Research Note

Detection of *Salmonella enterica* subsp. *enterica* Serovar Cubana from Naturally Contaminated Chick Feed

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**ABSTRACT**

Because some significant outbreaks of human salmonellosis have been traced to contaminated animal feed, the rapid and efficient detection of *Salmonella* in feed is essential. However, the current U.S. Food and Drug Administration Bacteriological Analytical Manual (BAM) culture method that uses lactose broth as a preenrichment medium has not reliably supported the results of real-time PCR assays for certain foods. We evaluated the BAM culture method and a quantitative real-time PCR (qPCR) assay using two preenrichment media, modified buffered peptone water and lactose broth, to detect *Salmonella enterica* subsp. *enterica* serovar Cubana in naturally contaminated chick feed. After 24 h of incubation, the qPCR method was as sensitive as the culture method when modified buffered peptone water was used as the preenrichment medium but less sensitive than culture when lactose broth was used. After 48 h of incubation, detection of *Salmonella Cubana* by qPCR and by culture in either preenrichment medium was equivalent. We also compared the performance of the traditional serotyping method, which uses pure cultures of *Salmonella* grown on blood agar, to two molecular serotyping methods. The serotyping method based on whole genome sequencing also requires pure cultures, but the PCR-based molecular serotyping method can be done directly with the enriched culture medium. The PCR-based molecular serotyping method provided simple and rapid detection and identification of *Salmonella Cubana*. However, whole genome sequencing allows accurate identification of many *Salmonella* serotypes and highlights variations in the genomes, even in tight genomic clusters. We also compared the genome of the chick feed isolate with 58 *Salmonella* Cubana strains in GenBank and found that the chick feed isolate was very closely related to an isolate from a foodborne outbreak involving alfalfa sprouts.

Key words: Chick feed; Molecular serotyping; Preenrichment broth; Quantitative real-time PCR; *Salmonella Cubana*

Animal feed and feed ingredients contaminated with *Salmonella* can transmit this pathogen to animals and the food chain (13) and facilitate infection among humans (8, 10). *Salmonella enterica* subsp. *enterica* serovar Cubana was first identified in 1946 (21) and has been associated with chicken feed and salmonellosis outbreaks associated with alfalfa sprouts and pet food (18, 20, 22).

During method validation studies for detection of *Salmonella* in feed, we identified a commercial chick feed that was naturally contaminated with *Salmonella* Cubana. Because this is an unusual serovar with few reported cases, we previously evaluated multiple methods to detect the organism and characterized the strain by traditional serotyping methods, pulsed-field gel electrophoresis (PFGE), in vitro antimicrobial susceptibility testing, and whole genome sequencing (WGS) (3).

In this study, we compared the genome of our chick feed isolate to other *Salmonella* Cubana genomes in the National Center for Biotechnology Information (NCBI) GenBank (https://www.ncbi.nlm.nih.gov/). We also compared the performance of a previously reported quantitative real-time PCR (qPCR) *Salmonella* detection method (5, 7, 8) with U.S. Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) culture methods (2) because this method had not been evaluated for use with animal feed. We also compared the performance of the traditional serotyping method, which uses pure cultures of *Salmonella* grown on blood agar, to two molecular serotyping methods.

**MATERIALS AND METHODS**

Chick feed and *Salmonella* Cubana isolate. Chick feed was purchased in an impervious sealed bag from a commercial source,
and in the course of a method validation study this feed was found to be naturally contaminated with *Salmonella* Cubana. The organism was cultured repeatedly from the feed, and because it was an unusual serotype, the isolate was sequenced and characterized (3).

**Preenrichment culture.** Unless specified otherwise, all media were prepared according to the BAM (2). Sixty 25-g samples taken from the bag of naturally contaminated chick feed were weighed aseptically into sterile Whirl-Pak filter bags (Fisher Scientific, Pittsburgh, PA). Thirty samples were preenriched in 225 mL of modified buffered peptone water (mBPW; 3M, Maplewood, MN), and 30 samples were preenriched in 225 mL of lactose broth (LB; Difco, BD, Sparks, MD). All samples were mixed thoroughly by hand to obtain homogeneity and incubated at 37°C for 24 h. The flap of the Whirl-Pak bag was folded over to form a secure but not air-tight closure during incubation. The positive control for the preenrichment step was approximately 30 CFU of the chick feed, which was preenriched with the other samples.

**BAM culture method.** After preenrichment, the BAM *Salmonella* culture procedure (2) was followed. Aliquots of 1.0 and 0.1 mL from the incubated preenrichment broth cultures were subcultured in 10 mL of tetrathionate (TT) broth and 10 mL of Rappaport-Vassiliadis (RV) medium, respectively. The TT broth was incubated for 24 h at 43 ± 0.2°C, and the RV medium was incubated for 24 h at 42 ± 0.2°C. After incubation, each selective enrichment broth was streaked onto bismuth sulfite (BS; Difco, BD), Hektoen enteric (Difco, BD), and xylose lysine deoxycholate (Difco, BD) agar plates, which were incubated for 24 h at 35 ± 2°C. Presumptive-positive colonies were transferred to triple sugar iron agar (TSI; Difco, BD) and lysine iron agar (LIA; Difco, BD) slants and incubated for 24 h at 35 ± 2°C. Growth from presumptive-positive TSI slants was confirmed as *Salmonella* with somatic group antisera (Statens Serum Institute, Copenhagen, Denmark) and by biochemical analysis using the VITEK 2 compact automated system (bioMérieux, Hazelwood, MO). The BS agar plates that had no colonies that resembled *Salmonella* at 24 h were incubated for an additional 24 h at 35°C (48 h total). When colonies resembling *Salmonella* appeared by 48 h of incubation, these colonies were transferred to TSI and LIA slants for confirmation of identity.

**Determination of contamination level.** A three-tube most-probable-number (MPN) analysis was performed to determine the level of the *Salmonella* Cubana contamination in the chick feed before preenrichment (4, 19). The amounts of feed used were 100, 10, 1.0, and 0.1 g, with three replicates for each amount. The 100-and 10-g samples of chick feed were added to 900 and 90 mL, respectively, of mBPW in sterile Whirl-Pak filter bags. For the 1.0- and 0.1-g amounts, 1.0 and 0.1 g of chick feed were added to 9.0 and 9.9 mL, respectively, of mBPW broth in sterile 150-mL test tubes (12 tubes). These mixtures were not blended and were incubated for 24 ± 2 h at 35 ± 2°C. Total aerobic plate counts for the chick feed were determined according to the BAM method (19).

**DNA extraction.** DNA was extracted from both 24-h preenriched broth cultures (1-mL samples) and 48-h selective enrichment broth cultures (250-μL samples). Samples were centrifuged at 10,000 × g for 5 min, and the pellets were suspended in 250 μL of DNase- and RNase-free water, heated for 10 min at 100°C, cooled on ice for 5 min, and centrifuged at 16,000 × g for 5 min. The resulting supernatants (2 μL) were used as templates for qPCR.

**qPCR assay.** We used primers and TaqMan probes targeting a 262-bp fragment of the *Salmonella*-specific invA gene (6, 7) and a validated method for qPCR detection of *Salmonella* in food (5). Each PCR was performed in a 20-μL volume. Each reaction contained 1.8 μL of rehydrated *Salmonella* master mix containing primers (10 μM) and probe (2.5 μM), the internal amplification control, 2 μL of extracted DNA template, and 10 μL of ready-to-use master mix (VeriQuest Fast Probe qPCR master mix 2X, Affymetrix, Santa Clara, CA). The qPCR was performed on the 7500 Fast instrument (Applied Biosystems, Life Technologies, Grand Island, NY) at 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 5 min, and 50 cycles of 95°C for 3 s and 60°C for 30 s. The threshold was set manually at 0.05, and the baseline was set at 3 for the start cycle and 15 for the end cycle. Data were collected and analyzed by instrument software version 1.04. The threshold cycle (CT) value was the fractional PCR cycle number at which the reporter fluorescence was greater than the threshold and was calculated for each PCR for the targets and the internal amplification control. For each sample, PCR results were considered valid only when amplification of the internal amplification control indicated no inhibition of the reactions. A standard curve was constructed using DNA template from serial dilutions of *Salmonella* Cubana CVM42234 yielding 1 × 10^0 to 10 CFU/mL (Fig. 1). The cutoff CT value was set at 39 fluorescence units. Samples with CT < 39 were considered *Salmonella* positive and samples with CT ≥ 39 were considered *Salmonella* negative based on the limit of detection (LOD) of this qPCR assay (5) (Table 1).

**Molecular serotyping.** PCR serotyping from preenriched broth cultures was performed only when the sample tested positive for *Salmonella* with the qPCR assay. The primers and methods used for PCR serotyping have been previously described (12, 15). WGS-based serotyping was done using the SeqSero database (26) and the same genomic sequences as previously reported (3).

**Comparative genomics.** WGS data from about 70 *Salmo- nella* Cubana isolates were downloaded from the NCBI short read archive database and the FDA GenomeTrakr Project data sets (1), de novo assemblies were created with SPAdes software (http://cab.spbu.ru/software/spades/), and 58 genomes were selected for analysis based on the assembly quality. The NCBI BioSample database records were used to annotate the genomes with metadata for each strain. A local blast database of *Salmonella* genomes and
perl scripts were used to screen for plasmids and selected loci. The fully closed Salmonella Cubana genome CFSAN0002050 from the isolate recovered from the 2012 outbreak associated with alfalfa sprouts (11) was used for all comparisons.

An in-house bioinformatics workflow was used to determine alleles in 2,780 Salmonella core genes based on a published data set (17) and informative single nucleotide polymorphisms in the genomes were identified. Of the 2,400 core genes present in all the genomes with one or more alleles, 1,000 random core gene loci were selected to build a maximum likelihood phylogenetic tree. This evolutionary reconstruction was bootstrapped on MEGA7 suite (16) (n = 500), and the resulting tree was reannotated with isolate metadata. Unique isolates are shown in Figure 2.

Statistics. McNemar’s test was performed using SAS version 9.3 (SAS Institute, Cary, NC) to compare the differences between results obtained with the mBPW and LB cultures.

RESULTS AND DISCUSSION

According to the FDA “Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds” (23), to validate a molecular detection method such as the qPCR-based assay in this study, results must be confirmed by use of a reference method, and the proposed method must perform as well as or better than the reference method. For detection of bacteria, reference methods are generally culture based and result in a pure isolate. The FDA qPCR can detect

<table>
<thead>
<tr>
<th>Dilution no.</th>
<th>Salmonella level (CFU/mL)</th>
<th>C_T (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5 x 10⁹</td>
<td>14.9 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>1.5 x 10⁸</td>
<td>16.7 ± 0.3</td>
</tr>
<tr>
<td>3</td>
<td>1.5 x 10⁷</td>
<td>20.6 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>1.5 x 10⁶</td>
<td>24.1 ± 0.8</td>
</tr>
<tr>
<td>5</td>
<td>1.5 x 10⁵</td>
<td>27.4 ± 0.5</td>
</tr>
<tr>
<td>6</td>
<td>1.5 x 10⁴</td>
<td>30.6 ± 0.1</td>
</tr>
<tr>
<td>7</td>
<td>1.5 x 10³</td>
<td>34.6 ± 0.8</td>
</tr>
<tr>
<td>8</td>
<td>1.5 x 10²</td>
<td>38.1 ± 0.3</td>
</tr>
<tr>
<td>9</td>
<td>1.5 x 10</td>
<td>NA</td>
</tr>
</tbody>
</table>

a Three replicate samples were tested for each Salmonella Cubana level. NA, not applicable.

Dissicated coconut | Phililines | unknown
Sprouts | 2010 | USA:OH | unknown
animal feed, wheat millrun | 11/8/2012 | Mexico | JDGX01.0017
sesame seeds, hulled | 3/30/2005 | Lebanon | JDGX01.0030
tahini | 2/3/2012 | India | unknown
pistachio halva | 5/5/2005 | Lebanon | JDGX01.0048
frozen raw octopus | 1/2/2003 | Philippines | JDGX01.0035
sesame candy with pistachio | 6/12/2011 | Lebanon | unknown
senna tea | 7/2/2009 | Mexico | JDGX01.0059
klunji powder (mashoq habbe sauda) | 7/28/2010 | India | JDGX01.0035
Environmental Samples | 5/24/2016 | USA:TX | unknown
ground shrimp | 3/24/2013 | Mexico | JDGX01.0040
ground shrimp | 3/24/2013 | Mexico | JDGX01.0045
environmental swab | 2/11/2009 | USA:WI | JDGX01.0073
environmental swab | 2/11/2009 | USA:WI | JDGX01.0017
Asiago Cheese Sauce | 2/24/2016 | USA:TX | unknown
swab | 8/7/2010 | USA:NM | unknown
swab | 7/11/2011 | USA:NM | unknown
alpha barf fishes | 9/28/2001 | Thailand | unknown
alfalfa sprouts | 9/20/2012 | USA:AZ | JDGX01.0077
chick feed | 6/6/2012 | USA:MD | JDGX01.0018
alfalfa sprouts | 8/21/2006 | USA:FL | unknown
Sprouts | 2010 | USA:OH | unknown
Swine | unknown | unknown | unknown
animal feed, dairy cow feed | 9/9/2010 | USA:KS | unknown
short grain rice | 8/6/2007 | Mexico | JDGX01.0058
cottonseed | 8/28/2002 | USA:AZ | JDGX01.0051
swabs | 3/5/2009 | USA:UT | JDGX01.0044
Bovine (feed) | 1/9/2007 | USA:WA | unknown
animal feed, turkey feed | 8/7/2012 | USA:UT | JDGX01.0044
Sprouts | 9/8/2010 | USA:NY | JDGX01.0018
Salmonella enterica enterica serovar Typhimurium Reference
Urine | 9/8/2011 | USA:Lehigh PA | unknown
swab | 3/4/2009 | USA:UT | JDGX01.0056

FIGURE 2. Salmonella enterica subsp. enterica serovar Cubana core genome single nucleotide polymorphism analysis based on 1,000 nonduplicative core genes. All 58 Salmonella Cubana isolates from many different sources cluster together with minimal whole genome differences. Salmonella Cubana 2050 is the only outbreak strain from alfalfa sprouts, and this isolate has the same PFGE pattern as the strain isolated from chick feed (dots on phylogenetic tree).
Salmonella Cubana at as low as 10^2 CFU/mL (CT = 38.1) in
pure culture (Table 1 and Fig. 1), which was 10-fold more
sensitive than culture methods and was similar to the LOD
for this method as previously noted for other Salmonella
serotypes (5–7). Therefore, a sample result could be qPCR
positive but culture negative.

Table 2 shows comparative results for the detection of
Salmonella in naturally contaminated chick feed in mBPW
and LB by culture and qPCR methods. The
Salmonella contamination level in chick feed was 0.004 MPN/g, which
corresponds to the low number of positive cultures found in
30 replicates for each preenrichment culture; i.e., six positive
results from the mBPW cultures and five positive results
from the LB cultures. The 24-h qPCR was as sensitive as the
culture method using mBPW, with six positive assay results
from both the culture and qPCR methods. However, when
LB was used for the preenrichment, only two positive 24-h
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cultures (27.5) was lower than that for the LB cultures
(36.7), suggesting that Salmonella multiplied more rapidly
in mBPW. Bacteria in the feed may have used lactose to
produce acids that lowered the pH of the LB during
incubation, which has been reported to suppress the growth
of Salmonella to the LOD of this qPCR (~10^3 CFU/mL).

Detection failures with the 24-h qPCR assays of LB
preenrichment cultures have been reported previously for
foods (14, 15). After selective enrichment, the detection
performance of the 48-h qPCR for both mBPW and LB
cultures was the same as that of the culture methods (Table
2).

In Table 2, replicate 6 had a positive qPCR result at 48
h of incubation that was not confirmed by culture. The
reason for this result is unknown but could be related to the
low level of Salmonella in the RV and TT broth cultures as
indicated by the CT of 36.1, which is lower than that for
cultures of 10^3 CFU/mL (Fig. 1). There also may have been
nonspecific binding of the probe to other small, similar
sequences from other microflora present in the feed. The
positive qPCR result also could be due to cross-contamina-
tion during the DNA template preparation or the PCR or due

| TABLE 2. Performance of BAM culture methods and qPCR
assays for the detection of Salmonella Cubana in chick feeda |
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Preenrichment sample no.</td>
<td>BAM result</td>
<td>qPCR CT</td>
</tr>
<tr>
<td>mBPW (n = 30)</td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>1</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>22.5</td>
</tr>
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<td>3</td>
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<tr>
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<td>30</td>
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<td>29.3</td>
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<tr>
<td>Total no. positive</td>
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<td>6</td>
</tr>
<tr>
<td>Mean CT</td>
<td>27.5</td>
<td>7</td>
</tr>
</tbody>
</table>

| Lactose broth (n = 30) |
| 31 | − | − | − |
| 32 | + | 38.4 | 23.1 |
| 33 | + | 34.9 | 20.1 |
| 34 | − | − | − |
| 35 | + | 21.9 | 21.9 |
| 36 | − | − | − |
| 37 | − | − | − |
| 38 | − | − | − |
| 39 | − | − | − |
| 40 | − | − | − |
| 41 | − | − | − |
| 42 | + | − | 21.2 |
| 43 | + | − | 27.7 |
| 44 | − | − | − |
| 45 | − | − | − |
| 46 | − | − | − |
| 47 | − | − | − |
| 48 | − | − | − |
| 49 | − | − | − |
| 50 | − | − | − |
| 51 | − | − | − |
| 52 | − | − | − |
| 53 | − | − | − |

a +, positive test result; −, negative test result. For total number
positive, values with different letters are significantly different (P < 0.05).

TABLE 2. Continued

<table>
<thead>
<tr>
<th>Preenrichment sample no.</th>
<th>BAM result</th>
<th>qPCR CT</th>
</tr>
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<tbody>
<tr>
<td>54</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>55</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>56</td>
<td>−</td>
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<tr>
<td>57</td>
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<td>−</td>
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<td>58</td>
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<td>59</td>
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<td>−</td>
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<tr>
<td>60</td>
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<tr>
<td>Total no. positive</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Mean CT</td>
<td>36.7</td>
<td>22.8</td>
</tr>
</tbody>
</table>

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sequences from other microflora present in the feed. The
positive qPCR result also could be due to cross-contamina-
tion during the DNA template preparation or the PCR or due
to the presence of dead cells in the selective broth culture that would be detected by qPCR but would not be culturable. Other explanations for the negative culture result are (i) only 10 μL of the preenrichment culture was plated onto the selective agars, and there may have been no organisms in the 10-μL sample and (ii) overgrowth of competing microorganisms may have masked the Salmonella colonies on the agar plates (9).

The PCR serotyping method has been used to detect S. enterica serotypes from environmental samples and various food matrices (12). At 24 h of incubation, the PCR serotyping method was able to identify the Salmonella serotype as Cubana from five of six culture-positive mBPW samples but from only two of five culture-positive LB samples. The PCR molecular serotyping method is dependent upon the level of Salmonella in the sample, with an LOD of about 10^5 CFU/mL (12). Traditional and WGS-based serotyping methods correctly identified the Salmonella serotype as Cubana. However, the PCR serotyping method was able to identify Salmonella Cubana directly from the 24-h preenrichment broth culture, whereas the traditional serotyping method takes 5 to 14 days. Although the WGS serotyping approach is rapid once the WGS sequence is obtained, it takes several days to obtain a pure culture of the organism and thus obtain the sequence data. The PCR serotyping method has the potential of identifying specific Salmonella serotypes much more rapidly than can the other methods.

Core genome analysis and metadata analysis (Fig. 2) revealed many sources of Salmonella Cubana in produce, meat, fish, and the environment and tight clustering of the isolates. The chick feed isolate had the same PFGE profile (JDGX01.0018) as does strain SRR1106187, which was isolated from alfalfa sprouts in 2010 (Fig. 2, dots on phylogenetic tree). Alfalfa sprouts have been involved in meat, fish, and the environment and tight clustering of the genomes, even in tight clusters. Additional information regarding the presence and virulence potential of plasmids also can be gleaned from WGS data.

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ERRATUM

In the article “Synergistic Antimicrobial Combinations Inhibit and Inactivate *Listeria monocytogenes* in Neutral and Acidic Broth Systems” by S. M. Kozak, K. M. Margison, and D. J. D’Amico, which appears in the *Journal of Food Protection* 80(8):1266–1272, 2017, doi:10.4315/0362-028X.JFP-17-035, the correct concentration of ACSL (antimicrobial 1) in combination with SC (antimicrobial 2 at 1,250 ppm) in the first row of Table 3 on page 1269 should be 1,500 ppm. The correct concentration of ACSL (antimicrobial 1) in combination with BRA (antimicrobial 2 at 1,500 ppm) in row 7 of the same table should be 936 ppm.

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ERRATUM

In the article “Detection of *Salmonella enterica* subsp. *enterica* Serovar Cubana from Naturally Contaminated Chick Feed” by Faiza Benahmed, Hua Wang, Junia Jean-Gilles Beaubrun, Gopal R. Gopinath, Chorg-Ming Cheng, Darcy E. Hanes, Thomas S. Hammack, Mark Rasmussen, and Maureen K. Davidson, which appears in the *Journal of Food Protection* 80(11):1815–1820, 2017, doi:10.4315/0362-028X.JFP-16-344, contributing author Sherry Ayers was accidentally left off the author byline. The correct author byline and affiliations are as follows:

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