SHORT COMMUNICATION

Incidence and molecular detection of *Salmonella enterica* serogroups and *spvC* virulence gene in raw and processed meats from selected wet markets in Metro Manila, Philippines

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Abstract—A simple and specific detection method for *Salmonella enterica* was applied to determine its incidence in raw and processed meats purchased from selected wet markets in Metro Manila, Philippines. A total of 320 raw and processed meat samples were analysed for the presence of *S. enterica* and *S. enterica* possessing *spvC* gene of the virulence plasmid. Polymerase chain reaction (PCR) revealed that 30.63% (98/320) were positive for *S. enterica* and 2.81% (9/98) of *S. enterica*-positive samples were also positive for the *spvC* gene. *S. enterica* was identified from chicken samples (67.5%), ground pork (65%), beef (52.5%), sausage (*longganisa*) (25%), cured pork meat (*tocino*) (20%), burger patty (12.5%), and meatloaf (*embotido*) (2.5%). Positive samples were further analysed for O-serogrouping targeting *S. enterica* serogroups A, B, C1, C2, D, and E1. Our findings revealed that the raw and processed meats tested were contaminated with more than one serogroup in a sample. Samples were found positive for *S. enterica* serogroups E1 (78.57%), C1 (29.59%), C2 (20.41%), B (17.35%) and D (6.1%). No samples were found positive for *S. enterica* serogroup A. This is the first report on the use of multiplex PCR for the detection and characterization of *S. enterica* in raw and processed meats in the Philippines. Data on incidence of *S. enterica* and its serogroups found in raw and processed meats in selected Philippine wet markets gathered from this study can be used for further research on epidemiology and related topics.

Keywords—*Salmonella enterica*, serogroups, *spvC* gene, raw and processed meats, wet markets, Metro Manila

INTRODUCTION

Potential biological hazards in meat include bacteria, toxins, viruses, protozoa and parasites. Of these, bacteria cause a large proportion (approximately 90%) of all foodborne illnesses. The bacterial pathogens that are most likely to be found in commonly slaughtered livestock (cattle, sheep, and swine) and poultry (chicken and turkey) include *Salmonella enterica*, *Campylobacter* sp., and *Listeria monocytogenes*. These pathogens have been implicated in widely publicized foodborne disease outbreaks associated with the consumption of meat and poultry products (Food Safety and Inspection Service 1999). Salmonellosis, which is caused by *Salmonella*, is one of the leading causes of foodborne bacterial enteritis in many countries (Centers for Disease Control and Prevention 2004a; Tirado and Schmidt 2001). Outbreaks of diseases are reported frequently, implicating different kinds of food contaminated with *S. enterica* (Centers for Disease Control and Prevention 2004b; Ethelberg et al. 2004; Fielding et al. 2003; Matsui et al. 2004). *S. enterica* is a common cause of human gastroenteritis and bacteremia worldwide (Hendriksen et al. 2009; Morpeth et al. 2009; Schlundt et al. 2004; Voetsch et al. 2004). A wide variety of animals, particularly food animals, have been identified as reservoirs of non-typhoidal *Salmonella* (Bangtrakulnonth et al. 2004; Guard-Petter 2001; Vindigni et al. 2007). Currently, *Salmonella* consists of 2,659 different serovars (Issenhuth-Jeanjean et. al. 2014) and based on the *Salmonella* Serotype Statistics, approximately 60% belong to subspecies I, *S. enterica* subsp. Enterica (Centers for Disease Control and Prevention 2008).

Raw and processed meats in the Philippines are typically sold in two types of markets, supermarkets and wet markets. The supermarkets, which are indoor markets, often display pre-packaged raw meat under refrigeration. In contrast, the wet markets usually display unwrapped raw and processed meats at ambient temperatures which are easily contaminated with foodborne pathogens. Reports of *S. enterica* contamination on raw and processed meats in the country are limited. Reliable detection methods are therefore required for diagnosis and prevention of food contamination and foodborne outbreaks. Traditional microbiological techniques, such as the International Organization for Standardization (ISO) method 6579 for detecting *Salmonella* in food, take up to 5 days to obtain a positive result. This ISO method includes pre-enrichment and selective enrichment in liquid culture, and biochemical and serological confirmation of colonies grown on agar plates (International Organization for Standardization 2002). Delays caused by the identification procedure can hinder the appropriate response to an outbreak of disease. For a more reliable and fast analysis, polymerase chain

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Submitted: May 15, 2015
Revised: August 7, 2015
Accepted: August 16, 2015
Published: September 30, 2015

reaction (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. PCR employs specific primers to detect the presence of pathogens in a shorter period of time. Various studies used this technique for the detection of *Salmonella* spp. (Chiu and Ou 1996; Kawasaki et al. 2009; Pritchett et al. 2000). Furthermore, studies on the application of molecular methods in detection and characterization of *S. enterica* isolates in the Philippines are limited. Multiplex PCR of *S. enterica* isolates, for instance, is not widely performed in the Philippines. As such, known serogroups are not accurately documented.

This study aimed to determine the presence of *S. enterica* in raw and processed meats from four wet markets in Metro Manila, Philippines: Commonwealth, Balintawak, Cubao, and Caloocan. Samples included raw meat such as cut chicken, minced pork, cut beef and processed meats, such as homemade meatloaf (locally called *embotido*), sausage (locally called *longganisa*), cured pork meat (locally called *tocino*), burger patty, and ham. Specifically, this study aimed to: (1) detect *S. enterica* via multiplex PCR targeting the *invA* and *spvC* genes; and (2) characterize *S. enterica* isolates via multiplex PCR detection of the somatic (O) antigen.

MATERIALS AND METHODS

Collection of samples

Three hundred twenty (320) raw and processed meat samples were randomly obtained from selected wet markets in Metro Manila, Philippines: Balintawak, Caloocan, Commonwealth, and Cubao (Table 1). Ten samples of each type of meat were collected in each of the four wet markets. All samples were placed in a cooler (approximately 5 °C) during transport and immediately processed in the laboratory for pre-enrichment. Samples were labeled according to their location, sample type and numbered from 1 to 10.

Table 1. Incidence of *Salmonella enterica* in raw and processed meat samples collected in selected wet markets in Metro Manila, Philippines.

Meat Sample	Type of Meat	Code	Number of samples per site	Number of samples in 4 wet markets	Number of culture-positive samples	Number of PCR-positive samples	
						invA	*spvC*
Raw	Cut chicken	C	10	40	26	27	6
	Ground pork	GP	10	40	26	26	0
	Cut beef	B	10	40	20	21	0
Processed	Burger patty	P	10	40	5	5	3
	Embotido	E	10	40	1	1	0
	Ham	H	10	40	0	0	0
	Longganisa	L	10	40	10	10	0
	Tocino	T	10	40	8	8	0
		Total	**80**	**320**	**96**	**98**	**9**

Cultivation of S. enterica

To determine the presence of *S. enterica* in the samples, conventional culture method was used. Twenty five (25) grams of sample was suspended into 225 ml buffered peptone water (BPW, Difco) and incubated at 37 °C for 18-24 h. One hundred microliters of pre-enriched culture was simultaneously transferred to 10 ml Rappaport Vassiliadis Soy enrichment broth (RVS, Merck) and incubated at 42 °C for 18-24 h. Enriched RVS culture was inoculated in xylose lysine deoxycholate agar (XLD, Merck) and brilliant green agar (BGA, Difco), and then incubated at 37 °C for 24-48 h at aerophilic condition. Growth on either BGA or XLD was considered culture-positive. *S. enterica* serovar Typhimurium UPCC 1360 obtained from the University of the Philippines-Culture Collection (UPCC) in the Microbiological Research and Services Laboratory (MRSL) of the Natural Sciences Research Institute (NSRI), was used as positive control.

DNA extraction

In each sample, 500 μl of the RVS-enriched culture was collected from triplicate tubes into a microfuge tube and centrifuged at 15,330 × g for 5 min. The supernatant was discarded, and the cell pellets were suspended in 1 ml phosphate buffered saline (PBS), centrifuged at 15,330 × g for 5 min. The supernatant was again discarded, and the pellets were suspended in 50 µl sterile distilled water for DNA extraction (O'Regan et al. 2008). The suspension was heated at 99 ºC for 10 min (Shanmugasamy et al., 2011), and then pelleted by centrifugation at 2,656 × g for 5 min. The DNA extracts were immediately cooled on ice.

Multiplex PCR detection of invA and spvC genes

DNA amplification was performed in a reaction volume of 25 µl. Each reaction mixture contained 12.5 µl of 2× GoTaq® Green Master Mix (Promega), 1 µl each of 10 µM forward and reverse primers of *invA* and *spvC* genes (Table 2), 7 µl of sterile double distilled water, and 1.5 µl DNA template extracted from RVS broth. PCR was performed under the following conditions: initial denaturation at 95 °C for 2 min, denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s and final extension at 72 °C for 5 min. Amplicons were visualized by a UV transilluminator (BioRad®) after electrophoresis on 1.5% agarose gel stained with ethidium bromide (0.5 μg/ml). The *Salmonella* Typhimurium UPCC 1360 obtained from MRSL was used as positive control, while sterile double distilled water was used as negative control.

Table 2. Primer sequences used for multiplex PCR methods for the detection of *Salmonella enterica*.

Primer	Sequence (5'-3')	Amplification target	Amplicon length (bp)	Tm (°C)	Reference
F-*invA*	ACAGTGCTCGTTT ACGACCTGAAT	*S. enterica*	244	50.6	Chiu and Ou 1996
R-*invA*	AGACGACTGGTAC TGATCGATAAT			48.9	
F-*spvC*	ACTCCTTGCACAA CCAAATGCGGA	*S. enterica* plasmid virulence	571	65.6	Chiu and Ou 1996
R-*spvC*	TGTCTTCTGCATTT CGCCACCATCA			65.2	
F-abe1	GGCTTCCGGCTTT ATTGG	B group	561	45.2	Hong et al. 2008
R-abe1	TCTCTTATCTGTTC GCCTGTTG			47.9	
F-tyv	GAGGAAGGGAAAT GAAGCTTTT	D group	614	46.0	Hirose et al. 2002
R-tyv	TAGCAAACTGTCT CCCACCATAC			50.2	
F-prt	CTTGCTATGGAAG ACATAACGAACC	A & D group	256	50.9	Hirose et al. 2002
R-prt	CGTCTCCATCAAA AGCTCCATAGA			50.6	
F-wbaD-manC	ATTTGCCCAGTTC GGTTTG	C1 group	341	43.8	Hong et al. 2008
R-wbaD-manC	CCATAACCGACTT CCATTTCC			47.3	
F-abe2	CGTCCTATAACCGA GCCAAC	C2 group	397	48.7	Hong et al. 2008
R-abe2	CTGCTTTATCCCTC TCACCG			48.7	
F-wzx–wzy	GATAGCAACGTTC GGAAATTC	E1 group	281	45.3	Hong et al. 2008
R-wzx–wzy	CCCAATAGCAATAA ACCAAGC			45.3	

Multiplex PCR detection of O-antigen genes

DNA amplification was performed in a reaction volume of 25 µl. Each reaction mixture contained 12.5 µl of 2× KAPA2G Fast multiplex mix (KAPA Biosystems), 0.5 µl (0.2 µM each) of forward and reverse primers: prt, abe1, tyv, wzx-wzy, wbaD-manC, abe2 (Table 2), 3.5 µl of PCR grade water, and 2 µl DNA template. Multiplex PCR of the O-antigen was performed under the following cycling conditions: an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 60 s. Final extension was done at 72 °C for 10 min. The positive control used is a mixture of DNA extracts consisting of *Salmonella* serogroups B (abe1-positive), C1 (wbaD-manC-positive), C2 (abe2-positive), D (tyv- and prt-positive), and E1 (wzx-wzy-positive), which were previously confirmed by DNA sequencing (Ng and Rivera, 2015), while sterile double distilled water was used as negative control.

Agarose Gel Electrophoresis

For the *invA-spvC* assay, amplicons were visualized by a UV transilluminator (BioRad®) after electrophoresis (Mupid®-2 Plus) at 100 V for 25 min. These were run on 1.5% agarose gel stained with ethidium bromide (0.5 μg/ml). The sizes of the target sequences were estimated using a Universal DNA Ladder (KAPA

Biosystems). The amplicons for the O-antigen assay were viewed similarly, but on 2% agarose gel and using a 100 bp DNA ladder (Vivantis) to estimate band sizes.

RESULTS

Incidence of S. enterica

A total of 320 raw and processed meat samples were included for the detection of *S. enterica*. Ninety-six (96) out of 320 samples were culture-positive for *S. enterica* (30%), while PCR detection showed that 98 samples were positive for *S. enterica* (30.63%).

Distribution of S. enterica among raw and processed meat samples

Notably, a higher incidence of *S. enterica* was found in raw meats compared to processed meats. A large percentage of chicken samples (67.5%) was positive for *S. enterica*, followed by ground pork (65%), beef (52.5%), longganisa (25%), tocino (20%), burger patty (12.5%), and embotido (2.5%). *S. enterica* was not detected in ham samples (Figure 1).

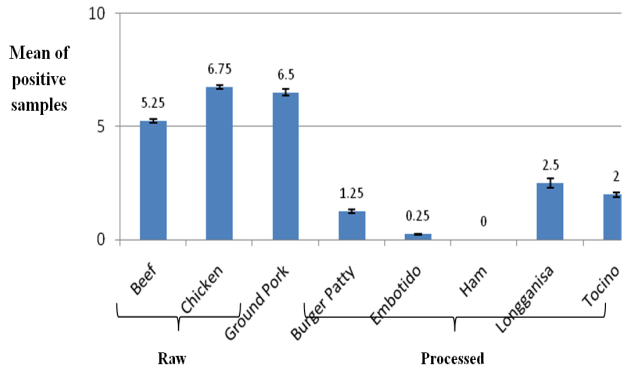

Figure 1. Incidence of *S. enterica* in raw and processed meat products from selected wet markets in Metro Manila, Philippines.

Molecular identification of S. enterica by PCR targeting invA and the virulence gene, spvC

In this study, *invA* gene was present in 98 samples. The *spvC* gene was present in 2.8% of the *S. enterica*-positive samples. *S. enterica* possessing *spvC* was found in six chicken and three burger patty samples only.

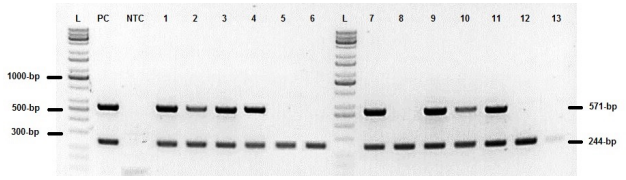

Figure 2. Presence of *spvC* (571-bp) and *invA* (244-bp) in selected *S. enterica* isolates. L –KAPA Universal DNA ladder, PC - positive control, NTC – no template control, 1-13 - isolates.

S. enterica serogroups and their distribution among raw and processed meats

The amplicons produced intense bands of different expected sizes, representing five targeted serogroups (Table 2). Of the 98 *invA*-positive *S. enterica* samples, 78.57% were positive for serogroup E1, followed by C1 (29.59%), C2 (20.41%), B (17.35%) and D (6.1%). There were no samples positive for serogroup A.

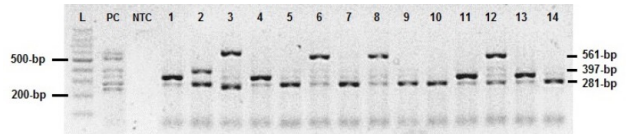

Figure 3. *S. enterica* O-serogroups (614-bp: tyv for D, 561-bp: abe1 for B, 397-bp: abe2 for C2, 341-bp: wbaD-manC for C1, 281-bp: wzx-wzy for E1, and 256-bp: prt for A and D). L –Vivantis VC 100bp DNA ladder, PC - positive control, NTC - no template control, 1-14 - isolates.

DISCUSSION

S. enterica was detected in raw and processed meat samples from selected wet markets using culture and multiplex PCR methods. These methods yielded high incidence of *S. enterica*, which supported the results of previous studies that showed the sensitivity of targeting the *invA* gene of *S. enterica* as a reliable detection method (Amini et al. 2010; Chiu and Ou 1996; Shanmugasamy et al. 2011). The *invA* gene has been recognized as international standard for detection of genus *Salmonella*. The expression of this gene enables *Salmonella* to invade the epithelial cells of its host (Shanmugasamy et al. 2011). Ninety-six (96) out of 320 samples were culture-positive for *S. enterica* (30%), while PCR detection showed 98 samples positive for *S. enterica* (30.63%). Nine samples harbored the virulence plasmid *spvC* gene, which reflects a relatively lower incidence than those in other published reports (Amini et al. 2010; Chiu and Ou 1996). Nevertheless, the presence of this gene indicates that these isolates may cause systemic disease (Heithoffet al. 2008).

The positive result for *invA* in two samples but negative for *S. enterica* in culture-based method suggests that the latter methods may overlook the presence and underestimate the number of *S. enterica* in a sample. El Shamy et. al. (2008) noted that conventional culture media may not be universally sensitive for the detection of *Salmonella*, depending on sample type or serotype.

The PCR-based detection used in this study is simpler, more rapid, and more specific for the detection of *S. enterica* compared to culture/traditional method of detection of *S. enterica*. The traditional method normally takes 7 days. With the multiplex PCR used in this study, identification of serogroups of *S. enterica* could be done in 3 days. Boiling method was selected for the preparation of the DNA template because of the convenience of the method. Centrifugation and washing steps were sufficient to remove the PCR inhibitors from the culture medium.

A large percentage of chicken (67.5%), ground pork (65%), and beef (52.5%) samples were positive for *S. enterica*. Several outbreaks and studies of salmonellosis in chicken/poultry have been reported (Rajagopal and Mini 2013; Wegener and Baggesen 1996). Ground meat (e.g. ground pork, burger patty) is among the food items frequently associated with outbreaks of salmonellosis. It is predisposed to contamination because many processing steps are involved in its manufacture (e.g. grinding), which potentially contribute to an increase of *S. enterica* counts in the final product (Stock and Stolle 2001). Thus, the incidence of *S. enterica* in ground pork could come from improper handling and use of unsanitary mechanical grinder in meat processing in wet markets.

S. enterica was also present in processed meat: *longganisa* (25%), *tocino* (20%), burger patty (12.5%), and *embotido* (2.5%), despite the presence of preservatives and additives (Table 1). Handling and transport were the most probable reasons for cross-contamination. The absence of *S. enterica* in ham samples could be attributed to thermal processing during the manufacturing process.

The highest incidence of 78.57% observed for *S. enterica* serogroup E1 was similar to the results of Balala et al. (2006) and Lee et al. (2009), which also showed the predominance of serogroup E1 among isolates from clinical and food samples collected in the Philippines. The multiplex PCR also detected *S. enterica* from serogroups C1 (29.59%), C2 (20.41%), B (17.35%) and D (6.1%). Four isolates were not characterized in multiplex PCR for O-serogrouping. Only six serogroups were targeted using the primers abe1, tyv, prt, wbaD-manC, abe2 and wzx-wzy in the O-serogrouping assays. Hence, these isolates could belong to other 40 *Salmonella* serogroups (Grimont and Weill 2007) that were not tested in this study.

Obtaining DNA extracts from the RVS-enriched medium allows for the detection of multiple *S. enterica* serogroups in one sample. It eliminates the need for a pure isolate of every serotype of *S. enterica* in a sample, thus saving time and effort. The multiplex PCR assay used for the serogrouping of the isolates may also be considered less expensive compared to conventional serotyping assuming that PCR and post-PCR equipment are available.

CONCLUSION

In this study, a simple and specific detection method for *S. enterica* was applied in raw and processed meat samples collected from selected wet markets in Metro Manila, Philippines. Through molecular characterization by targeting a portion of the *invA* and *spvC* genes, *S. enterica* isolates were detected and successfully characterized serologically via PCR-based detection of somatic (O) antigen. More than one serogroup were present in a sample; 78.57% (77 samples) with the predominance of *S. enterica* serogroup E1. The methods used in this study can greatly reduce the reliance on the costly and tedious conventional serotyping. They can be applied by any facility that lacks the expensive typing sera and expertise needed for conventional serotyping but is equipped with basic PCR facilities. Likewise, multiplex PCR can be useful especially during outbreaks when rapid detection is necessary.

This is the first report on the use of multiplex PCR for the detection and characterization of *S. enterica* in raw and processed meats in the Philippines. This study, likewise, was able to establish incidence data for *S. enterica* and its serogroups found in raw and processed meats in selected Philippine wet markets which could be used for further research in epidemiology and related topics. The relatively high incidence of *S. enterica* in raw and processed meats illustrates the risk for *Salmonella* infection due to poorly processed meat.

ACKNOWLEDGEMENT

We thank Phyllis Anne P. Paclibare, Pauline Dianne M. Santos, and Alyzza Marie B. Calayag for the technical assistance. This study was supported by research grants from the Department of Agriculture-Biotechnology Program Implementation Unit (Project Code DABIOTECH-R1212) and the Office of the Vice-Chancellor for Research and Development of the University of the Philippines Diliman (Project Code 151515 PNSE).

CONFLICT OF INTERESTS

The authors declare no conflict of interests regarding the publication of this article.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

SASDR and WLR conceptualized this study. The experiments were conducted by SASDR. SASDR and WLR prepared the manuscript.

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