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Detection and Identification of Salmonella enterica, Escherichia coli, and Shigella spp. via PCR-ESI-MS: Isolate Testing and Analysis of Food Samples

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Pierce SE, Bell RL, Hellberg RS, Cheng C-M, Chen K-S, Williams-Hill DM, Martin WB, Allard MW. 2012. Detection and identification of Salmonella enterica, Escherichia coli, and Shigella spp. via PCR-ESI-MS: isolate testing and analysis of food samples. *Applied and Environmental Microbiology* 78(23): 8403-8411.
doi: 10.1128/AEM.02272-12

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This is a pre-copy-editing, author-produced PDF of an article accepted for publication in *Applied and Environmental Microbiology*, volume 78, issue 23, in 2012 following peer review. The definitive publisher-authenticated version is available online at DOI: 10.1128/AEM.02272-12.

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AEM Accepts, published online ahead of print on 21 September 2012
Appl. Environ. Microbiol. doi:10.1128/AEM.02272-12
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5 Detection and Identification of *Salmonella enterica*, *Escherichia coli*, and
6 *Shigella* spp. via PCR-ESI-MS: Isolate Testing and Analysis of Food
7 Samples

8 Running Title: Characterization of Foodborne Bacteria by PCR-MS

9
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22 **Abstract**

23

24 An assay to identify the common foodborne pathogens, *Salmonella*, *Escherichia*
25 *coli*, *Shigella* and *Listeria monocytogenes,* was developed in collaboration with Ibis
26 Biosciences (a division of Abbott Molecular) for the PLEX-ID Biosensor system, a
27 platform that uses electrospray ionization mass spectroscopy (ESI-MS) to detect the base
28 composition of short PCR amplicons. The new foodborne pathogen (FBP) plate has been
29 experimentally designed using four gene segments for a total of eight amplicon targets.
30 Initial work built a DNA basecount database that contains over 140 *Salmonella enterica*,
31 139 *E. coli*, 11 *Shigella*, 36 *Listeria* patterns and 18 other *Enterobacteriaceae* organisms.
32 This assay was tested to determine the scope of the assay's ability to detect and
33 differentiate the enteric pathogens as well as to improve the reference database associated
34 with the assay. Over 800 bacterial isolates of *S. enterica, E. coli*, and *Shigella* species
35 were analyzed. Overall, 100% of *S. enterica*, 99% of *E. coli*, and 73% of *Shigella* spp.
36 were detected using this assay. The assay was also able to identify 30% of *S. enterica*
37 serovars to the serovar level. To further characterize the assay, spiked food matrices and
38 food samples collected during regulatory fieldwork were also studied. While analysis of
39 pre-enrichment media was inconsistent, identification of *S. enterica* from selective
40 enrichment media resulted in serovar-level identifications for 8 of 10 regulatory samples.
41 The results of this study suggest that this high-throughput method may be useful in
42 clinical and regulatory laboratories testing for these pathogens.

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46 **Introduction**

47 Mass spectrometry is an established analytical technique with growing
48 applications within microbiology. With high sensitivity and high resolution, mass
49 spectrometry can be used to differentiate microbial species based on sub-cellular
50 variations. Recently, several papers have been published concerning the application of
51 either matrix-assisted laser desorption ionization (MALDI) (6,14,36) or electrospray
52 ionization (ESI) mass spectrometry (MS) (13,21,30,37) to the detection and identification
53 of microbes. While some methods examine protein expression, this work centers on the
54 use of nucleic acid information to identify bacteria.

55 MS techniques involving the analysis of DNA take advantage of the difference in
56 mass between strands with different base compositions. In order to utilize MS for DNA-
57 based identification of bacteria, a region of DNA that varies between species or
58 subspecies is amplified by PCR and the mass of this amplicon is then determined. Since
59 the exact masses of the individual bases in DNA are known, the quantity of each of these
60 bases within the amplified sequence can be calculated based on the exact mass of the
61 strand. While the exact sequence is not obtained through this method, the base
62 compositions, or basecounts, can provide enough information to discriminate between
63 species, subspecies, and even serovars depending on the organism and the assay (15,19).

64 This technique is comparable to other methods that differentiate between
65 microbes using nucleic acid information, such as 16S rRNA gene sequencing. 16S
66 sequencing is widely used for bacterial identification and classification, and a library of
67 data has been amassed for reference and support (11). While DNA sequencing does

68 provide actual sequence information that can be translated to labs using other instruments
69 and methods, it is relatively time-consuming and requires a pure sample, unless PCR
70 products are cloned prior to sequencing. Although new sequencing technology can
71 provide results from mixed cultures, sample preparation and data analysis remains time
72 and resource intensive. PCR-MS can analyze samples containing mixtures of bacteria
73 with minimal sample preparation. DNA can be extracted directly from the enrichment
74 broth, thereby eliminating the need to isolate individual colonies; and results can be
75 obtained from extracted DNA in under 5 hours and from culture in well under 8 hours.
76 While PCR-MS is similar to real-time PCR in these respects, it has the advantage of
77 being able to provide both breadth and depth in the identification of organisms. Real-
78 time PCR methods can often detect multiple species or can provide sub-species
79 characterization of one species, but few methods can do both in one assay
80 (10,25,26,28,31).

81 PCR-MS has been successfully applied to clinical microbial characterization
82 (2,29,33), and it would also be a welcome addition to other fields including food safety.
83 The U.S. Food and Drug Administration (FDA) analyzed 206,723 import food lines and
84 25,214 domestic food facilities in 2010 for signs of adulteration or mishandling of foods
85 and a multitude of samples were tested for microbial contamination as a result of these
86 inspections (16). Samples analyzed for microbial contamination at FDA are processed
87 using culturing techniques that take days to weeks to provide isolate confirmation (1).
88 Traditional serology testing on *Salmonella enterica*, for example, takes place once an
89 isolate is confirmed as *S. enterica* and can require up to an additional month to complete
90 the characterization. High-throughput screening methods that could provide serotype

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91 information in just a few days, including time for the initial enrichment, would greatly
92 enhance the ability of the agency to conduct real-time monitoring and outbreak
93 investigations.

94 To this end, a PCR-MS assay for use on the PLEX-ID Biosensor system was
95 developed with the aim of detecting and differentiating between *Salmonella* spp.,
96 *Escherichia coli*, *Shigella* spp. and *Listeria monocytogenes*. Along with speciating these
97 four important foodborne pathogens, this assay was designed to simultaneously
98 differentiate the six subspecies of *S. enterica* (I, II, IIIa/IIIb, IV, and VI), and provide
99 some serotyping of *S. enterica* and *E. coli*. Despite the promise of this assay, it is heavily
100 reliant on a reference database, and the current database requires the inclusion of
101 numerous important serovars. Furthermore, the strength of this assay has yet to be tested
102 with regulatory samples.

103 The goal of this work was to improve the size and scope of the database while
104 assessing the detection and identification abilities of the assay. To enlarge the database,
105 over 800 pure culture isolates were analyzed with the assay. The isolates consisted of *S.*
106 *enterica*, pathogenic *E. coli*, and *Shigella* species. These species are all in the
107 *Enterobacteriaceae* family, are found in foods, and cause illness in humans. *Listeria* spp.
108 isolates were not tested. Non-target bacterial cultures were also analyzed to test the limit
109 of assay specificity. In addition, several foods were spiked with *S. enterica*, and samples
110 of the pre-enrichment broth were analyzed to determine the effects of food matrices and
111 background microflora on assay performance. Finally, enrichment broth aliquots from
112 regulatory samples positive for *S. enterica* were examined to compare results obtained by
113 PCR-MS to serological identification methods.

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115 **Materials and Methods**

116 **Bacterial strains and template preparation.** *S. enterica* isolates (n = 437) were
117 taken from the collection at the U.S. FDA Pacific Regional Lab-Southwest (PRL-SW) in
118 Irvine, CA. These cultures had previously been serotyped at FDA laboratories in
119 Arkansas and Colorado and were held at -70°C in motility test media. The cultures were
120 thawed, plated on trypticase soy agar (TSA), and incubated for 18-24 hours at 37°C. Two
121 methods were used for DNA extraction of the isolated colonies. A boiling method
122 described previously was used for the extraction of most isolates (9). An automated
123 method was also employed for some samples as follows. The PrepSEQ nucleic acid
124 extraction kit (Life Technologies, Foster City, CA) was used on the MagMax sample-
125 handling system (Life Technologies) according to the manufacturer's instructions with
126 two exceptions: rather than using the proteinase K in the kit, the samples were heated at
127 95°C for 20 minutes at the start of extraction. In addition, the elution volume was
128 reduced to 150 µl. Both methods provided suitable DNA for PCR-MS analysis. During
129 testing, two isolates were determined not to be *S. enterica* and removed from further data
130 analysis. This was confirmed via Vitek 2 GN cards or API 20E strips (bioMerieux,
131 Durham, NC) and a real-time PCR method (8,9). In addition, two isolates identified by
132 serology as subspecies II and one identified as subspecies IV were reclassified as
133 subspecies I based on 16S sequencing results obtained with a MicroSEQ 500 16S rDNA
134 Bacterial Identification Kit (Applied Biosystems, Foster City, CA) combined with a
135 3500xl Genetic Analyzer (Applied Biosystems). These isolates were also excluded from
136 the results described here.

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137 *E. coli* isolates (n = 234) were obtained from the Orange County Public Health
138 Lab (OCPHL) in Santa Ana, CA and the U.S. FDA Center for Food Safety and Applied
139 Nutrition (CFSAN). *Shigella* spp. (n = 207) isolates were obtained from OCPHL,
140 CFSAN, the Los Angeles County Public Health Lab (LACPHL), and the American Type
141 Culture Collection (ATCC). Serology for *E. coli* was performed at the originating labs.
142 Species-level identification of *Shigella* spp. isolates were obtained by real-time PCR
143 and/or microarray analysis. DNA was extracted using the boiling method described by
144 Cheng et al. (7).

145 Non-target bacterial strains analyzed for exclusivity were obtained from ATCC.
146 These included *Enterobacter aerogenes* ATCC 13048, *Klebsiella pneumoniae* ATCC
147 13883, *Pseudomonas aeruginosa* ATCC 9027, *P. aeruginosa* ATCC 27853, *Proteus*
148 *hauseri* ATCC 13315, *Rhodococcus equi* ATCC 6939, *Staphylococcus aureus* ATCC
149 6538, *S. aureus* ATCC 25923, and *S. epidermidis* ATCC 14990. In addition, two
150 *Citrobacter freundii* isolates were obtained from the culture collection at PRL-SW. The
151 control cultures were plated on TSA and incubated for 24 hours at 37°C, and DNA was
152 extracted via the boiling protocol used for *S. enterica* isolates.
153
154 **Template preparation for regulatory samples**. Regulatory samples were
155 analyzed to determine the ability of the instrument to detect the target organisms in food
156 samples containing normal background microflora. As part of the microbiological
157 workflow at FDA, samples are incubated in a pre-enrichment broth for 24 h followed by
158 selective enrichment in both Rappaport-Vassiliadis (RV) medium and Tetrathionate (TT)
159 broth (1). After pre-enrichment, a 1 ml aliquot of the pre-enrichment broth was set aside

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160 and held either at -70°C or 4°C. A 1 ml aliquot was also taken following selective
161 enrichment with RV and TT broths. The aliquots from RV/TT broths were combined in a
162 1:1 ratio for each sample and held either at -70°C or 4°C. If samples were found to be
163 presumptive positive for *S. enterica* using the VIDAS assay (bioMerieux), the enrichment
164 broths were frozen at -70°C. Broths were held at 4°C for a maximum of 1 week. Prior to
165 PCR-MS, the broths were thawed and DNA was extracted directly from the broths using
166 a modified boiling protocol (9). No enumeration was performed on regulatory samples.
167
168 **Template preparation for spiked food matrices.** Food matrices spiked with *S.*
169 *enterica* were also analyzed with PCR-MS. *S. enterica* was spiked at two levels: 2-3.8
170 cfu/25 grams of food and 20-30 cfu/25 grams of food. Samples were spiked in replicates
171 of six subsamples for each level. Other samples were unspiked, and the samples were all
172 blinded. The spiking levels were chosen to be consistent with other evaluations of rapid
173 detection methods (12,32,34). After PCR-MS results were obtained and interpreted, the
174 identities of the spiked samples were revealed. The foods and their unspiked controls
175 were sampled and incubated as described in the BAM (1) except that the pre-enrichment
176 broth for all foods was modified buffered peptone water (mBPW). For these samples,
177 only the mBPW pre-enrichment broth and not the RV or TT enrichment broths were
178 analyzed by PCR-MS. The boiling protocol described above for regulatory samples was
179 used to extract DNA from the mBPW post-incubation.
180
181 **Foodborne pathogen (FBP) plate design**. PCR primer sets were developed that
182 were capable of amplifying all known species, sub-species and serotypes of *Salmonella*

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183 and their close relatives of *E. coli* and *Shigella*. Primers were chosen that would be able
184 to distinguish essential molecular lineages using base composition signatures. The Gram-
185 negative enteric primer designs were based upon analysis of previous sequence
186 alignments of *mut*S and *mdh* genes across a large selection of *Salmonella* diversity (4). A
187 surveillance panel of eight primer pairs was selected, comprising three Gram-negative
188 enteric primer pairs targeting variable *mut*S gene fragments and three primer pairs
189 targeting variable *mdh* gene fragments. Two additional gene targets, to detect *L.*
190 *monocytogenes*, were designed, the invasion-associated secreted endopeptidase gene
191 (*iap*) for the p60 protein gene (27) and the *prf*A gene, which positively regulates the
192 expression of listeriolysin for use in the identification of *Listeria* samples (35) (Fig. 1).
193 The eight sets of forward/reverse primer sequences (1 set in each well) are listed in Table
194 1. All primers used in this study had a thymine nucleotide at the 5' end to minimize
195 addition of non-templated adenosines during amplification using Taq polymerase (5).
196 The sensitivity of each PCR primer pair was determined using known quantities of a
197 synthetic calibrant DNA template as described previously (22). Each of the primer pairs
198 was sensitive to as little as twenty copies of the calibrant DNA, and several primers were
199 sensitive to five copies. Additionally, an ultra-clean DNA polymerase, Immolase, was
200 used for amplification due to the ability of these primers to pick up residual *E. coli* DNA
201 commonly found in some commercial preparations of polymerase (data not shown). The
202 FBP Plate comes pre-loaded with 0.2 mM dNTPs, 0.5 µM PCR primers and 100 copies
203 of calibrant in each well. The DNA basecount database to which sample results are
204 matched includes over 140 *Salmonella enterica*, 139 *E. coli*, 11 *Shigella*, 36 *Listeria*
205 patterns and 18 other *Enterobacteriaceae* organisms.

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207 **PCR/ESI-MS.** After DNA extraction, template DNA was diluted 1:10 with
208 PCR-grade water. The template was distributed onto the FBP assay plate (Abbott
209 Molecular, Abbott Park, IL) using an automated liquid handler (Abbott Molecular). The
210 liquid handler added 5 µl of diluted template DNA and 1 unit of Immolase DNA
211 polymerase in PCR buffer to each well of the plate. The plate was then heat-sealed with
212 foil on a Thermo Scientific ALPS microplate heat sealer (Rockford, IL). Each sealed
213 plate was loaded onto a Mastercycler Pro thermocycler (Eppendorf, Hauppauge, NY) and
214 PCR-amplified under the following conditions: 95°C for 10 min; then 8 cycles of 95°C
215 for 30 s, 48°C for 30 s, and 72°C for 30 s; then 37 cycles of 95°C for 15 s, 56°C for 20 s,
216 and 72°C for 20 s; followed by 72°C for 2 min, and 99°C for 20 min. The plate was then
217 loaded onto the PLEX-ID system (Abbott Molecular) for amplicon desalting and
218 analysis. The PLEX-ID has a desalting carousel and a dual-sprayer ESI quadrupole time-
219 of-flight mass spectrometer. Analysis of each plate took about an hour.

220

221 **Data analysis**. The PLEX-ID instrument software analyzed the spectra for each
222 sample and determined the basecounts of the peaks in the spectra. For the software to
223 confirm a specific basecount, both complementary DNA strands had to be detected.
224 Spectra were manually analyzed to confirm the results. The software then compared
225 these basecounts to known organisms in the PLEX-ID FBP database containing base
226 compositions from over 700 validated isolates from across a diversity of genera. To
227 consider an isolate as validated, the identity must have been confirmed using an
228 alternative method to standard serology including pulsed-field gel electrophoresis

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229 (PFGE), Vitek (bioMerieux), molecular serotyping, multilocus sequence typing (MLST),
230 or whole genome sequencing.

231 For the *S. enterica* isolates studied, identifications made by PCR-MS were
232 compared to those made by serology. If the serovar determined by serology was one of
233 the top identifications in the software, the serology and PCR-MS results were called
234 concordant for that isolate. It should be noted that for some serovars, the top
235 identification resulted in an identical match to more than one serovar. If the correct
236 serovar was included in this group, the isolate was still called concordant. If all isolates
237 with the same serovar were found to be concordant, the serovar was considered
238 concordant. In some cases, different isolates with the same serovar had different PCR-
239 MS identifications. For these serovars, if more than one isolate was found to be
240 concordant the serovar was described as semi-concordant. Isolates that did not match
241 serological results were described as discordant. *E. coli* isolates were compared to
242 serological results in a manner similar to the *S. enterica* isolates; however, the isolates
243 were only compared at the serogroup level (O-antigens) rather than the serovar level (O-
244 and H-antigens).
245
246 **Results**

247 **Common disease-causing *S. enterica*.** While there are over 2,500 serovars of *S.*
248 *enterica*, 85% of human illness caused by *Salmonella* is associated with only 40 serovars
249 (Table 2) (7). Of those 40 serovars, 32 were represented in the collection at PRL-SW and
250 were analyzed on the assay plate (n = 178 isolates). Up to nine isolates of each serovar
251 were analyzed. For 18 of the 32 serovars, all isolates had identifications concordant with

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252 serology. An additional 10 serovars were semi-concordant. For the semi-concordant
253 serovars, discordant isolates often had single nucleotide polymorphisms (SNPs) or
254 differences in one or two amplicons (out of six) that resulted in a different identification.
255 Only 4 of the top 40 serovars tested did not have concordant identifications for any
256 isolates. Some of the discordant serovars, such as Hartford, appear to be missing from
257 the database while others, like Mbandaka, do not match the database strains. A full list of
258 serovars tested can be found in Supplemental Table S1.
259
260 **Less common *S. enterica* subspecies I isolates**. To determine the range of the
261 identification capabilities of the assay, a collection of *Salmonella* serovars that are
262 uncommon agents of human disease were also investigated. The isolates (n = 232) were
263 from 116 different subspecies I serovars. While all of these serovars were isolated from
264 food samples, not all of them have been implicated in human illness. Although these
265 isolates are less clinically relevant than those discussed above, detecting and identifying
266 these *S. enterica* is an important food safety effort as these serovars still have the
267 potential to cause illness and may be agents in future outbreaks.
268 Of the 116 non top-40 serovars tested, only 8 were concordant. An additional 4
269 serovars were semi-concordant, and the remaining 104 serovars were discordant. While
270 the results were discordant at the serovar level, all isolates but one were classified as
271 subspecies I indicating the assay can subspeciate well. The rate of discordance is
272 strikingly different from the results obtained with clinically common *S. enterica*. This
273 discrepancy highlights the large number of validated isolates already populating the
274 PLEX-ID database for the top 40 *Salmonella* serotypes causing foodborne illness, and the

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275 need for a stronger reference database for many of the remaining serovars. Adding the
276 isolates under investigation herein to the database should improve serovar-level
277 identification for *S. enterica* serovars that are found in foods yet might not have been
278 implicated in prior human illness.
279
280 ***S. enterica* subspecies II-VI**. *S. enterica* from five other subspecies were also
281 investigated with this technique (n=22): subspecies II (n = 4), subspecies IIIa (n = 2),
282 subspecies IIIb (n = 7), subspecies IV (n = 8), and subspecies VI (n = 1). While
283 subspecies IV bacteria occasionally cause human illness - often through handling reptiles
284 and other carrier animals - *S. enterica* from other subspecies are infrequent disease agents.
285 While the assay was not able to provide serovar-level identifications with these isolates, it
286 was capable of providing resolution at the subspecies level. All isolates tested were
287 concordant to the subspecies identifications (data not shown). Although the sample size
288 is small, the results show that the assay is capable of classifying all six subspecies.
289 As the majority of serovars analyzed were uncommon and not found in the
290 database, the rate of concordance was low when all *S. enterica* results are considered
291 (n=432 isolates). In total, 108 serovars were discordant, 14 serovars were semi-
292 concordant, and 31 serovars were fully concordant with conventional serology. As
293 discussed above, the difference in rates of concordance between common and uncommon
294 *S. enterica* suggests that increasing the database size will improve the serovar level
295 identifications.
296

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297 **Diarrheagenic *E. coli* isolates**. The PRL-SW *E. coli* isolate collection included
298 11 enteropathogenic *E. coli*, 33 general diarrheagenic *E. coli*, 72 non-O157 shiga-toxin
299 producing *E. coli*, and 112 O157 enterohemorrhagic *E. coli* (Table 3). Of the 228 isolates
300 analyzed, 226 were detected by this assay. The majority of isolates in the collection have
301 the serotype O157:H7 or O157:H unknown. The O157:H7 isolates (n = 66) were all
302 concordantly identified. Among the O157:H unknown isolates, one was unidentified by
303 the instrument and the remaining isolates (n = 45) were identified as O157:H7. *E. coli*
304 O55:H7 (n = 7), an evolutionary precursor to *E. coli* O157:H7, could not be separated
305 from *E. coli* O157:H7 with this assay and showed equivalent matches to O55:H7 and
306 O157:H7.
307 The results from non-O157 diarrheagenic *E. coli* are included in Table 3. While
308 isolates of several serogroups were concordant at the serogroup level, isolates from other
309 serogroups were inconsistently identified. For example, all five O45 isolates and 10 of
310 11 O103 isolates were concordant at the serogroup level. However, only 16 of 32 O26
311 isolates and 12 of 47 O111 isolates were concordant with serology.
312
313 ***Shigella* spp. and EIEC isolates**. *Shigella* spp. isolates obtained from local
314 public health laboratories as well as national collections at FDA/CFSAN and ATCC were
315 analyzed with PCR-MS (n = 201) (Fig. 2). *S. sonnei* isolates had the strongest correlation
316 between speciation data and PCR-MS results: all 52 isolates were concordant.
317 Identification of other *Shigella* spp. was less successful. Of 64 *S. flexneri* isolates, 46
318 were concordant at the species level, and 36 of 46 *S. boydii* were concordant. Only 16 of
319 38 *S. dysenteriae* isolates were concordant, with discordant isolates being identified as *E.*

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320 *coli* or other *Shigella* species. One *Shigella* isolate, the species of which could not be
321 determined by other methods, was identified as either *S. flexneri* or *S. boydii* by PCR-MS,
322 but that identification was also observed with several *S. dysenteriae* isolates, thereby
323 preventing a conclusive species identification. *Shigella* spp. isolates that were not
324 detected to the species level were frequently detected as various serotypes of *E. coli*. One
325 isolate of *S. boydii* was identified as *E. albertii*. *E. albertii*, which causes diarrhea, is a
326 recent addition to the *Escherichia* genus and has been shown to be closely related to
327 several strains of *S. boydii* (23).

328 The pathogenic *E. coli* sub-group that is the most closely related to *Shigella* is the
329 enteroinvasive *E. coli* (EIEC). Both EIEC and *Shigella* spp. cause similar illness in
330 humans. Four of five EIEC isolates analyzed were identified as *E. coli*. The fifth isolate
331 was identified as *S. dysenteriae*. Only one of the five isolates, an EIEC from the O111
332 serogroup, was concordant at the serogroup level. Two O124 EIEC isolates were
333 identified as belonging to the O78 serogroup while the final isolate was from an unknown
334 serogroup.

335

336 ***S. enterica* in spiked food samples**. In addition to bacterial isolates, DNA from
337 the pre-enrichment broth of 72 blind food samples spiked with *S. enterica* was analyzed
338 by PCR-MS (Table 4). Results from spiked tomato and chili pepper samples were
339 relatively poor. Only 4 of the 12 spiked chili powder samples and 6 of the 12 spiked
340 tomato samples were identified with high confidence as *S. enterica*. While all 4 of the
341 chili powder *S. enterica* identifications listed the correct serovar, Weltevreden, only 4 of
342 the 6 tomato *S. enterica* identifications listed the correct serovar (i.e., Newport). Cheese

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343 samples spiked with Typhimurium were more promising: the serovar was identified with
344 high confidence in all 12 of the spiked samples. *S. enterica* was also identified in all 12
345 spiked fish samples; however, the serovar used for spiking, Senftenberg, is one in a
346 cluster of over 15 serovars that cannot be distinguished on this assay. In general,
347 unspiked foods resulted in no *S. enterica* identification. However, five of the twenty-four
348 controls did have low quality matches to *S. enterica* in which only one of six primer pairs
349 matched. While not all of the spiked food samples were well characterized by PCR-MS,
350 results from the regulatory samples described below indicate that results from the tomato
351 and chili powder samples may have improved if the selective enrichments rather than the
352 pre-enrichments were analyzed.
353
354 ***S. enterica* in regulatory samples**. Aliquots of pre-enrichment and selective
355 enrichment broths were taken from food samples during the normal regulatory workflow.
356 Only samples that were confirmed positive for *S. enterica* were analyzed for this project.
357 Results were poor when analyzing the pre-enrichment broth (n = 16) (Table 5). While
358 identifications were made from several samples, they were of low quality with
359 amplification at 2 of 6 primer sites or less.
360 Results were more successful when analyzing the selective enrichments (n = 10).
361 Nine samples showed identifications of *S. enterica* at a high confidence level. In two of
362 these cases, the identification in the PCR-MS database matched the identification made
363 by serology. In five additional cases, comparing the samples to an external database
364 containing basecount signatures from isolates studied in this work resulted in a
365 concordant identification. An additional *S. enterica* isolated from silver fish was

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366 identified as a rare subspecies I serovar, Fulica, by serology. This bacterium had no
367 perfect match in the FBP database or in the isolates studied for this work, but the closest
368 result was a subspecies II isolate. The final sample was identified by serology as a
369 monophasic C2 serovar. The PCR-MS identification was Hadar or Blockley, both of
370 which are C2 serovars. When the results from the isolates studied at PRL-SW are
371 included, the monophasic C2 matched five serovars which are all in the O:8 serogroup
372 (either C2 or C3). While neither PCR-MS nor serotyping could identify a serovar for this
373 isolate, they are concordant in the serogroup identification. Among the six regulatory
374 samples for which both the pre-enrichment and enrichment broths were analyzed, *S.*
375 *enterica* was detected in five of the enrichment broths, but not in any of the pre-
376 enrichment broths.
377
378 **Non-target bacteria**. To determine the selectivity of the assay, bacterial isolates
379 other than *S. enterica*, *E. coli*, or *Shigella* spp. were analyzed (n = 11). This set included
380 enteric bacteria as well as other Gram-negative and Gram-positive bacteria. Ideally, any
381 non-target bacteria would not amplify during PCR in order to limit the effects of PCR
382 inhibition. However, since the PCR primers were designed to broadly target enteric
383 bacteria, some non-target bacteria will also be amplified. When this occurs, the non-
384 target bacteria should not be confused with the target organisms. To help prevent false
385 positives in the case of non-target amplification, several common non-target bacteria are
386 present in the database.
387 As expected, most Gram-negative bacteria amplified and were detected by the
388 instrument (Table 6). *E. aerogenes*, *K. pneumoniae*, and *P. aeruginosa* all amplified in at

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389 least one well and were identified to the species level. *P. hauseri*, *R. equi*, *S. epidermidis*,
390 and two strains of *S. aureus* did not amplify. The results with *P. hauseri* are surprising
391 given that it is a Gram-negative enteric bacterium. Two *C. freundii* isolates were
392 identified as non-pathogenic *E. coli*.
393
394 **Discussion**
395 Recent advances in ESI-MS using mass spectrometers have enabled analysis of
396 PCR amplicons with sufficient mass accuracy that the nucleotide base composition (the A,
397 G, C, and T count) of the PCR amplicon can be unambiguously determined for 120-140
398 base pair fragments (12). The measured base compositions allow identification of the
399 bacterial species with a high degree of resolution. Broad-range primers targeted to highly
400 conserved sites within *Salmonella*, *E. coli*, *Shigella*, and *Listeria* which flank highly
401 variable, information-rich regions were used to amplify DNA from these bacteria and
402 their close relatives out of mixed samples in the same assay. For this FBP plate, 8 primer
403 pairs, each targeting the variable and informative gene segments within *mutS* and *mdh*
404 (for Gram-negative enterics), and *prf*A and *iap* (for *Listeria*), were designed where the
405 resultant base compositions provide species, sub-species, and some serotype information.
406 While the *E. coli* serotype O157:H7 is the most well known shiga-toxin producing *E. coli*
407 (STEC), *E. coli* with other serotypes are increasingly found to produce toxins and cause
408 illness (17,20,24). Thus, the ability to determine *E. coli* serotypes is a growing need for
409 many labs. *Shigella* are genetically similar organisms to *E. coli* (3,18), and the genetic
410 resemblance can cause misidentification of *Shigella* as *E. coli*, especially in DNA-based
411 testing schemes.

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412 The plate was able to detect and identify the target organisms to varying degrees
413 of success. In analyzing pure samples, *S. enterica* were detected 100% of the time and
414 30% of the serovars had identifications concordant with serology (95% CI 23-37%). *E.*
415 *coli* were detected 99% of the time and were concordant to the serogroup level in 72% of
416 the isolates (95% CI 65-77%) although the majority of those isolates were from the O157
417 serogroup. Since many *Shigella* spp. were identified as *E. coli*, the *Shigella* spp. isolates
418 were detected as *Shigella* 81% of the time and were identified to the species level 73% of
419 the time (95% CI 66-78%).

420 The results of this study indicate that expanding the current reference database
421 will enhance the potential of this assay to provide serovar-level information for *S.*
422 *enterica* and should enhance the detection of *E. coli* and *Shigella* spp. Of 432 *S. enterica*
423 isolates tested, 181 did not match an entry in the reference database at all six primer sites.
424 These non-matching isolates had serovar identifications that were concordant with
425 serology only 9% of the time. In comparison, of the 251 isolates that did match a
426 database entry at all six primer pairs, 69% had identifications concordant with serology.
427 Most of the isolates that did not match a database entry at all six sites still had PCR
428 products in all six wells. The presence of PCR amplicons in wells that were not used for
429 the identification indicates that these isolates have the potential to be uniquely identified
430 once their basecounts are added to the database.

431 The results for *E. coli* isolates did not always match serology. While *E. coli*
432 O157:H7 was reliably identified, the assay inconsistently determined the serogroups of
433 non-O157 pathogenic *E. coli*. Similarly, *Shigella* spp. were frequently identified as *E.*
434 *coli*. Improving the size of the reference database with regard to *E. coli* and *Shigella* spp.

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435 will likely improve the detection and identification abilities of the assay. However, *E.*
436 *coli* and *Shigella* spp. only amplify in five or fewer wells in this assay while *S. enterica*
437 amplifies in six. It is possible that this will limit the ability to improve differentiation of
438 *E. coli* and *Shigella* spp.

439 When analyzing the regulatory and spiked food samples, it was clear that
440 extracting DNA from pre-enrichment media frequently resulted in no or low quality
441 identifications. Analyzing samples from selective enrichment media was more successful.
442 While the difficulty in detecting *S. enterica* in pre-enrichment media does necessitate an
443 additional day to incubate in selective media, it indicates that the *S. enterica* detected are
444 viable. Since PCR techniques amplify DNA from both living and dead organisms, it can
445 be difficult to determine whether the detected organisms are viable and capable of
446 reproduction. The ability to detect *S. enterica* only after selective enrichment suggests
447 that the bacteria replicated in this media- an important distinction for regulatory action.

448 The results with non-target bacteria indicate that the assay is selective: Gram-
449 positive bacteria and some Gram-negative species do not amplify, and many of the
450 bacteria that do amplify are identified correctly. One exception was *C. freundii*. *C.*
451 *freundii* can biochemically and serologically resemble atypical lactose-fermenting *S.*
452 *enterica* but is unlikely to be confused with *E.coli*, the organism identified via PCR-MS.
453 The false positive *E. coli* identification would be ruled out by further testing with
454 procedures outlined in the BAM.

455 While this work greatly improves the body of knowledge for the use of the FBP
456 assay for the identification of bacteria, there are several limitations present in this study.
457 Perhaps the biggest limitation is the small size of the pure culture exclusivity panel.

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458 Ideally, many more relevant isolates from both Gram-positive and Gram-negative species
459 would have been tested with the assay in order to provide an adequate assessment of the
460 false positive rate of the assay. However, the culture collection used in this work did not
461 support further testing. In addition, the effects of the food matrix on sensitivity would be
462 better understood by performing spiking experiments in a wider range of foods than was
463 attempted here. Continued research, including single lab and multi-lab validation studies,
464 will provide the best opportunity to address these concerns.
465
466 **Conclusions**
467 This work indicates that PCR-MS in general and this assay in particular could
468 reduce the time necessary to detect and identify foodborne pathogenic bacteria. The
469 assay was successful at detecting all *S. enterica* subspecies and some serovars. This was
470 observed in pure culture testing and in the analysis of regulatory samples and spiked food
471 matrices. Although it was common to observe more than one serovar listed as a possible
472 identification, reducing the list from thousands down to a few possible serovars is very
473 useful. That information could easily be used to narrow focus during an outbreak
474 traceback procedure. This assay has the potential to significantly reduce the time
475 necessary to characterize bacteria in food samples and greatly improve the response time
476 to foodborne bacterial outbreaks.
477 Many of the noted deficiencies can be improved upon through expansion of the
478 current reference library. In addition, planned changes to the assay plate design are
479 expected to improve the ability of the assay to differentiate between closely-related
480 bacteria such as *Shigella* and *E. coli* as well as improve the serotyping resolution. New

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481 primer pairs are designed to interrogate more regions of the *S. enterica* genome to
482 improve discrimination power. For *E. coli*, added primers will provide information on
483 virulence factors including the ability to express Shiga toxins and hemolysin. Due to the
484 extensive number of serovars, for *Salmonella* in particular, it is unlikely that all serovars
485 will be differentiated on one assay plate. However, changes to the database and to the
486 assay plate design may permit the identification of many of the bacterial pathogens
487 important to the protection of the public health. To date, hundreds of known validated
488 bacterial cultures have been run on the FBP plates in order to database the variations
489 found in these cultures and to determine the specificity and discriminating power of these
490 particular primer pairs. The addition of new genetic targets for better serotyping will
491 improve the accuracy of calls from the instrument. Using genetic targets other than the O
492 and H antigen genes overcomes the limitations of directly targeting these gene segments,
493 which require specific primer pairs for each known type. Further, since the H and O
494 antigen gene segments evolve rapidly, newly emerging strains might not be readily
495 detected by the traditional approach if the primer regions differentiate.

496

497 **Acknowledgements**

498 Funding was provided by FDA/CFSAN and the FDA Commissioner's Fellowship
499 Program.

500 We thank the microbiology analysts at PRL-SW for providing sample aliquots.
501 We are grateful to Erik Burrows and Jeffrey Blazar for insightful comments and editing
502 which improved the manuscript and to Jim Bono, Yi Chen, Rachel Binet, Keith Lampel,
503 Thomas Hammack, Peter Feng and Chris Keys for access to strains used to populate the

504 original basecount database. We greatly appreciate the technical support provided by Erik
505 Burrows, Melinda McFarland and John Callahan; and all of the team at Ibis
506 biosciences/Abbott Molecular including James Hannis, Sheri Manalili, Heather Matthews,
507 Roberta Housley and Ranga Sampath who jointly created and designed these primers and
508 database upon which this study rests.
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645 Figure legend

646

647 Figure 1. Layout of the FBP assay plate. The 96-well plate can accommodate 12

648 samples.

649 Table 1. Primer sequences on the FBP assay plate.

650 Table 2. List of the 40 most common disease-causing *S. enterica* serovars from the U.S.

651 in 1996-2006 (5).

652 Supplemental Table S1. List of all *S. enterica* serovars studied and related PCR-MS

653 identifications.

654 Table 3. PCR-MS identifications for diarrheagenic *E. coli* isolates.

655 Figure 2. Chart of PCR-MS identifications for *Shigella* spp. and EIEC isolates.

656 Table 4. Results from PCR-MS analysis of spiked foods[a].

657 Table 5. Comparison of serology and PCR-MS results for regulatory food samples.

658 Table 6. PCR-MS results for non-target bacteria[a].

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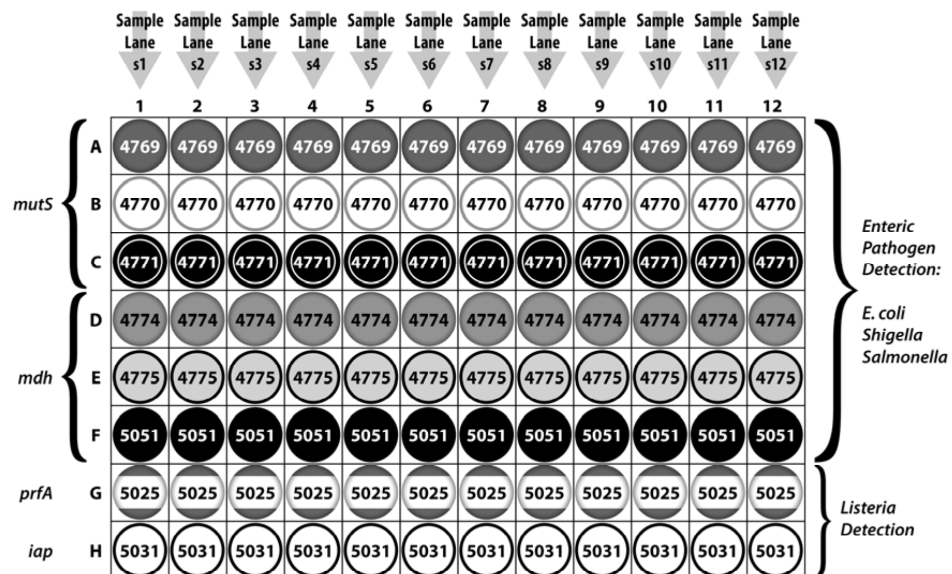

Figure 1. Layout of the FBP assay plate. The 96-well plate can accommodate 12 samples.

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Table 1. Primer sequences on the FBP plate.

Well	Forward Primer (5'-3')	Reverse Primer (5'-3')
1	TAT CAC CGA AGG TCG CCA CCC	TCG CAT ATA GGT ACT TTT ACC GCC CA
2	TGG GCG GTA AAA GTA CCT ATA TGC G	TCCGACGCGGGTAAAAATACGGTC
3	TAT TTT TAC CCG CGT CGG CGC AGC	TAC CGC GTC CGA TTT CAT CCA TCA
4	TAC GAC AAA AAC AAA CTG TTT GGC GT	TCA GAA TAG TCA CGC CGG AGT G
5	TCG GAT CGG CAA CCC TTT CTA TG	TGA GAG AAG AAG CGG GCA TAC TG
6	TGC ACT TGA AGG CGC TGA CG	TCG GGC AGG TTT TAG CAA TCT G
7	TCA ATG GGA GCC ACA CGA ATA TTG T	TGA AAG CGC CTT TGT AGT ATT GTA AAT TCA
8	TCG TGG AAT AAT TTA TCT GCT TCT TCT ATT TAT GT	TTG TTT TTC AGC TGC TGG AGC TT

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Table 2. List of the 40 most common disease-causing *S. enterica* serovars from the U.S. in 1996-2006 (5).

Rank[a]	% Human Disease	Serovar[b]	Concordance[c]
1	19.2	Typhimurium (+1)	Concordant
2	17.8	Enteritidis	Semi-concordant
3	8.4	Newport	Concordant
4	5.2	Heidelberg	Concordant
5	3.4	Javiana (+1)	Concordant
6	2.4	Typhimurium var. 5- (+1)	Concordant
7	2.4	Montevideo (+3)	Concordant
8	2.0	Muenchen (+1)	Concordant
9	1.7	Oranienburg	Semi-concordant
10	1.6	Saintpaul	Semi-concordant
11	1.5	Infantis	Semi-concordant
12	1.5	Thompson	Semi-concordant
13	1.5	Braenderup	Concordant
14	1.5	Agona (+1)	Concordant
16	1.1	Hadar (+1)	Concordant
19	1.0	Paratyphi B var. Java (+1)	Concordant
20	0.8	Poona	Concordant
22	0.6	Stanley (+1)	Concordant
23	0.6	Anatum (+1)	Concordant
24	0.5	Bareilly	Concordant
25	0.5	Mbandaka	Discordant
26	0.5	Paratyphi B	Semi-concordant
27	0.5	Hartford	Discordant
28	0.4	Panama	Semi-concordant
29	0.4	Derby	Semi-concordant
30	0.4	Litchfield	Discordant
31	0.4	Schwarzengrund	Semi-concordant
32	0.4	Senftenberg (+10)	Concordant
33	0.4	Brandenburg (+10)	Concordant
34	0.3	Sandiego	Discordant
36	0.3	Give	Semi-concordant
38	0.2	Rubislaw	Semi-concordant

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[a]The following serovars, with their corresponding ranks, were not available for analysis: 15- I 4,[5],12:i:-; 17- Mississippi; 18- Typhi; 21- Berta; 35- Paratyphi A; 37- Reading; 39- Norwich; and 40- Miami.

[b]Numbers in parentheses are the number of other serovars identified at the top confidence level along with the concordant serovar.

[C]Concordant – the serology and PCR-MS results matched for all isolates. Semi-concordant – the serology and PCR-MS results matched for some but not all isolates. Discordant – the serology and PCR-MS results did not match for any isolates.

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Table 3. PCR-MS identifications for STEC isolates.

Serotype	Total (n)	Isolates (n)	PCR-MS ID[a]
O157:H7	66	**66**	**O157:H7 (and O55:H7)**
O157:H unknown	46	**45**	**O157:H7 (and O55:H7)**
		1	Unidentified
O55:H7	7	**7**	**O55:H7 (and O157:H7)**
O45:H2 or O45:HND	5	**5**	**O45:H2 (and O103, O111, O121)**
O26:H11 or O26:HND	32	11	*Shigella* spp., O111
		10	**O26:H2 and O26:H11**
		6	**O26:H11 and O111**
		4	Others
		1	Unidentified
O103:H2, O103:H6, O103:H(25)	11	**6**	**O103 (and O45, O111, O139, O128, O91)**
		4	**O103, O45**
		1	O104:H21
O104:H4	1	1	O111/EAEC and *Shigella* spp.
O111:H8, O111:H11, O111:HNM	47	17	O26:H2 and O26:H11
		8	O103
		8	**O111 (and O26, O103)**
		5	O113:H21
		5	**O111:H8, *S. flexneri***
		4	O45
O113:H21	1	**1**	**O113:H21**
O118:H16	1	1	O26:H2 and O26:H11
O121:H19, O121:HND	5	**1**	**O121 (and O45, O143)**
		1	O26:H2 and O26:H11
		1	O157:not H7
		2	*E. coli* (unknown serotype)
O143:H4	1	1	O138
O145:H(25), O145:HND	6	3	O157:H7
		1	**O145:HND**
		1	O111
		1	*E. coli* (unknown serotype)

[a]Bold results indicate PCR-MS identification was concordant with serology at the serogroup level (O antigen).

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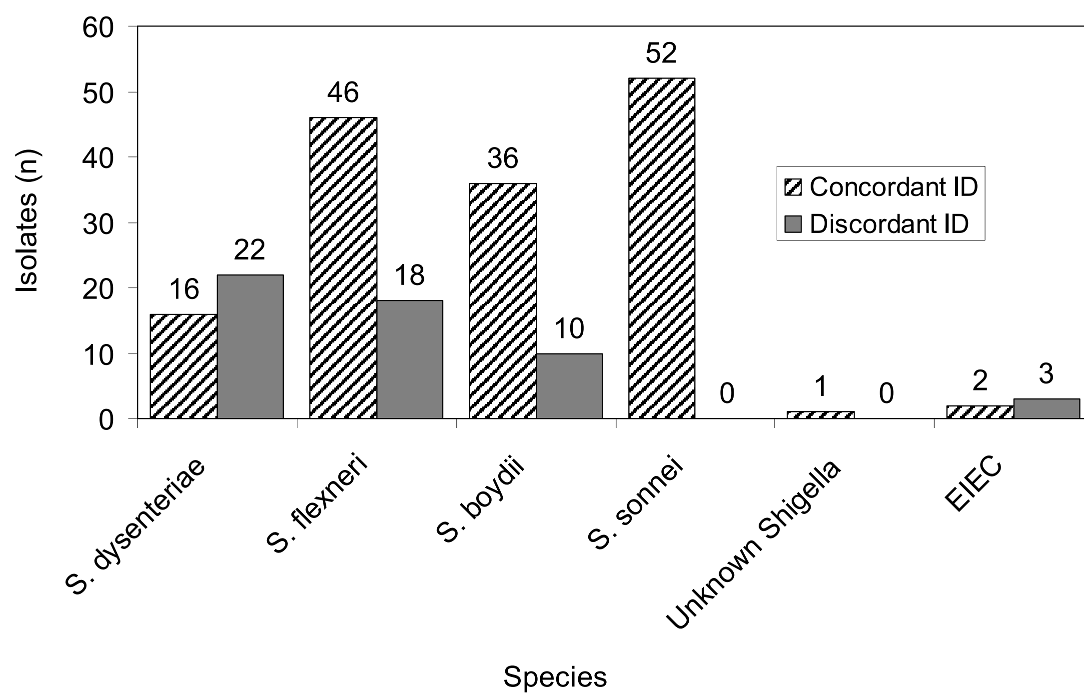

Figure 2. Chart of PCR-MS identifications for *Shigella* spp. and EIEC isolates.

Table 4. Results from PCR-MS analysis of spiked foods[a].

Chili Powder			Tomato		
Spike (cfu/25g)	Serovar	PCR-MS results[b]	Spike (cfu/25g)	Serovar	PCR-MS results[b]
3	Weltevreden	-	2.4	Newport	-
3	Weltevreden	LQ	2.4	Newport	-
3	Weltevreden	LQ	2.4	Newport	-
3	Weltevreden	LQ	2.4	Newport	LQ
3	Weltevreden	LQ	2.4	Newport	Mgulani (+1)
3	Weltevreden	LQ	2.4	Newport	Newport (+7)
30	Weltevreden	LQ	24	Newport	-
30	Weltevreden	LQ	24	Newport	-
30	Weltevreden	Weltevreden (+8)	24	Newport	Telaviv (+2)
30	Weltevreden	Weltevreden (+8)	24	Newport	Newport (+1)
30	Weltevreden	Weltevreden (+2)	24	Newport	Newport
30	Weltevreden	Weltevreden (+2)	24	Newport	Newport
Soft Cheese			Fish		
Spike (cfu/25g)	Serovar	PCR-MS results[b]	Spike (cfu/25g)	Serovar	PCR-MS results[b]
3.8	Typhimurium	Typhimurium (+1)	2	Senftenberg	LQ
3.8	Typhimurium	Typhimurium (+1)	2	Senftenberg	LQ
3.8	Typhimurium	Typhimurium (+1)	2	Senftenberg	Butantan
3.8	Typhimurium	Typhimurium (+1)	2	Senftenberg	Senftenberg (+22)
3.8	Typhimurium	Typhimurium (+1)	2	Senftenberg	Senftenberg (+16)
3.8	Typhimurium	Typhimurium (+1)	2	Senftenberg	Senftenberg (+15)
38	Typhimurium	Typhimurium (+1)	20	Senftenberg	Senftenberg (+16)
38	Typhimurium	Typhimurium (+1)	20	Senftenberg	Senftenberg (+16)
38	Typhimurium	Typhimurium (+1)	20	Senftenberg	Senftenberg (+15)
38	Typhimurium	Typhimurium (+1)	20	Senftenberg	Senftenberg (+15)
38	Typhimurium	Typhimurium (+1)	20	Senftenberg	Senftenberg (+15)
38	Typhimurium	Typhimurium (+1)	20	Senftenberg	Senftenberg (+15)

[a]Only the pre-enrichment broth (mBPW) was tested for each sample. Results from unspiked samples are not shown.

[b]Values in parentheses indicate the number of other serovars listed at the same confidence level. LQ= Low quality match to *S. enterica* (match in 1 of 6 wells).

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Table 5. Comparison of serology and PCR-MS results for regulatory food samples.

Sample[a]	Media[b]	Serology	PCR-MS results[c,e]	PCR-MS:PRLSW[d]
Anis seeds I	TSB	Montevideo	No ID	No ID
Anis seeds II	TSB	Montevideo	Montevideo (+3 others) (2 of 6)	No ID
Fennel seeds I	TSB	Emek	Poona (1 of 6)	No ID
Fennel seeds II	TSB	Emek	II/IIIa/Chester (1 of 6)	Poona/II/IIIa/ Sandiego
Papaya I	LB	Duesseldorf	No ID	No ID
Papaya II	LB	Mbandaka	No ID	No ID
Papaya III	LB	Mbandaka	No ID	No ID
Papaya IV	LB	Untypable C2	No ID	No ID
Papaya V	LB	Typhimurium	No ID	No ID
Papaya VI	LB	Mbandaka	No ID	No ID
Papaya VII	LB	Meleagridis	No ID	No ID
Shrimp I	LB	Agona	No ID	No ID
Silver fish	LB	Fulica	No ID	No ID
Tuna I	LB	Weltevreden	No ID	No ID
Tuna II	LB	Monophasic C2	No ID	No ID
Tilapia I	LB	Albany	No ID	No ID
Papaya V	RV/TT	Typhimurium	Typhimurium/ Typhimurium 5-	Typhimurium/ Typhimurium 5-
Papaya VI	RV/TT	Mbandaka	Senftenberg (4 of 6)	Mbandaka
Papaya VII	RV/TT	Meleagridis	Pullorum/ Enteritidis	Meleagridis
Shrimp II	RV/TT	Weltevreden	Mbandaka/Virch ow	Weltevreden/ Virchow
Shrimp III	RV/TT	Weltevreden	Mbandaka/Virch ow	Weltevreden/ Virchow
Silver fish	RV/TT	Fulica	Salamae (5 of 6)	Salamae (5 of 6)
Tuna I	RV/TT	Weltevreden	No ID	No ID
Tuna II	RV/TT	Monophasic C2	Hadar/Blockley	Hadar (+4 others)
Tilapia II	RV/TT	Tennessee	Agona/Lac -	Tennessee
Tilapia III	RV/TT	Enteritidis	Enteritidis (+3 others)	Enteritidis/ Thompson

[a]Samples in grey boxes were extracted from pre-enrichment media.

[b]TSB = trypticase soy broth; LB = lactose broth; RV/TT = Rappaport-Vassiliadis medium

and tetrathionate medium

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[c]Results refer to identifications made from the database.

[d]Results refer to identifications made by comparing the basecounts for the samples to the *S. enterica* isolates studied in this work.

[e]Indicates number of amplicons out of six that match the listed serotype.

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Table 6. PCR-MS results for non-target bacteria[a].

Species (including strain)	PCR-MS ID
C. freundii	*E. coli*
C. freundii	*E. coli*
***E. aerogenes* 13048**	***E. aerogenes***
***K. pneumoniae* 13883**	***K. pneumoniae***
***P. aeruginosa* 9027**	***P. aeruginosa***
***P. aeruginosa* 27853**	***P. aeruginosa***
P. hauseri 13315	no detection
R. equi 6939	no detection
S. aureus 6538	no detection
S. aureus 25923	no detection
S. epidermidis 14990	no detection

[a]Bold font indicates a concordant identification at the species level.