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Full Length Research Paper

Rapid detection and isolation of *Salmonella* sp. from amphibians and reptiles

Sang Phil Shin, Hyo Jin Yang, Ji Hyung Kim, Casiano H. Choresca Jr., Jee Eun Han, Jin Woo Jun, Sang Yoon Han and Se Chang Park*

Laboratory of Aquatic Animal Medicine and Research Institute for Veterinary Science, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Republic of Korea.

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The principal objective of this study was to assess the presence of *Salmonella* sp. in imported amphibians and reptiles. A total of 49 samples were collected from commercial pet shops and life science museums in Korea and examined via the traditional detection method (TDM) and the PCR method. *Salmonella typhimurium* and *Salmonella enteritidis* were screened via multiplex PCR (mPCR). The results of the TDM showed a positive isolation in 18.4% of the samples, whereas the PCR method had a 28.6% positive detection rate. These results indicate the possibility that humans can contact *Salmonella* infections from amphibians and reptiles. Our findings are also important from a public health standpoint. Additionally, the presence of *Salmonella* sp. in these samples indicates the necessity of appropriate quarantine practices to be established.

Key words: Amphibian, PCR, reptile, *Salmonella* sp.

INTRODUCTION

Salmonella species live within the intestinal tracts of both warm-blooded and cold-blooded animals. The genus *Salmonella* belongs to the family Enterobacteriaceae and is divided taxonomically into two species, *Salmonella enterica* and *Salmonella bongori*. *S. enterica* comprises six subspecies: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enteric* subsp. *houtenae* (IV) and *S. enterica* subsp. *indica* (VI) (Tindall et al., 2005). However, for simplicity's sake, *S. bongori* is generally commonly referred to as subsp. V. The most frequently encountered subspecies is *S. enterica* subsp. I. found primarily in mammals; this subspecies is the most common cause of human disease (Centers for Disease Control and Prevention, 2005). The other five subspecies of *S. enterica*, as well as *S. bongori*, are detected primarily in nonhuman hosts and only occasionally cause disease in humans (Bopp et al., 2003; McQuiston et al., 2008). Among the 2,541 total serotypes, 1,504 are in *S. enterica* subsp. I (Popoff et al., 2004). *Salmonellae* from serogroups B and D account for approximately two-thirds of all reported *Salmonella* infections (Mermin et al., 2004). The common serotypes causing disease in humans are *Salmonella enteritidis* (serogroup D) and *Salmonella typhimurium* (serogroup B) (Baggesen et al., 2000; Aktas et al., 2007). These two species are classed as *Salmonella enterica* subsp. I.

Salmonellae are naturally detected in the gastro-intestinal tracts of reptiles and amphibians (Kaufmann and Morrison, 1966; Kourany et al., 1970; Sharma et al., 1977). A recent increase in the popularity of exotic reptile pets has resulted in an increase in the number of cases of reptile-associated salmonellosis (Centers for Disease Control and Prevention, 2003). Clinical cases of reptile-associated salmonellosis have been on an uptick reported worldwide (Sanyal et al., 1997; Woodward et al., 1997; De Jong et al., 2005; Kaibu et al., 2006). In Korea, 5,000 and 127,000 kg of amphibians and reptiles,

*Corresponding author. E-mail: parksec@snu.ac.kr. Tel: +82 2 880 1282. Fax: +82 2 880 1213.

Abbreviations: **TDM**, Traditional detection methods; **mPCR**, multiplex polymerase chain reaction; **BPW**, buffered peptone water; **RV**, Rappaport Vassiliadis; **XLD**, xylose lysine desoxycholate agar; **BGA**, brillant green agar; **TSB**, tryptic soy broth.

respectively, were imported at in 2009 (Korea Customs Service, 2010). Presently, there are no specific regulations regarding quarantine practices on the importation of amphibians and reptiles in Korea.

Traditional detection methods (TDM) for *Salmonella* are based on cultures using selective media and the characterization of suspicious colonies via biochemical and serological tests. These methods generally require 6 days and generate many false positive results. However, there have been a few published studies that have described alternative or complementary methods for the identification of *Salmonella* at the subspecies level. With regard specifically to *S. enterica* subsp. I, several studies have been published that describe identifications at the serovar level using multiplex polymerase chain reaction (mPCR) assays (Herrera-León et al., 2004; Kim et al. 2006). In *S. enterica* subspecies other than subsp. I, a great deal of sequence data have recently been published. It would clearly be useful to develop a practical multiplex PCR assay for *Salmonella* detection using those data. Therefore, in this study, we report and describe a newly-developed PCR assay for the rapid, reliable and easy detection of *Salmonella* from amphibians and reptiles, and evaluate the presence of *S. typhimurium* and *S. enteritidis* using specific mPCR assays for these two *Salmonella* serovars.

MATERIALS AND METHODS

Samples collection

From April to September 2010, sterile swabs from 49 skin and anus samples (from 27 species) of imported amphibians and reptiles were aseptically collected from commercial pet shops and life science museums in Seoul, Korea. Thirty swab samples were taken from 20 reptile species and 19 swab samples originated from 7 amphibian species.

Traditional detection methods (TDM) and PCR method

The TDM assays for the detection of different *Salmonella* subsp. were conducted as previously described (Hendriksen, 2003). In brief, swab samples were cultured in buffered peptone water (BPW) at 37°C for 24 h followed by selective enrichment of 0.2 in 2 ml of Rappaport Vassiliadis (RV) broth. After 24 h, Xylose lysine desoxycholate agar (XLD), and brillant green agar (BGA) (BD Difco, Spark, MD, U.S.A) were used as selective agar. The presumptive positive colonies were grown for 24 h in 2 ml of tryptic soy broth (TSB) at 37°C. The DNA of bacterial cultures was employed as template in PCR. The GapA (encoding for glyceraldehyde-3-phosphate dehydrogenase) housekeeping gene was amplified and sequenced as previously reported for the identification of *Salmonella* via BLAST search (McQuiston et al., 2008).

In an effort to evaluate the efficacy and accuracy of the PCR *Salmonella* detection method, we selected a *Salmonella* enterotoxin gene (stn) primer set (Makino et al., 1999). Swab samples were cultured for 24 h in RV broth at 37°C, followed by buffered peptone water (BPW). The BPW was incubated for 24 h at 37°C. DNA was extracted from the BPW as described previously. PCR was carried out as described previously (Ziemer and Steadham, 2003). The BPW was then subcultured onto XLD agar and BGA. The presumptive positive colonies were identified via gapA gene amplification and sequencing.

Multiplex PCR for detection of *S. typhimurium* and *S. enteritidis*

In order to detect *S. typhimurium* and *S. enteritidis*, multiplex PCR (mPCR) was carried out as previously described, using DNA extracted from the cultured BPW and isolates (Moussa et al., 2010). We selected 3 target genes--*stn*, *sefA* (*S. Enteritidis* fimbrial antigen) (Doran et al., 1996), and *fliC* (H1-i antigen specific for *S. Typhimurium*) (Soumet et al., 1999). The primers (Macrogen, Seoul, Republic of Korea) employed in this study are listed in Table 1.

Statistical analysis

The data obtained from TDM and PCR methods were compared using Wilcoxon signed ranks test. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

While the TDM revealed positive isolation of 9 *Salmonella* subsp. (18.4%) in 49 examined samples, the PCR method detected 14 *Salmonella* subsp. (28.6%). All isolates isolated by TDM were also detected by PCR. However, 5 isolates detected by the PCR method were not isolated via the TDM. Three of these originated from the amphibians and 11 originated from reptiles. The *Salmonella* subsp. identification of the isolates revealed that 6 subsp. I, 2 subsp. II, 1 subsp. IIIa, 3 subsp. IIIb, 1 subsp. IV, and 1 subsp. V. Subsp VI were not isolated from any of the samples examined. *S. typhimurium* was detected in the Savanna monitor (*Varanus exanthematicus*)-1 sample, but *S. enteritidis* was not detected (Figure 1).

In this study, the presence of *Salmonella* from amphibians and reptiles was evaluated via TDM and PCR methods. The predominant subsp. identified was *S. enterica* subsp. I, a major source of *Salmonella* infection in humans. Additionally, *S. typhimurium* was isolated from the Savanna monitor (*Varanus exanthematicus*). These findings reflect the possibility of human *Salmonella* infection from amphibians and reptiles, which are important from a public health perspective, as an estimated 3 to 5% of all cases of salmonellosis in humans are associated with exposure to exotic pets (Woodward et al., 1997).

Presently, there are no specific regulations concerning quarantine practices on the importation of amphibians and reptiles into Korea. Consequently, amphibian and reptile samples may constitute a source of amphibian- and reptile-associated salmonellosis. Amphibian-reptile-associated salmonellosis has been increasingly reported worldwide, and this problem makes an important consideration about the importation restrictions on amphibian and reptiles (Sanyal et al., 1997; Woodward et al., 1997; De Jong et al., 2005; Kaibu et al., 2006). In

Table 1. Primers sequences used for amplification of DNA for the detection of *Salmonella* species in this study.

Primer name	Sequence (5'-3')	Annealing temperature (°C)	Cycle	Size of amplified product (bp)	Specificity
gapA	TAT GAC TAT CAA AGT AGG TA GTT GGA GTA ACC GGT TTC GT	55	35	924	-
stn	CTT TGG TCG TAA AAT AAG GCG TGC CCA AAG CAG AGA GAT TC	55	35	260	*Salmonella* sp.
fliC	CGG TGT TGC CCA GGT TGG TAA T ACT GGT AAA GAT GGC T	55	35	620	*S. typhimurium*
sefA	GAT ACT GCT GAA CGT AGA AGG GCG TAA ATC AGC ATC TGC AGT AGC	55	35	488	*S. enteritidis*

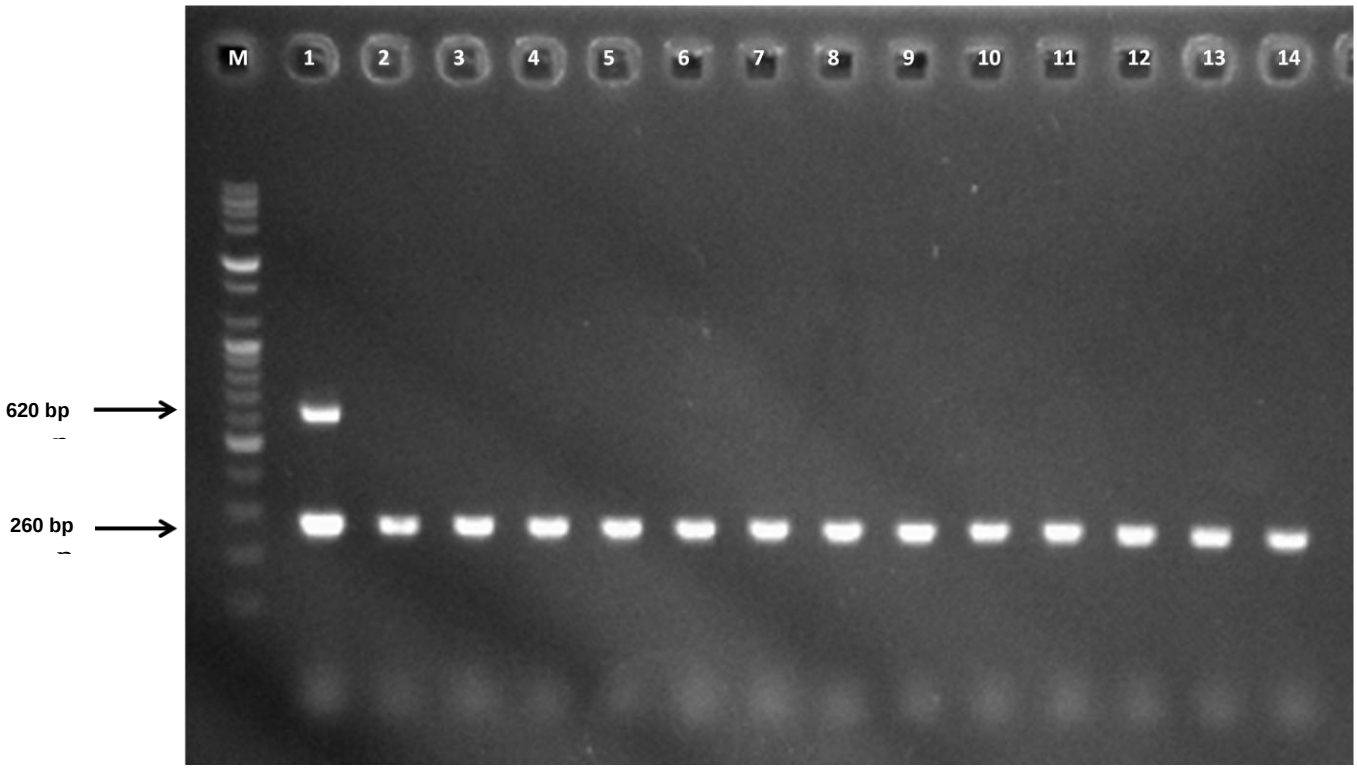

Figure 1. Amplification products obtained using the mPCR for detection of *S. typhimurium* and *S. enteritidis* in isolates from imported amphibians and reptiles. Lane M shows molecular weight marker using 100 bp ladder; lane 1, *Varanus exanthematicus*; lane 2, *Testudo graeca ibera*; lane 3, *Tiliqua nigrolutea*; lane 4, *Pogona henrylawsoni*; lane 5, Litoria *caerulea*; lane 6, *Python curtus*; lane 7, *Ceratophrys cranwelli*; lane 8, *Chelydra serpentine*; lane 9, *Python molurus bivittatus*; lane 10, *Python reticulates*; lane 11, *Pseudemys* sp.; lane 12, *Varanus exanthematicus*; lane 13, *Pituophis catenifer*; lane 14, *Polypedates leucomystax*.

Canada, pet turtle–associated salmonellosis was recognized as a serious health problem in the 1960s and 1970s, and the country banned the importation of turtles in 1975 (Koopman and Janssen, 1973). Sweden has a long tradition of combating and controlling *Salmonella* in feed, animals, and humans, dating back to a large outbreak of salmonellosis in 1953 that affected > 9,000 persons and caused 90 deaths (Bengtsson et al., 1955; Lindberg et al., 1999). From 1970 to 1994, these control measures also included import restrictions on reptiles; anyone who wished to import reptiles or turtles required a certificate stating that the animals were free of *Salmonella* (De Jong et al., 2005).

The PCR method described herein required 2 days to

complete, was reliable and easy to perform, and had significantly ($p < 0.05$) higher detection of *Salmonella* (28.6%) than the TDM (18.4%) in our 49 amphibian and reptile samples. Conventional biochemical-based detection methods generally require at least 6 days to complete using several media, and these methods do not always distinguish *Salmonella* spp. from other bacteria. A previous study conducted with XLD agar and BGA revealed false positive results of *Citrobacter* spp. and *Proteus* spp. (Rall et al., 2005). We also isolated these bacterial species on the XLD agar and BGA. The PCR method is significantly ($p < 0.05$) more sensitive than TDM and reduces detection time; additionally, the PCR method generated no false positives and was more effective than TDM in detecting *Salmonella* spp. Moreover, the mPCR method employed in the detection of *S. enteritidis* or *S. typhimurium* also generated specific results in both direct cultured samples and isolated *Salmonella* spp. These PCR and mPCR methods are clearly useful, particularly for the rapid screening of large numbers of samples.

Based on these results, we believe that these *Salmonella*-infected samples may constitute a persistent potential source of salmonellosis; therefore, there is a clear need for a quarantine system to be established in Korea. However, imported amphibians and reptiles should be assessed randomly by PCR-based techniques until such laws are implemented. When *S. enteritidis* or *S. typhimurium*-infected amphibians or reptiles are discovered, that particular batch of samples should not be accepted into the country. Import regulations and public information campaigns have repeatedly proven to be effective public health measures against amphibian- and reptile- associated salmonellosis.

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