*Journal of Food Protection, Vol. 78, No. 5, 2015, Pages 873–880*
doi:10.4315/0362-028X.JFP-14-342


# Multiplex PCR–Based Serogrouping and Serotyping of *Salmonella enterica* from Tonsil and Jejunum with Jejunal Lymph Nodes of Slaughtered Swine in Metro Manila, Philippines

KAMELA CHARMAINE S. NG[1] AND WINDELL L. RIVERA[1,2]*

[1]*Institute of Biology, College of Science, and* [2]*Natural Sciences Research Institute, University of the Philippines, Diliman, Quezon City 1101, Philippines*

MS 14-342: Received 20 July 2014/Accepted 6 January 2015

## ABSTRACT

Food poisoning outbreaks and livestock mortalities caused by *Salmonella enterica* are widespread in the Philippines, with hogs being the most commonly recognized carriers of the pathogen. To prevent and control the occurrence of *S. enterica* infection in the country, methods were used in this study to isolate and rapidly detect, differentiate, and characterize *S. enterica* in tonsils and jejuna with jejunal lymph nodes of swine slaughtered in four locally registered meat establishments (LRMEs) and four meat establishments accredited by the National Meat Inspection Services in Metro Manila. A total of 480 samples were collected from 240 animals (120 pigs from each type of meat establishment). A significantly higher proportion of pigs were positive for *S. enterica* in LRMEs (60 of 120) compared with meat establishments accredited by the National Meat Inspection Services (38 of 120). More *S. enterica*–positive samples were found in tonsils compared with jejuna with jejunal lymph nodes in LRMEs, but this difference was not significant. A PCR assay targeting the *invA* gene had sensitivity that was statistically similar to that of the culture method, detecting 93 of 98 culture-confirmed samples. Multiplex PCR–based O-serogrouping and H/*Sdf I* typing revealed four *S. enterica* serogroups (B, C1, D, and E) and six serotypes (Agona, Choleraesuis, Enteritidis, Heidelberg, Typhimurium, and Weltevreden), respectively, which was confirmed by DNA sequencing of the PCR products. This study was the first to report detection of *S. enterica* serotype Agona in the country.

Food poisoning outbreaks and livestock infection caused by *Salmonella enterica* are widespread in the Philippines, as evidenced by cases of food poisoning reported in Benguet, Tondo, Manila, and Bulacan and cases of hog morbidity and mortality in Tacloban and Leyte *(25, 31, 41, 47)*. Moreover, the study of Azanza *(2)* on 60 reported Philippine foodborne outbreaks from 1995 to 2004 revealed that meat-containing dishes were the most common causes of the outbreaks evaluated, with spaghetti as the leading food vehicle and *S. enterica* as the primary cause of infection *(1)*. Among the pathogenic bacteria that cause approximately 90% of all foodborne illnesses, *S. enterica* is one of the most frequently reported and is recognized as one of the leading causes of gastroenteritis and enteric fever, leading to millions of cases of diarrheal illness and thousands of hospitalizations and deaths worldwide each year *(6, 11, 14, 34)*. A wide variety of animals have been identified as reservoirs of *S. enterica*. These include domestic and wild mammals, reptiles, birds, and insects, of which swine are the most commonly recognized carriers *(19)*.

The demand for the production of quality and wholesome livestock meat is increasing. However, the hog livestock production system, despite being the top livestock industry in the Philippines *(5)*, is constantly challenged with various microbial diseases such as salmonellosis that lead to huge monetary losses due to morbidity-linked reduction in productivity and increased costs of disease treatment *(38)*. Moreover, the threat and prevalence of this disease in the country continue to be high *(25)*. Swine slaughter offers many opportunities for contamination of pork carcasses with *S. enterica*, with contamination occurring through fecal, pharyngeal, or environmental sources *(38)*. Good sanitary practices and proper waste disposal in the slaughterhouse are, thus, vital to prevent carcass contamination by *S. enterica*.

Early detection of *S. enterica* in food products protects consumers from contaminated products and outbreaks of food poisoning. Detection of *S. enterica* in swine, however, is challenging because infection does not always manifest clinical symptoms. Furthermore, the number of bacterial cells shed by asymptomatic carrier swine is generally below the detection limit for standard culture methods, which may take 3 to 10 days to accomplish, resulting in an underestimation of herd prevalence *(20)*. The International Organization for Standardization method 6579, which includes preenrichment and selective enrichment in liquid culture and biochemical and serological confirmation of colonies grown on agar plates, takes about 5 days to complete *(43)*. In clinical cases, a delay in detecting the

* Author for correspondence. Tel/Fax: +63-2-9205471; E-mail: wlrivera@science.upd.edu.ph.

organism may lead to serious, if not fatal, consequences for patients. Thus, reliable tools must be used to reduce the number of carrier swine and decrease the incidence of salmonellosis in both humans and animals.

Molecular detection and characterization allow rapid detection and identification of emerging serotypes and new mechanisms of *S. enterica* transmission. These are important prerequisites to identify sources of *S. enterica* contamination and to control outbreaks. A molecular method developed for sensitive and specific detection of *Salmonella* species is the PCR *(10, 23, 27)*. PCR has been applied in various stages of the diagnostic procedure: confirmation of suspected colonies grown on agar plates, analysis of enrichment broths, and direct analysis of suspected foodstuffs. Various researchers have employed this technique to detect *Salmonella* species *(7, 9, 22, 30, 35)*.

Serotyping has been a fundamental measure in the epidemiological surveillance of *S. enterica*. The traditional method is based on the Kaufmann-White serotyping scheme. However, this method is time-consuming and tedious, as well as subjective in interpretation. Moreover, it requires well-trained technicians and high-quality antisera, both of which could be difficult to obtain consistently and are very costly in resource-limited settings. Such limitations have led to the development of multiplex PCR to identify *S. enterica* serotypes, a method which has been found to be highly sensitive, very specific, fast, and reproducible *(10, 23, 27, 32, 36)*. Application of such molecular methods as multiplex PCR–based serogrouping and serotyping for detection, differentiation, and characterization of *S. enterica* isolates in the Philippines is limited. Hence, serogroups and serotypes of *Salmonella* species documented in the country do not accurately represent the actual number of serogroups and serotypes existing in the country.

Tonsils, digestive tracts, and lymph nodes are the organs in which *S. enterica* is most likely to be found. This study, thus, aimed to rapidly detect, differentiate, and characterize *S. enterica* isolates from tonsils and jejuna with jejunal lymph nodes (JLN) of swine at slaughter in selected meat establishments in Metro Manila. Specifically, this study aimed (i) to isolate and rapidly detect *S. enterica* by enrichment culture followed by a PCR assay targeting the *invA* gene and (ii) to determine the serogroup and serotype of *S. enterica* isolates through multiplex PCR–based detection of somatic (O), capsular (Vi), and flagellar (H) antigens, and *Sdf I* regions. This pioneer study, which involved rapid detection, differentiation, and characterization of *Salmonella* species in slaughtered swine in Metro Manila, contributes to epidemiological data focused on the prevalence of, and baseline data about, serogroup and serotype diversity in the country.

## MATERIALS AND METHODS

**Sample collection.** Thirty hogs from each of the four locally registered meat establishments (LRMEs) in Quezon City and four National Meat Inspection Services (NMIS)–accredited slaughterhouses in Malabon, Makati, Pasig, and Quezon City in Metro Manila, Philippines, were selected for sample collection. Tonsil tissue and a 15-cm-long segment of jejunum with JLN were collected from each hog, using sterile forceps and a butcher's knife during evisceration. Samples were immediately transferred to a sterile bag that was cooled during transport to the laboratory. Afterward, 25 g of jejunum with JLN was weighed on a sterile foil and was preenriched with 225 ml of buffered peptone water (Merck, Darmstadt, Germany) in a sterile bottle, agitated for 2 min, and incubated for 18 to 24 h at 37°C. The tonsil tissues were preenriched in the same way as the intestinal samples.

**Single-enrichment broth culture method.** One-hundred-microliter aliquots of the samples of preenriched tonsil tissue and jejunum with JLN were inoculated into Rappaport-Vassiliadis broth (10 ml; Difco, BD, Sparks, MD), and 1 ml of the preenriched samples was inoculated into tetrathionate broth (10 ml); these were incubated at 37°C for 24 h. After incubation, broth cultures were streak plated onto selective, chromogenic medium, Rainbow agar *Salmonella* (Biolog, Hayward, CA) *(3)*.

**DNA extraction.** Three presumptive colonies of *S. enterica* cells from Rainbow agar *Salmonella* were suspended in 150 μl of sterile distilled water. The suspension was heated at 100°C for 10 min and was cooled to room temperature afterward. The cell debris was pelleted by centrifugation at 15,856 × *g* for 2 min. The clear supernatant obtained was used as DNA template in PCR *(37)*. The DNA concentration of the extracts was measured using NanoDrop 2000 (Thermo Fisher Scientific, Inc., Waltham, MA) following manufacturer's instructions.

**PCR-based identification of *S. enterica* isolates.** Primers *invA*-F and *invA*-R, which amplify a 244-bp fragment of the *invA* gene specific for *S. enterica,* were used to confirm suspected *S. enterica* isolates *(7)*. Promega GoTaq Green Master Mix, consisting of GoTaq DNA polymerase, 2× Green GoTaq reaction buffer, 3 mM $MgCl_2$, and 0.4 mM deoxynucleoside triphosphates (dNTPs), was used for PCR amplification of the *invA* region. DNA amplification was performed in a reaction volume of 25 μl. PCR was performed under the following cycling conditions: an initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 2 min. Final extension was done at 72°C for 5 min. For each run, DNA from *S. enterica* serotype Typhimurium was used as the positive control, and sterile water as template was included as the negative control. Amplicons were checked by separating PCR products through agarose gel electrophoresis in 1× Tris-acetate-EDTA buffer at 100 V for 30 to 40 min. All PCR products were analyzed in a 1.5% agarose gel stained with 0.5 μg/ml ethidium bromide for 20 min and were visualized on a UV transilluminator. The sizes of the bands were estimated using a 1,000-bp DNA ladder (Vivantis, Selangor Darul Ehsan, Malaysia) as molecular weight marker.

**Primers for multiplex PCR.** Six sets of primers targeting O-antigen synthesis genes in *rfb* gene cluster specific for *S. enterica* serogroups A, B, C1, D, and E and *viaB* gene for Vi-positive strains were employed for O-serogrouping multiplex PCR. These primers were F-rfbJ and R-rfbJ, which amplify a 662-bp fragment of the *rfbJ* gene and target the B group *(29)*; F-tyv and R-tyv, which amplify a 614-bp fragment of the *tyv* gene and target the D group *(18)*; F-vi and R-vi, which amplify a 439-bp fragment of the *vi* gene and target the Vi strains *(18)*; F-prt and R-prt, which amplify a 256-bp fragment of the *prt* gene and target the A and D group *(18)*; F-wzxC1 and R-wzxC1, which amplify a 483-bp fragment of the *wzxC1* gene and target the C1 group *(17)*; and F-wzxE1 and R-wzxE1, which amplify a 345-bp fragment of the *wzxE1* gene and target the E group *(17)*. Primers that were used in

TABLE 1. *Culture and* invA *PCR–positive* Salmonella enterica *isolates from tissue samples of slaughtered swine in selected meat establishments in Metro Manila*[a]

| Detection method[b] | Tissue sample | No. positive | | Total no. ($n = 480$) |
|---|---|---|---|---|
| | | NMIS-accredited meat establishments | Locally registered meat establishments | |
| Culture | Tonsil | 19 | 34 | 53 |
| | Jejunum with JLN | 19 | 26 | 45 |
| Total | | | | 98 |
| *invA* PCR | Tonsil | 19 | 33 | 52 |
| | Jejunum with JLN | 16 | 25 | 41 |
| Total | | | | 93 |

[a] Swine tissue samples ($n = 480$) of tonsils and jejuna with jejunal lymph nodes (JLN) came from locally registered meat establishments and NMIS-accredited meat establishments. Positive tissue samples for both detection methods were not necessarily from the same hog.
[b] Values for the culture detection method represent presumptive *S. enterica* isolates isolated from the corresponding tissue sample, whereas those for the *invA* PCR assay represent confirmed *S. enterica* isolates.

the second-step multiplex PCR for H/*Sdf I* typing were H-for and H:i, which amplify a fragment of the *fliC* gene present in *S. enterica* Typhimurium *(17, 27),* and sdfF and sdfR, which amplify a 333-bp fragment of the *Sdf I* region uniquely present in *S. enterica* serovar Enteritidis *(1).* In both multiplex PCR assays, internal controls (P1–P2 primers that amplify *oriC* gene) were incorporated to avoid false-negative results *(46).*

**O-serogrouping multiplex PCR.** KAPA2G Fast multiplex mix (2×), consisting of 1 U of KAPA2G Fast HotStart DNA polymerase, 1.5× KAPA2G buffer A, 3 mM $MgCl_2$, and 0.2 mM dNTPs, was used for O-serogrouping multiplex PCR. DNA amplification was performed in a reaction volume of 25 μl. Each reaction mixture contained 12.5 μl of KAPA2G Fast multiplex mix (2×); 0.5 μl (0.2 μM) each of primers F-prt, R-prt, F-rfbJ, R-rfbJ, F-vi, R-vi, F-wzxC1, R-wzxC1, F-tyvD, R-tyvD, F-wzxE, R-wzxE, and positive control primers (P1–P2); 3.5 μl of PCR grade water; and 2 μl of DNA template. O-serogrouping multiplex PCR was performed under the cycling conditions described by Lim and Thong *(28).* Amplicons were analyzed as described above. For each PCR experiment, DNA from *S. enterica* Typhimurium was used as positive control. A negative control (using sterile water as template) was included in each run.

**H/*Sdf I*–typing multiplex PCR.** KAPA2G Fast multiplex mix (2×), consisting of 1 U of KAPA2G Fast HotStart DNA polymerase, 1.5× KAPA2G buffer A, 3 mM $MgCl_2$, and 0.2 mM dNTPs, was used for H/*Sdf I*–typing multiplex PCR. DNA amplification was performed in a reaction volume of 25 μl. Each reaction mixture contained 12.5 μl of KAPA2G Fast multiplex mix (2×); 0.5 μl (0.2 μM) each of primers H-for, H:i, *Sdf,* and positive control primers (P1–P2); 7.5 μl of nuclease-free PCR grade water; and 2 μl of DNA template. H/*Sdf I*–typing multiplex PCR was performed under the cycling conditions described by Lim and Thong *(28).* Amplicons were analyzed as described above. For each PCR experiment, DNA from *S. enterica* Typhimurium was used as positive control. A negative control (using sterile water as template) was included in each run.

**DNA sequencing of selected amplicons.** Selected PCR products from each serogroup were sent to Macrogen, Inc. (Seoul, South Korea) for purification and DNA sequencing to validate their identities. Nucleotide sequence data obtained were checked in BioEdit v. 7.0.9.0 Sequence Alignment program *(13)* and were compared with available sequences of *S. enterica* in GenBank using the Basic Local Alignment Search Tool available on the National Center for Biotechnology Web site (http://www.ncbi.nlm.nih.gov/BLAST).

**Data analysis.** The chi-square test of proportions was used to statistically evaluate any observable difference between the number of positive samples obtained from LRMEs and NMIS-accredited meat establishments. Subsequent analysis of the swine tissue from which *Salmonella* strains were isolated per animal and the detection method employed was done by using McNemar's test.

## RESULTS

**Isolation and molecular detection of *S. enterica* from slaughtered swine in LRMEs and NMIS-accredited meat establishments.** Table 1 summarizes the number of *S. enterica*–positive samples detected by cultural and *invA* PCR methods from a total of 480 samples collected from meat establishments. Of the samples collected from NMIS-accredited meat establishments, 19 of 120 tonsils were positive by both the culture method and the PCR *invA* assay, whereas 19 of 120 jejuna with JLN were detected as positive by the culture method but only 16 of 120 by the PCR *invA* assay. On the other hand, testing of samples collected from LRMEs showed that 34 of 120 tonsils were detected as positive by the culture method, whereas 33 were positive by the PCR *invA* assay; of 120 jejuna with JLN, 26 were detected as positive by the culture method and 25 by the PCR *invA* assay. As seen in Table 1, the tissue with the highest number of *S. enterica*–positive samples was found to be the tonsils from LRMEs (34 of 120). There was no statistically significant relationship between the *S. enterica*–positive tonsils ($n = 120$) and jejuna with JLN ($n = 120$), regardless of the detection method used, for each type of meat establishment ($P = 0.37$). Nonetheless, the greater number of tissue samples detected as positive for *S. enterica* in LRMEs, by both culture (60 of 240) and PCR *invA* assay (58 of 240), possibly reflects a greater occurrence of cross-contamination attributed to unsatisfactory slaughtering facilities and practices. This was supported by chi-square test ($\chi^2 = 16.133$; df = 1) and two-tailed test ($P < 0.0001$), which revealed a statistically significant difference between the two types of meat establishments.

TABLE 2. *O-serogroup distribution of* Salmonella enterica invA *PCR–positive isolates*

| | Selected O-serogroups used in the study | | | | |
|---|---|---|---|---|---|
| | A or D | B | C1 | D | E |
| *invA* PCR–positive samples ($n = 93$) | 1[a] | 68 | 2 | 1[a] | 22 |

[a] Multiplex PCR essentially confirmed the presence of the antigen D from the same sample.

The PCR *invA* assay used in the study detected 93 of 98 presumptive *S. enterica* isolates from culture, and no statistically significant difference was found between the two methods supported by McNemar's test (two-tailed $P = 0.1336$).

**PCR-based serogrouping and serotyping of *S. enterica* isolates.** Differentiation of *S. enterica* into serogroups and serotypes is vital for its epidemiological surveillance. *S. enterica* isolates confirmed via PCR *invA* assay and subsequently subjected to multiplex PCR amplification of *S. enterica* serogroups revealed 68 *S. enterica* isolates classified under serogroup B (73%), 2 under serogroup C1 (2%), 1 under group D (1%), and 22 under group E (24%) (Table 2). The subsequent H/*Sdf I*–typing multiplex PCR performed demonstrated that 64 isolates classified under serogroup B corresponded to *S. enterica* serotype Typhimurium (69%), whereas the sole isolate under serogroup D corresponded to *S. enterica* serotype Enteritidis (100%). Twenty randomly selected isolates that are representative of the four serogroups were characterized further through DNA sequencing to confirm their identities: 12 from serogroup B (including the 4 isolates that did not correspond to *S. enterica* serotype Typhimurium), 2 from serogroup C1, 1 from serogroup D (corresponding to *S. enterica* serotype Enteritidis), and 5 from serogroup E.

**DNA sequencing of *S. enterica* O-serogrouping multiplex PCR amplicons.** DNA sequencing confirmed the results of O-serogrouping and H/*Sdf I*–typing multiplex PCR. Sequences confirmed the identities of eight isolates classified under serogroup B and the sole isolate under serogroup D and revealed that they corresponded to *S. enterica* serotypes Typhimurium and Enteritidis reference strains in GenBank, with 99 to 100% and 99% sequence similarities, respectively (Table 3). This implies that the subsequent two-step multiplex PCR used and the results obtained are consistent, reliable, and reproducible. Further, DNA sequencing revealed 99% sequence similarity of the three isolates classified under serogroup B (that did not correspond to serotype Typhimurium) with serotype Agona, whereas the remaining serogroup B isolate was found to have 99% sequence similarity with *S. enterica* serotype Heidelberg reference strain in GenBank. Sequencing also showed that five isolates classified under serogroup E corresponded to *S. enterica* serotype Weltevreden reference strain in GenBank: one had 100% sequence similarity, two isolates had 99% sequence similarity, and the other two had 98% sequence similarity. In addition, sequencing of the two isolates classified in serogroup C1 revealed that these isolates corresponded to *S. enterica* serotype Choleraesuis reference strain in GenBank; both had 99% sequence similarity. A total of six *S. enterica* serotypes have been detected by O-serogrouping and H/*Sdf I*–typing multiplex PCR and DNA sequencing, namely, *S. enterica* serotypes Agona, Choleraesuis, Enteritidis, Heidelberg, Typhimurium, and Weltevreden. To our knowledge, this is the first report on detection of *S. enterica* serotype Agona in the country.

TABLE 3. *Sequence similarities of isolates and reference* Salmonella enterica *sequences obtained from GenBank*

| Isolate | Region | Serogroup | *Salmonella enterica* subsp. *enterica* strain | Accession no. | Query length and cover, E value | % maximum identity | Reference |
|---|---|---|---|---|---|---|---|
| Lt16 | rfbJ | B | Typhimurium U288 | CP003836.1 | 677, 97%, 0.0 | 99 | *36* |
| Lt21 | rfbJ | B | Typhimurium U288 | CP003836.1 | 671, 98%, 0.0 | 99 | *36* |
| Lt24 | rfbJ | B | Typhimurium U288 | CP003836.1 | 653, 99%, 0.0 | 99 | *36* |
| Lt30 | rfbJ | B | Typhimurium U288 | CP003836.1 | 686, 95%, 0.0 | 99 | *36* |
| Lai1 | rfbJ | B | Agona SL483 | CP001138.1 | 674, 98%, 0.0 | 99 | *12* |
| Lai27 | rfbJ | B | Typhimurium U288 | CP003836.1 | 672, 95%, 0.0 | 99 | *36* |
| Lat23 | rfbJ | B | Agona SL483 | CP001138.1 | 678, 97%, 0.0 | 99 | *12* |
| Lat27 | rfbJ | B | Agona SL483 | CP001138.1 | 665, 97%, 0.0 | 99 | *12* |
| Lbt30 | rfbJ | B | Typhimurium U288 | CP003836.1 | 684, 97%, 0.0 | 99 | *36* |
| Lct47 | rfbJ | B | Typhimurium U288 | CP003836.1 | 674, 93%, 0.0 | 100 | *36* |
| Nt4 | rfbJ | B | Typhimurium U288 | CP003836.1 | 684, 94%, 0.0 | 99 | *36* |
| Pt26 | rfbJ | B | Heidelberg B182 | CP001120.1 | 662, 98%, 0.0 | 99 | *12* |
| Lat20 | wzxC1 | C1 | Choleraesuis SC-B67 | AE017220.1 | 490, 99%, 0.0 | 99 | *8* |
| Lbt18 | wzxC1 | C1 | Choleraesuis SC-B67 | AE017220.1 | 487, 99%, 0.0 | 99 | *8* |
| Lt25 | tyv | D | Enteritidis P125109 | AM933172.1 | 619, 99%, 0.0 | 99 | *44* |
| Li16 | wzxE1 | E | Weltevreden 2007-60-3289-1 | FR775224.1 | 344, 100%, 2e−176 | 99 | *44* |
| Lt3 | wzxE1 | E | Weltevreden 2007-60-3289-1 | FR775224.1 | 357, 69%, 4e−115 | 98 | *4* |
| Mbi8 | wzxE1 | E | Weltevreden 2007-60-3289-1 | FR775224.1 | 354, 99%, 3e−171 | 98 | *4* |
| Mbi25 | wzxE1 | E | Weltevreden 2007-60-3289-1 | FR775224.1 | 348, 99%, 3e−180 | 100 | *4* |
| Pt12 | wzxE1 | E | Weltevreden 2007-60-3289-1 | FR775224.1 | 253, 96%, 3e−119 | 99 | *4* |

## DISCUSSION

**Isolation and molecular detection of *S. enterica* from slaughtered swine in LRMEs and NMIS-accredited meat establishments.** Use of PCR-based methods has revolutionized molecular detection of *S. enterica* through the provision of ultrasensitive amplification and detection of specific nucleic acid sequences. In the Philippines, however, PCR-based detection and characterization of *S. enterica* isolates is not widely employed due to budgetary limitations and lack of access to equipment and materials needed. Researchers in the Philippines rely more on the conventional culture method of isolation of *S. enterica,* which takes about 5 days to complete, is tedious, and requires substantial manpower. Hence, there is a need for a rapid, reliable, and cost-effective tool for detection and characterization of *S. enterica* to prevent outbreaks of salmonellosis and to administer appropriate treatment to those affected by it.

Tonsils, lymph nodes, feces, and the digestive tract are the most likely locations from which *S. enterica* can be isolated from swine. Among these, tonsils are one of the first organs to come in contact upon ingestion of contaminated feedstuff or feces *(48),* and could be important sites for invasion and dissemination of *S. enterica*; hence, they should always be examined. As seen in Table 1, for the PCR *invA* assay in LRMEs, more tonsil tissues were positive for *S. enterica* compared with tissues from jejuna with JLN. Despite the observable difference in the number of positive tonsil and JLN tissues from both types of meat establishments and through both culture method and PCR amplification of the *invA* region, no statistically significant relationship was found ($P = 0.37$). A greater number of tonsil and jejunum with JLN samples were positive for *S. enterica* in LRMEs (Table 1), possibly reflecting a greater occurrence of cross-contamination in this type of abattoir. This was supported by *t* test ($P = 0.034$), which revealed a statistically significant difference between the two types of meat establishments. To directly assess this factor, additional samples (i.e., equipment swabs, water samples, swabs from butchers) should be obtained.

The implicated higher percentage occurrence of *S. enterica* contamination detected in LRMEs by data obtained in this study (Table 1) could be attributed not only to unsanitary practices during slaughter but also to the diet of the swine. Hogs slaughtered in LRMEs are mostly grown in backyards and are fed with anything available, leftovers or sometimes commercial feeds or root crops, whereas hogs grown on commercial farms and slaughtered in NMIS-accredited meat establishments are given formulated feeds. This could make a difference in the microflora of their gastrointestinal tract (unpublished data).

The PCR *invA* assay used in the study detected nearly all culture-confirmed samples (93 of 98), and *t* test ($P = 0.90$) showed no statistically significant difference between *S. enterica*–positive samples detected from the culture method and PCR *invA* assay (Table 1), confirming results from previous studies indicating that the PCR *invA* assay is a consistent and reliable molecular identification method for *S. enterica (21, 42).*

**Serogrouping and serotyping of *S. enterica* isolates.** The conventional method of *S. enterica* serotyping based on the Kauffman-White scheme *(12, 40)* is not 100% precise and is limited in its application, the production and quality of antisera, human error and subjectivity, and high cost. Such limitations have led to the development of multiplex PCR for identification of *S. enterica* serotypes, which has been found to be highly sensitive, very specific, fast, and reproducible *(10, 18, 23, 27, 29, 32, 36).* PCR-based serogrouping and serotyping allow determination of an antigenic profile for strains that cannot be serotyped, such as those that do not express antigens, those that are enclosed in a capsule, or those that possess a rough phenotype *(37).* Another advantage of using PCR-based serogrouping and serotyping is that the occurrence of cross-reactivity, which usually occurs in traditional serogrouping and serotyping assays, is prevented *(28).* In this study, two multiplex PCR assays were used; the first reaction classified the isolates into serogroups, whereas the second reaction determined the serotype. In both the O-serogrouping and H/*Sdf I*–typing multiplex PCR used in this study, an internal amplification control was included to avoid false-negative results. In this study, the published P1–P2 primer pair targeting the *oriC* gene, which is found in all *Salmonella* species *(46),* was included as an internal amplification control in all O-serogrouping and H-typing/*Sdf I* multiplex reactions. The 163-bp PCR product of P1–P2 primer was detected in all PCR *invA* assay–confirmed *S. enterica* isolates.

The combination of published primers into O-serogrouping and H/*Sdf I*–typing multiplex PCR used in the study allowed for identification of *S. enterica* Typhimurium and *S. enterica* Enteritidis based on amplification of the *fliC* alleles and *Sdf I* region, respectively. Only *S. enterica* serovars Typhimurium and Enteritidis were targeted in H-typing/*Sdf I* multiplex PCR because these are the two most commonly reported nontyphoidal *S. enterica* serovars in the country. *S. enterica* serovars Typhi and Paratyphi are likewise frequently reported in the country, but they are highly host-adapted pathogens, causing disease only in humans and higher primates *(33).*

The multiplex PCR–based O-serogrouping used in this study revealed four *S. enterica* serogroups, namely, B, C1, D, and E; this agrees with the results found by Vismanos and coworkers *(45).* The present study further showed that *S. enterica* isolates from slaughtered swine in Metro Manila classified in serogroup B had the highest occurrence (73%) (Table 2). In contrast, Lee et al. *(26)* found that the most prevalent serogroup of the *S. enterica* clinical isolates from Research Institute for Tropical Medicine, Manila, were classified in serogroup E (53.2%). The subsequent H/*Sdf I* typing and DNA sequencing detected six *S. enterica* serotypes, namely, Agona, Choleraesuis, Enteritidis, Heidelberg, Typhimurium, and Weltevreden.

Of the 68 isolates classified in serogroup B, 64 corresponded to *S. enterica* serotype Typhimurium. DNA sequencing revealed that the four serogroup B isolates corresponded to other serotypes, namely, Agona and Heidelberg. This is the first report of serotype Agona isolated from an animal source in the Philippines. Although

*S. enterica* serotype Heidelberg is more commonly reported in developed countries *(48)* and was not found among the 20 most common serovars in the African and Asian regions *(15),* Krauland and co-workers *(24)* detected this serotype in the Philippines. Importation of diseased animals from other countries may have caused the introduction of *S. enterica* serotypes Agona and Heidelberg among Philippine hogs.

Constant monitoring of *S. enterica* serovar distribution in many countries and implementation and evaluation of the effectiveness of *S. enterica* control measures throughout the food production chain is necessary. In developing countries such as the Philippines, serotype Typhimurium is one of the most commonly reported causes of human salmonellosis *(15).* This is consistent with the O-serogrouping and H/*Sdf I*-typing multiplex PCR results obtained in this study, in which 64 isolates, comprising 69% of all isolates classified under serogroup B, corresponded to *S. enterica* serotype Typhimurium reference strain in GenBank with high sequence similarities, 99 to 100% (Table 3).

Likewise, *S. enterica* serotype Enteritidis is one of the most commonly detected serotypes in the Philippines. However, only 1 of 93 *S. enterica* isolates detected in the present study corresponded to the *S. enterica* serotype Enteritidis reference strain in GenBank with 99% sequence similarity (Table 3); this may indicate a decrease in the occurrence of this serotype in the country, particularly in Metro Manila, perhaps due to the intense focus on this particular serovar and the introduction of specific monitoring and control programs against it. Hendriksen and coworkers *(15)* did indeed observe a steady decrease in the prevalence of *S. enterica* serotype Enteritidis isolated from humans during an observation of *S. enterica* serovars in 37 selected countries, including the Philippines, from 2001 to 2007.

*S. enterica* serotype Choleraesuis primarily infects swine but can also cause bacteremia in humans. Two isolates classified under serogroup C1, comprising 2% of all isolates (Table 2), corresponded to *S. enterica* serotype Choleraesuis reference strain in GenBank with 99% sequence similarity (Table 3).

*S. enterica* serotype Weltevreden is prevalent in Southeast Asia *(11, 14, 16, 39).* In the Philippines, its prevalence decreased from 2003 to 2007 *(18, 30).* Despite this shift, this serotype showed increasing relative importance during the 2005 to 2007 observation of *S. enterica* serovar distribution in the Philippines. In the present study, O-serogrouping multiplex PCR and DNA sequencing showed that five isolates classified under serogroup E corresponded to *S. enterica* serotype Weltevreden reference strain in GenBank, with 100% (one isolate), 99% (two isolates), and 98% (two isolates) sequence similarities (Table 3). Isolates Lt16 and Li16, as well as Lat27 and Lai27, are noteworthy indicators of contamination in the slaughterhouse because sequences of these isolates gave interesting results.

The two-step multiplex PCR used in this study was adapted from previous studies but was modified by the use of a different combination of published primers. The modification used revealed a total of four *S. enterica* serogroups, namely, B, C1, D, and E, and six serotypes confirmed by DNA sequencing, namely, Agona, Choleraesuis, Enteritidis, Heidelberg, Typhimurium, and Weltevreden. To our knowledge, this is the first report on detection of serotype Agona in the country. This method was proven to generate consistent, reliable, and reproducible results. Thus, these methods, as well as findings of this study, should be conveyed to intended users and beneficiaries, i.e., meat inspection laboratories and the animal industry, through training seminars and distribution of manuals. Findings from this study will also be used to reiterate to local government units the need to renovate and improve LRMEs to comply with standards set by the NMIS, good manufacturing practices, and hazard analysis and critical control point programs.

Environmental samples, such as swabs from equipment and surroundings as well as from butchers who are in direct contact with pork carcasses, should be collected to directly assess the risk of cross-contamination in the abattoir. Also, additional primers specific for detection of *S. enterica* serotypes other than Enteritidis and Typhimurium are recommended for use in further H/*Sdf I* typing of isolates. Data generated from this study will not only contribute to the global data bank but will also aid in developing and implementing novel control strategies, determining appropriate disease treatment, and implementing and monitoring salmonellae control measures throughout the food production chain because control measures taken against specific serovars are not equally efficient against other serovars.

## ACKNOWLEDGMENTS

This work was supported by grants from the Office of the Vice Chancellor for Research and Development of the University of the Philippines Diliman, Office of the Vice President for Academic Affairs of the University of the Philippines System, and the Department of Agriculture-Biotechnology Program Implementation Unit (Project Code DABIOTECH-R1212).

## REFERENCES

1. Agron, P. G., R. L. Walker, H. Kinde, S. J. Sawyer, D. C. Hayes, J. Wollard, and G. L. Andersen. 2001. Identification by subtractive hybridization of sequences specific for *Salmonella enterica* serovar Enteritidis. *Appl. Environ. Microbiol.* 67:4984–4991.
2. Azanza, M. P. V. 2006. Philippine foodborne-disease outbreaks (1995–2004). *J. Food Saf.* 26:92–102.
3. Biolog. 2003. Rainbow® agar *Salmonella* technical information. Available at: http://www.biolog.com/docroot_biologco/wwwRoot/pdf/RBSalTech00P038A.pdf. Accessed 31 July 2011.
4. Brankatschk, K., J. Blom, A. Goesmann, T. H. Smits, and B. Duffy. 2011. Genome of a European fresh-vegetable food safety outbreak strain of *Salmonella enterica* subsp. *enterica* serovar Weltevreden. *J. Bacteriol.* 193:2066.
5. Bureau of Agricultural Statistics. Livestock and poultry database. Available at: http://countrystat.bas.gov.ph/index.asp?cont=tables&pageid=pxweb/database/m%20in/DETAILS/A_PRODUCTION/B%20LIVESTOCK/B%20LIVESTOCK.xml. Accessed 17 December 2010.
6. Centers for Disease Control and Prevention. 2007. *Salmonella* surveillance: annual summary, 2005. U.S. Department of Health and Human Services, Atlanta.
7. Chiu, C. H., and J. T. Ou. 1996. Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, invA and

spvC, by an enrichment broth culture-multiplex PCR combination assay. *J. Clin. Microbiol.* 34:2619–2622.
8. Chiu, C. H., P. Tang, C. Chu, S. Hu, Q. Bao, J. Yu, Y. Y. Chou, H. S. Wang, and Y. S. Lee. 2005. The genome sequence of *Salmonella enterica* serovar Choleraesuis, a highly invasive and resistant zoonotic pathogen. *Nucleic Acids Res.* 33:1690–1698.
9. Ferretti, R., I. Mannazzu, L. Cocolin, G. Comi, and F. Clementi. 2001. Twelve-hour PCR-based method for detection of *Salmonella* spp. in food. *Appl. Environ. Microbiol.* 67:977–978.
10. Fitzgerald, C., R. Sherwood, L. L. Gheesling, F. W. Brenner, and P. I. Fields. 2003. Molecular analysis of the rfb O antigen gene cluster of *Salmonella enterica* serogroup O:6,14 and development of a serogroup-specific PCR assay. *Appl. Environ. Microbiol.* 69:6099–6105.
11. Galanis, E., D. M. L. F. Wong, M. E. Patrick, N. Binsztein, A. Cieslik, T. Chalermchaikit, A. Aidara-Kane, A. Ellis, F. J. Angulo, and H. C. Wegener. 2006. Web-based surveillance and global *Salmonella* distribution, 2000–2002. *Emerg. Infect. Dis.* 12:381–388.
12. Hald, T., D. Vose, H. C. Wegener, and T. Koupeev. 2004. A Bayesian approach to quantify the contribution of animal-food sources to human salmonellosis. *Risk Anal.* 24:255–269.
13. Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41:95–98.
14. Hendriksen, R. S., M. Mikoleit, C. Kornschober, R. L. Rickert, S. Van Duyne, C. Kjelsø, H. Hasman, M. Cormican, D. Mevius, and J. Threlfall. 2009. Emergence of multidrug-resistant *Salmonella* Concord infections in Europe and the United States in children adopted from Ethiopia, 2003–2007. *Pediatr. Infect. Dis. J.* 28:814–818.
15. Hendriksen, R. S., A. R. Vieira, S. Karlsmose, D. M. Lo Fo Wong, A. B. Jensen, H. C. Wegener, and F. M. Aarestrup. 2011. Global monitoring of *Salmonella* serovar distribution from the World Health Organization Global Foodborne Infections Network Country Data Bank: results of quality assured laboratories from 2001 to 2007. *Foodborne Pathog. Dis.* 8:887–900.
16. Herikstad, H., Y. Motarjemi, and R. Tauxe. 2002. *Salmonella* surveillance: a global survey of public health serotyping. *Epidemiol. Infect.* 129:1–8.
17. Herrera-León, S., J. R. McQuiston, M. A. Usera, P. I. Fields, J. Garaizar, and M. A. Echeita. 2004. Multiplex PCR for distinguishing the most common phase-1 flagellar antigens of *Salmonella* spp. *J. Clin. Microbiol.* 42:2581–2586.
18. Hirose, K., K.-I. Itoh, H. Nakajima, T. Kurazono, M. Yamaguchi, K. Moriya, T. Ezaki, Y. Kawamura, K. Tamura, and H. Watanabe. 2002. Selective amplification of tyv (rfbE), prt (rfbS), viaB, and fliC genes by multiplex PCR for identification of *Salmonella enterica* serovars Typhi and Paratyphi A. *J. Clin. Microbiol.* 40:633–636.
19. Humphrey, T. 2000. Public-health aspects of *Salmonella* infection, p. 245–263. *In* C. Wray and A. Wray (ed.), *Salmonella* in domestic animals. CABI Publishing, Wallingford, UK.
20. Hurd, H., W. Schlosser, and E. Ebel. 1999. The effect of intermittent shedding on prevalence estimation in populations, p. 57–62. *In* Proceedings of the 3rd International Symposium on the Epidemiology and Control of *Salmonella* in Pork, Washington, DC, 5 to 7 August 1999.
21. Jamshidi, A., M. R. Bassami, and S. Afshari-Nic. 2009. Identification of *Salmonella* spp. and *Salmonella typhimurium* by a multiplex PCR-based assay from poultry carcasses in Mashhad, Iran. *Int. J. Vet. Res.* 3:43–48.
22. Kawasaki, S., P. M. Fratamico, N. Horikoshi, Y. Okada, K. Takeshita, T. Sameshima, and S. Kawamoto. 2009. Evaluation of a multiplex PCR system for simultaneous detection of *Salmonella* spp., *Listeria monocytogenes,* and *Escherichia coli* O157: H7 in foods and in food subjected to freezing. *Foodborne Pathog. Dis.* 6: 81–89.
23. Kim, S., J. G. Frye, J. Hu, P. J. Fedorka-Cray, R. Gautom, and D. S. Boyle. 2006. Multiplex PCR-based method for identification of common clinical serotypes of *Salmonella enterica* subsp. *enterica*. *J. Clin. Microbiol.* 44:3608–3615.
24. Krauland, M., L. Harrison, D. Paterson, and J. Marsh. 2010. Novel integron gene cassette arrays identified in a global collection of multi-drug resistant non-typhoidal *Salmonella enterica*. *Curr. Microbiol.* 60:217–223.
25. Labro, V. 2009. *Salmonella* infection in pigs rises. Available at: http://newsinfo.inquirer.net/inquirerheadlines/regions/view/20090201-186729/Salmonella-infection-in-pigs-rises. Accessed 3 November 2010.
26. Lee, H.-Y., L.-H. Su, M.-H. Tsai, S.-W. Kim, H.-H. Chang, S.-I. Jung, K.-H. Park, J. Perera, C. Carlos, and B. H. Tan. 2009. High rate of reduced susceptibility to ciprofloxacin and ceftriaxone among nontyphoid *Salmonella* clinical isolates in Asia. *Antimicrob. Agents Chemother.* 53:2696–2699.
27. Levy, H., S. Diallo, S. M. Tennant, S. Livio, S. O. Sow, M. Tapia, P. I. Fields, M. Mikoleit, B. Tamboura, and K. L. Kotloff. 2008. A PCR method to identify *Salmonella enterica* serovars Typhi, Paratyphi A and Paratyphi B among *Salmonella* isolates from the blood of patients with clinical enteric fever. *J. Clin. Microbiol.* 46: 1861–1866.
28. Lim, B. K., and K. L. Thong. 2009. Application of PCR-based serogrouping of selected *Salmonella* serotypes in Malaysia. *J. Infect. Dev. Ctries.* 3:420–428.
29. Lim, Y.-H., K. Hirose, H. Izumiya, E. Arakawa, H. Takahashi, J. Terajima, K.-I. Itoh, K. Tamura, S.-I. Kim, and H. Watanabe. 2003. Multiplex polymerase chain reaction assay for selective detection of *Salmonella enterica* serovar Typhimurium. *Jpn. J. Infect. Dis.* 56:151–155.
30. Löfström, C., R. Knutsson, C. E. Axelsson, and P. Rådström. 2004. Rapid and specific detection of *Salmonella* spp. in animal feed samples by PCR after culture enrichment. *Appl. Environ. Microbiol.* 70:69–75.
31. Lopez, A. 2008. DOH eyes *Salmonella* in Tondo food poisoning. Available at: http://newsinfo.inquirer.net/breakingnews/metro/view/20080925-162897/DoH-eyes-Salmonella-in-Tondo-food-poisoning. Accessed 10 January 2011.
32. Luk, J., U. Kongmuang, P. Reeves, and A. Lindberg. 1993. Selective amplification of abequose and paratose synthase genes (rfb) by polymerase chain reaction for identification of *Salmonella* major serogroups (A, B, C2, and D). *J. Clin. Microbiol.* 31:2118–2123.
33. Matthews, T. D., W. Rabsch, and S. Maloy. 2011. Chromosomal rearrangements in *Salmonella enterica* serovar Typhi strains isolated from asymptomatic human carriers. *mBio* 2:e00060-11.
34. Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5:841–842.
35. Menghistu, H. T., R. Rathore, K. Dhama, and R. K. Agarwal. 2011. Isolation, identification and polymerase chain reaction (PCR) detection of *Salmonella* species from field materials of poultry origin. *Int. J. Microbiol. Res.* 2:135–142.
36. Mortimer, C. K., T. M. Peters, S. E. Gharbia, J. M. Logan, and C. Arnold. 2004. Towards the development of a DNA-sequence based approach to serotyping of *Salmonella enterica*. *BMC Microbiol.* 4:31.
37. Nori, M. E. E., and K. L. Thong. 2010. Differentiation of *Salmonella enterica* based on PCR detection of selected somatic and flagellar antigens. *Afr. J. Microbiol. Res.* 4:871–876.
38. Nowak, B., T. von Müffling, S. Chaunchom, and J. Hartung. 2007. *Salmonella* contamination in pigs at slaughter and on the farm: a field study using an antibody ELISA test and a PCR technique. *Int. J. Food Microbiol.* 115:259–267.
39. Olsen, S. J., R. Bishop, F. W. Brenner, T. H. Roels, N. Bean, R. V. Tauxe, and L. Slutsker. 2001. The changing epidemiology of *Salmonella*: trends in serotypes isolated from humans in the United States, 1987–1997. *J. Infect. Dis.* 183:753–761.
40. Popoff, M. Y., and L. Le Minor. 2001. Antigenic formulas of the *Salmonella* serovars, 8th rev. WHO Collaborating Centre for Reference and Research on *Salmonella*. Institut Pasteur, Paris.
41. Reyes-Estrope, C. 2011. Salmonella found cause of food poisoning. Available at: http://newsinfo.inquirer.net/17168/Salmonella-found-cause-of-bulacan-food-poisoning. Accessed 14 August 2011.
42. Shanmugasamy, M., T. Velayutham, and J. Rajeswar. 2011. InvA gene specific PCR for detection of *Salmonella* from broilers. *Vet. World* 4:562–564.
43. Temelli, S., A. Eyigor, and K. Carli. 2012. *Salmonella* detection in poultry meat and meat products by the Vitek immunodiagnostic assay

system easy *Salmonella* method, a LightCycler polymerase chain reaction system, and the International Organization for Standardization method 6579. *Poult. Sci.* 91:724–731.

44. Thomson, N. R., D. J. Clayton, D. Windhorst, G. Vernikos, S. Davidson, and C. Churcher. 2008. Comparative genome analysis of *Salmonella* Enteritidis PT4 and *Salmonella* Gallinarum 287/91 provides insights into evolutionary and host adaptation pathways. *Genome Res.* 18:1624–1637. Available at: http://genome.cshlp.org/content/18/10/1624.full.pdf+html. Accessed 3 March 2012.
45. Vismanos, M. F. C., T. Sichann, M. A. Padilla, and L. R. Baldrias. 1999. Isolation and serotyping of *Salmonella* in cattle slaughtered in Laguna and Batangas [Philippines]. *Philipp. J. Vet. Med.* 36:55–60.
46. Widjojoatmodjo, M., A. Fluit, R. Torensma, G. Verdonk, and J. Verhoef. 1992. The magnetic immuno polymerase chain reaction assay for direct detection of salmonellae in fecal samples. *J. Clin. Microbiol.* 30:3195–3199.
47. Zabala, J. B., D. A. Mendoza, F. Caput, M. C. Roces, M. White, and M. M. Dayrit. 1993. *Salmonella* food poisoning in Benguet. *Philipp. J. Microbiol. Infect. Dis.* 22:5–7.
48. Zhao, S., D. White, S. Friedman, A. Glenn, K. Blickenstaff, S. Ayers, J. Abbott, E. Hall-Robinson, and P. McDermott. 2008. Antimicrobial resistance in *Salmonella enterica* serovar Heidelberg isolates from retail meats, including poultry, from 2002 to 2006. *Appl. Environ. Microbiol.* 74:6656–6662.