number of BAM-positive samples is different for assay B.
samples tested with assay A and 46 of 100 samples tested with assay D were confirmed positive for *Salmonella* (representing tests where the rapid assay and paired culture confirmation on the same enrichment were both positive); 57 of 100 samples tested positive with the BAM reference assay performed in parallel. For assay B, 68 of 100 samples were confirmed positive for *Salmonella*; 67 of 100 samples tested positive with the BAM reference assay performed in parallel with assay B (assay B was evaluated at a separate time and thus compared with the BAM data collected at the same time point). Unpaired analyses at the individual strain level also did not identify any significant differences between the POD for individual strains with each assay and the corresponding PODs for the reference assay (detailed data are given in supplemental Table 2).

Paired analysis revealed a number of false-negative (FN) results, i.e., tests where the rapid assay was negative but the paired culture confirmation on the same enrichment sample revealed the presence of *Salmonella* and thus was scored as positive (Table 3). Overall, the FN rates obtained across all five serotypes tested were 9, 4, and 26% for assays A, B, and D, respectively (Table 3). The FN rates obtained in this study are considerably higher than the FN rates reported (<2%) for the specific conditions evaluated in the AOAC validation studies for these three assays.

When paired results were evaluated separately for each of the five strains, diagnostic sensitivities differed widely among individual strains (Table 4; full details given in supplemental Table 3). For assays A and B, the FN rates ranged from 0 to 18% and from 0 to 14%, respectively. For each of these assays, one strain had an FN rate of >10%: 18% for *Salmonella* Kentucky in assay A and 14% for *Salmonella* Typhimurium in assay B. In the initial inclusivity study, with assay B detection of the *Salmonella* Typhimurium strain also was reduced (see Table 1). For assay D, all five strains had FN rates of >10%: from 12% for *Salmonella* Alachua to 37% for *Salmonella* Kentucky and 47% for *Salmonella* Minnesota. In the initial inclusivity study, with assay D *Salmonella* serotypes Alachua, Minnesota, and Kentucky were detected only at the LOD + 1 log unit (Table 1).

**Salmonella detection in dark chocolate.** Five strains representing five *Salmonella* serotypes (Alachua, Mississippi, Typhimurium, Virchow, and 13,22:b−−) were used to evaluate the ability of assays A, B, and D to detect *Salmonella* on dark chocolate contaminated at fractional positive levels (Tables 2 and 3). All of the uninoculated control samples tested negative for *Salmonella* by the culture-based method. The contamination level for individual strains ranged from 0.62 to 0.94 MPN/25 g (supplemental Table 2). Based on the AOAC POD model, the combined detection frequency for all five strains did not differ significantly among any of the three assays and the reference assay (Table 2). Overall, 61 of 100 samples tested with assay A, 56 of 100 samples tested with assay B, and 55 of 100 samples tested with assay D were confirmed positive for *Salmonella*; 60 of 100 samples tested positive with the BAM assay performed in parallel. Unpaired analyses at the individual strain level also did not identify any significant differences between the POD for individual strains with each assay and the corresponding PODs for the reference assay (detailed data are given in supplemental Table 2).

Overall paired analysis revealed FN rates across all five serotypes of 12, 16, and 15% for assays A, B, and D, respectively (Table 3). Similarly the diagnostic sensitivities for the individual strains differed widely (Table 4; full details given in supplemental Table 3); the FN rates ranged from 0 to 37% for assay A and 0 to 44% for both assays B and D. Multiple strains had FN rates of >10% with each of the assays evaluated. Diagnostic sensitivity was reduced for two strains with assays A, B, and D: the *Salmonella* Typhimurium strain (37, 18, and 44% FN rate, respectively) and the *Salmonella* 13,22:b−− strain (14, 44, and 20% FN rate, respectively).

---

**TABLE 3. Comparison of rapid assay results for detection of *Salmonella* in paired sample enrichment cultures**

<table>
<thead>
<tr>
<th>Matrix</th>
<th>No. of samples&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Assay</th>
<th>Assay pos, culture pos</th>
<th>Assay pos, culture neg</th>
<th>Assay neg, culture pos</th>
<th>Assay neg, culture neg</th>
<th>Sensitivity&lt;sup&gt;c&lt;/sup&gt;</th>
<th>False-negative rate&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Specificity&lt;sup&gt;e&lt;/sup&gt;</th>
<th>False-positive rate&lt;sup&gt;f&lt;/sup&gt;</th>
<th>Relative accuracy&lt;sup&gt;g&lt;/sup&gt;</th>
<th>P&lt;sup&gt;h&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry pet food</td>
<td></td>
<td>A</td>
<td>51</td>
<td>2</td>
<td>5</td>
<td>42</td>
<td>0.91</td>
<td>0.09</td>
<td>0.95</td>
<td>0.05</td>
<td>0.93</td>
<td>0.453</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>68</td>
<td>3</td>
<td>3</td>
<td>26</td>
<td>0.96</td>
<td>0.04</td>
<td>0.90</td>
<td>0.10</td>
<td>0.94</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>46</td>
<td>1</td>
<td>16</td>
<td>37</td>
<td>0.74</td>
<td>0.26</td>
<td>0.97</td>
<td>0.03</td>
<td>0.83</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Dark chocolate</td>
<td></td>
<td>A</td>
<td>61</td>
<td>1</td>
<td>8</td>
<td>30</td>
<td>0.88</td>
<td>0.12</td>
<td>0.97</td>
<td>0.03</td>
<td>0.91</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>56</td>
<td>4</td>
<td>11</td>
<td>29</td>
<td>0.84</td>
<td>0.16</td>
<td>0.88</td>
<td>0.12</td>
<td>0.85</td>
<td>0.119</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>55</td>
<td>2</td>
<td>10</td>
<td>33</td>
<td>0.85</td>
<td>0.15</td>
<td>0.94</td>
<td>0.06</td>
<td>0.88</td>
<td>0.039</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Assays were evaluated with 20 replicate samples for each of the five *Salmonella* serotypes tested per food matrix; thus, a total of 100 samples were tested for each assay for each matrix.

<sup>b</sup> Results from rapid assay sample enrichments were confirmed by secondary enrichment in selective media and isolation on selective agar plates; selected *Salmonella* colonies were confirmed by invA PCR assay.

<sup>c</sup> Number of confirmed assay positive results divided by the total number of culture positive results.

<sup>d</sup> Calculated as 1 – sensitivity.

<sup>e</sup> Number of confirmed assay negative results divided by the total number of culture negative results.

<sup>f</sup> Calculated as 1 – specificity.

<sup>g</sup> Number of confirmed rapid assay positive results divided by the number of confirmed rapid assay negative results.

<sup>h</sup> McNemar’s exact test; binomial distribution was used to test the tendency of an assay to yield false-negative or false-positive results.
Detection problems with assay performance for detection of individual strains associated with illness or the target food matrix; therefore, guidance is provided for selection of strains to be included in validation studies. These strain sets predominantly comprise Salmonella serotypes frequently associated with illness or the target food matrix; therefore, problems with assay performance for detection of individual strains would be restricted to only those strains included in the validation study. Our data revealed that some assays had reduced analytical detection sensitivity for some Salmonella strains; specifically, assay C did not detect two strains of S. bongori and S. enterica subsp. houtenae at 1,000 times the assay LOD. Although S. bongori and S. enterica subsp. houtenae are not typically associated with human disease and generally appear to be rare, foods that contain any Salmonella are considered by the FDA to be adulterated and would be subject to recall action. Our results are consistent with previously findings indicating that detection with rapid methods can differ for individual strains of pathogens. For example, the iQ-Check Salmonella II PCR and GeneDisc Salmonella assays gave poor detection results for several human clinical Salmonella Senftenberg strains and S. bongori, respectively (4, 23); FN results can be obtained for Salmonella strains that either lack the target genes or in which the target genes do not match the primer sequences. Studies with other pathogens also revealed that some rare variants (e.g., strains lacking the assay target gene) may not be detected with some assays. For example, DNA probes designed to detect Shiga toxin–producing Escherichia coli (STEC) via detection of stx1 and stx2 targets may fail to detect rare and divergent stx2f variant strains (5, 7, 16, 19, 39). Problems with detection of unusual pathogen variants are not limited to molecular and rapid assays. For example, some S. enterica subsp. diarizonae strains that are lactose positive and H2S negative produce colonies with atypical morphology on BAM differential agar media and:

### TABLE 4. Salmonella serotypes with for which diagnostic detection sensitivity was lowa

<table>
<thead>
<tr>
<th>Assay</th>
<th>Serotype</th>
<th>MPN/25 g</th>
<th>95% CI</th>
<th>n b</th>
<th>Assay pos, culture pos</th>
<th>Assay pos, culture neg</th>
<th>Assay neg, culture pos</th>
<th>Assay neg, culture neg</th>
<th>Sensitivity d</th>
<th>False-negative rate e</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Kentucky</td>
<td>0.62</td>
<td>0.37, 1.04</td>
<td>20</td>
<td>9</td>
<td>0</td>
<td>2</td>
<td>9</td>
<td>0.82</td>
<td>0.18</td>
</tr>
<tr>
<td>B</td>
<td>Typhimurium</td>
<td>0.62</td>
<td>0.37, 1.04</td>
<td>20</td>
<td>12</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>0.86</td>
<td>0.14</td>
</tr>
<tr>
<td>C</td>
<td>Alachua</td>
<td>0.94</td>
<td>0.57, 1.54</td>
<td>20</td>
<td>11</td>
<td>0</td>
<td>2</td>
<td>7</td>
<td>0.85</td>
<td>0.15</td>
</tr>
<tr>
<td>D</td>
<td>Kentucky</td>
<td>0.62</td>
<td>0.37, 1.04</td>
<td>20</td>
<td>14</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0.88</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Minnesota</td>
<td>0.75</td>
<td>0.45, 1.23</td>
<td>20</td>
<td>5</td>
<td>0</td>
<td>3</td>
<td>12</td>
<td>0.63</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>Typhimurium</td>
<td>0.74</td>
<td>0.44, 1.23</td>
<td>20</td>
<td>8</td>
<td>0</td>
<td>7</td>
<td>5</td>
<td>0.53</td>
<td>0.47</td>
</tr>
<tr>
<td>A</td>
<td>13,22:b:–</td>
<td>0.71</td>
<td>0.43, 1.18</td>
<td>20</td>
<td>12</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>0.86</td>
<td>0.14</td>
</tr>
<tr>
<td>B</td>
<td>13,22:b:–</td>
<td>0.71</td>
<td>0.43, 1.18</td>
<td>20</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>12</td>
<td>0.63</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>Virchow</td>
<td>0.90</td>
<td>0.60, 1.62</td>
<td>20</td>
<td>15</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>0.83</td>
<td>0.17</td>
</tr>
<tr>
<td>B</td>
<td>13,22:b:–</td>
<td>0.62</td>
<td>0.38, 1.08</td>
<td>20</td>
<td>9</td>
<td>1</td>
<td>2</td>
<td>8</td>
<td>0.82</td>
<td>0.18</td>
</tr>
<tr>
<td>D</td>
<td>13,22:b:–</td>
<td>0.71</td>
<td>0.43, 1.18</td>
<td>20</td>
<td>8</td>
<td>0</td>
<td>2</td>
<td>10</td>
<td>0.80</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>Alachua</td>
<td>0.89</td>
<td>0.54, 1.47</td>
<td>20</td>
<td>14</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>0.88</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Mississippi</td>
<td>0.94</td>
<td>0.58, 1.54</td>
<td>20</td>
<td>14</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>0.88</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Typhimurium</td>
<td>0.62</td>
<td>0.38, 1.08</td>
<td>20</td>
<td>5</td>
<td>0</td>
<td>4</td>
<td>11</td>
<td>0.56</td>
<td>0.44</td>
</tr>
</tbody>
</table>

a False-negative (FN) rates >10%. Detailed results for all strains, including strains with FN rates <10% are given in supplemental Table 3.
b Total number of samples tested per strain.
c Results from rapid assay sample enrichments were confirmed by secondary enrichment in selective media and isolation on selective agar plates; selected Salmonella colonies were confirmed by invA PCR assay.
d Number of confirmed assay positive results divided by the total number of culture positive results.
e Calculated as 1 – sensitivity.

Additional strains had high FN rates: the Salmonella Virchow strain with assay A (17%) and the Salmonella Alachua and Mississippi strains with assay D (12%). In the inclusivity study, with assay D three of the strains with FN rates >10% (Salmonella serotypes Alachua, Mississippi, and 13,22:b–) were detected only at the LOD + 1 log unit (see Table 1). None of these assays had FN results >2% in the respective AOAC validation studies for assay performance in chocolate under the specific conditions evaluated.

**DISCUSSION**

The comparative evaluation of the four rapid molecular assays for Salmonella revealed that (i) some rare Salmonella strains may not be detected equally well by all rapid assays, which may result in public health and/or regulatory risks, (ii) rapid assays may have FN rates >10% for detection of Salmonella, particularly when assays are evaluated with challenging matrices with low levels of injured Salmonella cells, and (iii) the end user may want to evaluate rapid assays with stringent protocols that reflect the challenging conditions unique to the target.

Analytical detection sensitivity (LOD) of rapid assays differs for rare Salmonella strains. Limited guidance is provided for selection of Salmonella strains to be included in validation studies. These strain sets predominantly comprise Salmonella serotypes frequently associated with illness or the target food matrix; therefore, problems with assay performance for detection of individual strains would be restricted to only those strains included in the validation study. Our data revealed that some assays had reduced analytical detection sensitivity for some Salmonella strains; specifically, assay C did not detect two strains of S. bongori and S. enterica subsp. houtenae at 1,000 times the assay LOD. Although S. bongori and S. enterica subsp. houtenae are not typically associated with human disease and generally appear to be rare, foods that contain any Salmonella are considered by the FDA to be adulterated and would be subject to recall action. Our results are consistent with previously findings indicating that detection with rapid methods can differ for individual strains of pathogens. For example, the iQ-Check Salmonella II PCR and GeneDisc Salmonella assays gave poor detection results for several human clinical Salmonella Senftenberg strains and S. bongori, respectively (4, 23); FN results can be obtained for Salmonella strains that either lack the target genes or in which the target genes do not match the primer sequences. Studies with other pathogens also revealed that some rare variants (e.g., strains lacking the assay target gene) may not be detected with some assays. For example, DNA probes designed to detect Shiga toxin–producing Escherichia coli (STEC) via detection of stx1 and stx2 targets may fail to detect rare and divergent stx2f variant strains (5, 7, 16, 19, 39). Problems with detection of unusual pathogen variants are not limited to molecular and rapid assays. For example, some S. enterica subsp. diarizonae strains that are lactose positive and H2S negative produce colonies with atypical morphology on BAM differential agar media and
may fail to be detected (41). Our findings underscore the importance of evaluating new detection assays with strain sets that cover the genetic diversity of the target organism and suggest that the use of standard strain sets in validation studies (e.g., the AOAC OMA) may be warranted. Our data also highlight for end users the importance of evaluating a given detection assay with diverse strains of the target pathogen and with (unusual) strains that may be expected in a specific commodity, product, or food supply chain. The end user must also have access to details about assay primers and probes (e.g., target nucleic acid sequences) because this information can help predict whether certain strains may not be detected with a given assay.

The one assay in our study that targeted bacterial surface molecules (assay D) had reduced analytical sensitivity for several of the Salmonella strains evaluated. The Salmonella Minnesota strain required 10-fold the LOD for detection in pure culture and produced the highest number of FN results (FN rate of 47%) in inoculated dry pet food. In other studies, assays targeting bacterial surface molecules had reduced sensitivity for detection of specific strains of the target organism (15, 17). Overall, these data suggest that assays that target cell surface structures (e.g., through antibodies or phage ligands) may have more variable LODs than molecular assays that target highly conserved genes. Variability in the LOD is more likely to be an issue when diverse pathogen groups (such as Salmonella) are targeted, e.g., the avidity of some surface molecule variants to the detection reagents may be reduced (13, 44).

Exposure to environmental stress can induce remodeling of bacterial surface structures (e.g., lipopolysaccharides), which may alter efficient detection by an assay ligand (31). In previous studies, Salmonella strains that were adapted to low temperatures in food samples were less likely to be detected with immunoassays (35, 43).

FN rates (diagnostic sensitivity) for commercial assays may exceed 10% under challenging conditions. Our data specifically indicate that under worst-case scenarios (e.g., injured cells, low levels of contamination, short enrichment times, or unusual and difficult-to-detect strains) commercial assays may have higher FN rates than those reported based on standard evaluations (i.e., validation studies) that may be conducted under more favorable conditions. The Salmonella Typhimurium strain we evaluated had an FN rate of >14% for five of the six assay-matrix combinations tested, and for the serotype 13,22:b:– one strain had an FN rate of >14% with all three assays for dark chocolate. Although detection probes may not be expected to target rare strains (e.g., 13,22:b:–) with high sensitivity, most probes will likely perform well with well-characterized and common strains (e.g., Salmonella Typhimurium) included in strain sets used for probe design. We hypothesized that low diagnostic sensitivity for some strains in food was due to slow growth during enrichment. This hypothesis is consistent with previous findings that isolation and detection of the foodborne pathogens Listeria and Salmonella can be affected by the culture medium used in the enrichment steps, indicating that strains of the same species may differ considerably in their ability to grow in different enrichment media (20, 26). Components (e.g., polyphenols, complex polysaccharides, and tannins) found in some food matrices often considered challenging can inhibit PCR amplification (38); however, inhibition should have been less of an issue in the present study because the results for the internal positive control included in both nucleic acid–based assays did not indicate inhibition.

The FN rates of >10% obtained with the rapid assays evaluated with multiple Salmonella strains in both matrices underscore the challenge of detecting target organisms in low-moisture foods, particularly when these types of food matrices contain low levels of pathogens that are adapted to the low-moisture environment and hence may have sustained sublethal injury. Several factors important for bacterial resuscitation and growth during enrichment to levels detectable with an assay include the severity of bacterial cell injury, the potentially low contamination level, sensitivity to selective agents in enrichment media, and the length of the enrichment period. The state of the target organism in the preenrichment sample (e.g., degree of bacterial cell injury), which varies for different strains and matrix formulations, greatly influences the chance of growing the pathogen to detectable levels in enrichment cultures. We found that reduction of Salmonella significantly varied for strains in dark chocolate but not dry pet food, which may indicate that strain biology and matrix effects play a role in the severity of injury and subsequent growth in enrichment cultures. In other studies, survival of Salmonella has been influenced by the local microenvironment (e.g., fat composition or water activity), which changes depending on the matrix formulation. For example, detection of Salmonella was poorer in foods with decreased moisture or sugar (milk powder and natural peanut butter, respectively) compared with various peanut butter formulations with higher moisture, sugars, and fats (8, 30). In some studies, survival of Salmonella and E. coli strains differed among several low-moisture foods (e.g., milk powder, milk chocolate, and dark chocolate) (6, 25, 32, 40).

The degree of injury and the contamination level influence the frequency of detection of the target organism with a specific enrichment strategy (e.g., enrichment medium composition and temperature and duration of enrichment). Similarly, strain and matrix variations are important factors; in several studies inoculation level and enrichment scheme (i.e., medium formulation and incubation time) were important for detection of sublethally injured foodborne pathogens (Salmonella, Listeria, Campylobacter, and STEC) (12, 26, 27). Jasson et al. (25) found that real-time PCR assays produced fewer positive results for chocolate samples inoculated with a low level of injured Salmonella Enteritidis compared with samples contaminated at higher levels. The severity of cellular injury also impacts the time needed for repair and resuscitation and differs for strains of several important foodborne pathogens (26, 27). These data suggest that standard enrichment schemes (which may differ depending on the detection method used) may not be suitable for recovery of injured pathogen cells, particularly those of unusual pathogen groups, found in
challenging food matrices; thus, evaluation of detection assays with a range of strains is recommended. Extended enrichment times may be necessary for low-water-activity food matrices, particularly with shelf-stable foods that permit time for additional testing.

Although our data indicate that molecular assays may have higher FN rates than expected when tested under challenging conditions, a major gap in our current understanding of alternative assays remains a lack of information on the performance measures (e.g., FN rates) of the “gold standard” reference assays (e.g., the BAM assay) under challenging conditions. This type of information is essential because alternative assays with FN rates such as those reported here may still be superior to the gold standard assays, which are unlikely to have FN rates of zero or close to zero, particularly under challenging scenarios such as those evaluated here. For example, using a metagenomics approach, Ottesen et al. (34) detected Salmonella in tomato samples that were negative with the BAM culture method. Future studies are thus needed to define FN rates for gold standard assays, for example by testing enrichments used in these gold standard assays after extended incubations beyond the standard enrichment time. This approach would likely yield additional positive samples when low levels of injured cells are present and these cells do not grow to detectable levels within the standard enrichment times.

End user evaluation of detection with stringent procedures reflective of conditions unique to the target foods is important. Our data indicate that the performance (e.g., diagnostic and detection sensitivity for some strains) of validated commercial assays in low-moisture matrices may differ from the performance expected based on data from validation studies. Thus, comparative evaluations under challenging conditions are particularly important when assays are to be used for detection of pathogens in foods, raw materials, or other samples that stress cells (leading to extended lag phases during enrichment) or that interfere with growth (e.g., due to presence of natural antimicrobial agents). The enrichment conditions (i.e., time, temperature, and medium) used in validation studies to recover the target pathogen from one matrix (e.g., milk chocolate) may not be sufficient to efficiently recover cells in another seemingly closely related matrix (e.g., dark chocolate) that presents a more stressful environment and increases pathogen injury. Reduced recovery of heat-injured Salmonella and Listeria strains has been reported in selective media compared with nonselective media (10, 11). Thus, end users must evaluate assays under worst-case scenario conditions, including difficult-to-detect strains, low inoculum levels, injured cells, and challenging matrices. Our data also indicate the importance of reporting the matrix used in assay evaluations with a high level of specificity (e.g., dark chocolate or milk chocolate versus just “chocolate”).

Although assays may have FN rates >10% under these types of challenging conditions, alternative assays with these high FN rates may still be suitable for routine use, especially when comparable data on the FN rates of gold standard traditional assays are lacking. Our comparative data clearly indicated that the assays evaluated differed considerably in their performance and that evaluation studies like the one reported here can be used to identify assays that may not be suitable for routine use with a given matrix.

Our data also provide baseline information that can be used to define key parameters for the adoption of new rapid assays. For example, end users may set a certain level of confidence for a key performance measure (e.g., some end users may require 95% confidence that an assay has less than a 10% FN rate). Alternatively, some end users may require a certain level of confidence that a new assay performs as well or better than a currently used assay. End users also may want to develop a risk assessment–based approach to determine an acceptable FN rate and assess the public health and business risks associated with FN results. For example, these types of risk assessments may allow end users to compare the risk associated with using different assays when one assay has an extremely high FN rate (e.g., >40%) for one rare pathogen strain and a low FN rate (e.g., <5%) for all other strains and another assay has consistent high FN rates of 10 to 15% across common strains.

ACKNOWLEDGMENT

This project was supported by a contract from Mars, Inc. (McLean, VA) to M. Wiedmann.

REFERENCES


