

Research Article

# Characterization of *Salmonella* spp. isolated from small turtles and human in Republic of Korea

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## Abstract

In 2013, the World Health Organization (WHO) reported that small, pet turtles had caused multistate *Salmonella* outbreaks in the United States, from where small turtles were subsequently exported into the Republic of Korea. We investigated cases of salmonellosis in South Korea associated with domestic small turtles and analysed genetic characteristics of *Salmonella* isolates in commercially-available small turtles. We traced six *Salmonella* serovars, known to have caused human infection in the United States (*S. Sandiego*, *S. Pomona*, *S. Poona*, *S. Newport*, *S. I 4,(5),12:i:-*, and *S. Typhimurium*), in isolates from suspected *Salmonella* infection cases in Korea from 2006 to 2015. Additionally, we conducted a pilot study of isolates from small turtles being sold in Korean markets, and performed molecular genetic analysis on the identified strains. *S. Pomona* was identified in one *Salmonella* infection case, while all strains isolated from small turtles belonged to either subspecies I (*S. enterica*,  $n = 10$ , 71.4%) or subspecies IIIb (*S. diarizonae*,  $n = 4$ , 28.6%). Two serovars (*S. Pomona* and *S. Sandiego*) that were highly associated with turtle-to-human transmission were identified with 100% homology to human isolates. Previous to this study, turtle-associated human *S. Pomona* infections were not well reported in Korea. We report *Salmonella* infection in small turtles in Korea, and confirm that small turtles should be considered the first infectious agent in *S. Pomona* infections. We therefore suggest quarantine measures for importing small turtles be enhanced in Korea.

## More Information

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Submitted: November 27, 2020

Approved: December 10, 2020

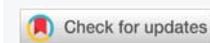
Published: December 11, 2020

**How to cite this article:** Chae SJ, Lim JS, Lee DY. Characterization of *Salmonella* spp. isolated from small turtles and human in Republic of Korea. Insights Vet Sci. 2020; 4: 051-055.

DOI: 10.29328/journal.ivs.1001027

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**Keywords:** *Salmonella* infection; Turtles; Zoonoses; Multilocus sequence typing (MLST); Pulsed-field gel electrophoresis (PFGE)



## Introduction

*Salmonella* spp. is a zoonotic pathogen often reported in humans where there was exposure to infected pets and livestock products [1-3]. *Salmonella* colonize the intestine of turtles, which are the main source of human infection due to shedding of bacteria in faeces [4]. In the United States, 15 outbreaks of *Salmonella* associated with small turtles were reported between 2006 and 2014 [5], with most patients exposed to small turtles (less than 4 inches long) raised on two farms in Louisiana. Thirty one percent of the patients were children under 10 years, of which 70% were under 1 year. In 2013, in accordance with the International Health Regulations, the World Health Organization (WHO) noticed the risk of *Salmonella* infections to 20 countries, including South Korea that imported turtles from the United States. Therefore, we conducted an epidemiological and microbiological survey on the risk of *Salmonella* infection from imported small turtles in Korea.

## Materials and methods

### Case study on human *Salmonella* infection

We examined six *Salmonella* serovars (*S. Sandiego*, *S. Pomona*, *S. Poona*, *S. Newport*, *S. I 4, [5], 12:i:-*, and *S. Typhimurium*) to investigate the possibility of *Salmonella* spp. infections through small turtle in Korea. We analysed the prevalence of the serovars in isolates from *Salmonella* infection cases in Korea during the last ten years. The six serovars were classified for inclusion in the epidemiological study according to three criteria: 1) the serovar was not reported in Korea before, 2) the serovar was only reported in the last decade, and 3) less than five isolates of the serovar were reported per year. Serovars not fitting any of the criteria were deemed candidates most likely transmitted from small turtles, and the epidemiological survey was then conducted on these.

### Samples from domestic turtles

Five small turtles were purchased from markets in four



randomly selected regions of Korea. Turtles were bred over 5 weeks in separate tanks to prevent cross-contamination, and breeding water was changed every 2-3 weeks. Samples from breeding water were collected weekly to analyse the colonisation of *Salmonella* in the turtles' intestines. In addition, samples of breeding water from turtles on Jeju Island (Jeju-si, Seogwipo-si) were collected weekly, as those turtles were associated with suspected human infection previously

### Isolation and identification of *Salmonella* spp. strains

Isolation and identification of *Salmonella* strains was performed following the Korea Centers for Disease Control and Prevention (KCDC) laboratory guidelines. 1 mL breeding water was inoculated into 9 mL Selenite Cysteine Broth (BD Diagnostic Systems™, Sparks, MD, US). The inoculum was then incubated overnight and a loopful of sample plated on MacConkey agar, Salmonella-Shigella agar, and Xylose Lysine Deoxycholate agar (BD™). The unique colonies were streaked on Kligler Iron agar (BD™), and their identification was performed using a biochemical test with API 20E Kit (Biomérieux™, Marcy-1 Etoile, France) and serological testing done as per Lee, et al. [6]. In addition, *invA* specific PCR was performed to confirm the microbiological identity [7]. Genomic DNA was extracted using the boiling method [8] and amplified by singleplex PCR with specific primers for the invasion of *Salmonella* into eukaryotic host cells (*invA* F; 5'-ATT AAT TAT GGA AGC GCT CGC ATT-3', *invA* R; 5'-GTA ATG AGA TCC ATC AAA TTA GCG-3'). Serovars were determined by the combination of somatic O and flagellar H antigens according to the Kauffmann-White classification scheme [9,10].

### PFGE, MLST & antimicrobial susceptibility of *Salmonella* strains

To investigate the epidemiological relationship, *S. Pomona* and *S. Sandiego* isolates (Group IV in our classification) were analysed by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) to compare with human isolates. PFGE was performed according to the PulseNet standard protocol (<http://www.pulsenetinternational.org/>)

protocols/). DNA of each isolate was digested with *Xba*I and *Bln*I restriction enzymes and separated by CHEF Mapper XA system (Bio-Rad, CA, USA). *Salmonella* Braenderup (ATCC BAA664) was used as a reference marker strain. PFGE patterns were analysed using BioNumerics v5.1 software (Applied Maths, Saint-Martens-Lartem, Belgium). The isolates were subjected to MLST by determination of the sequences for seven housekeeping genes: *aroC*, *dnaN*, *hemD*, *hisD*, *pure*, *sucA*, and *thrA* [11]. Sequencing of the nucleotides was performed with an automated sequencer (ABIPRISM 3730XL, Foster City, CA, USA), and the sequence type was determined using analysis tools on the MLST website (<http://mlst.warwick.ac.uk/mlst>).

An antimicrobial susceptibility test was performed using the VITEK 2 system with AST-N169 card (Biomérieux, Marcy-1 Etoile, France). Susceptibility of the following antimicrobial agents was tested: ampicillin, amoxicillin-clavulanic acid, amikacin, chloramphenicol, cephalothin, ciprofloxacin, ceftriaxone, cefotetan, cefotaxime, cefazolin, cefoxitin, gentamicin, imipenem, nalidixic acid, ampicillin-sulbactam, trimethoprim-sulfamethoxazole, and tetracycline. The *Escherichia coli* ATCC 25922 card was used for quality control. Results were interpreted as recommended by CLSI guidelines [12].

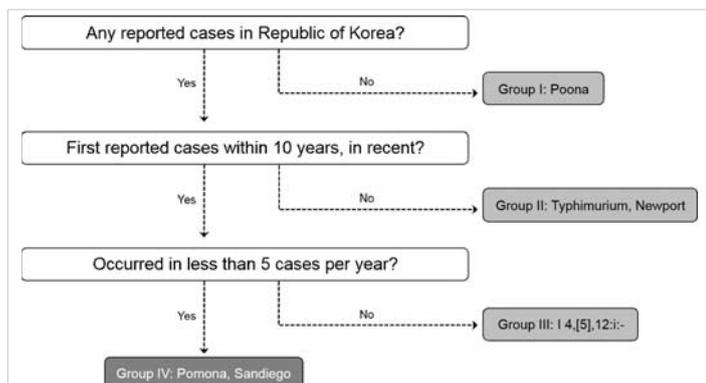
## Results

### Case study on human *Salmonella* infection

A total 5,867 *Salmonella* isolates were collected from diarrheal patients in Korea from 2006 to 2015 (Table 1). A total of 1,144 strains were identified belonging to six serovars which were thought to be related with small turtles, and were divided into four groups according to classification criteria (Figure 1). No isolates of Group I (Gr. I), *S. Pomona*, have been reported in Korea, and this serovar was therefore excluded from the case study. Group II, (Gr. II) including the *S. Typhimurium* ( $n = 745$ ) and *S. Newport* ( $n = 47$ ) serovars, were excluded from the study as there were too many cases to link logistically to small turtles. Group III (Gr. III) isolates, from the *Salmonella* I 4,[5],12:i:-, serovar ( $n = 346$ ), were also excluded; cases of this serotype, a variant strain of *S. Typhimurium*, have been continuously increasing since it was

**Table 1:** Prevalence of *Salmonella* serovars associated with small turtles imported from the USA to Korea, 2006-2015.

Number of organisms	Serovars							Total <i>Salmonella</i> spp.
	Gr. I	Gr. II		Gr. III	Gr. IV		Others	
	Poona	Newport	Typhimurium	I 4,[5],12:i:-	Pomona	Sandiego		
2006	0	1	38	0	0	0	332	371
2007	0	3	30	0	0	0	390	423
2008	0	4	49	11	0	0	306	370
2009	0	0	109	4	0	0	394	507
2010	0	2	72	18	0	0	605	697
2011	0	6	133	30	0	1	799	969
2012	0	5	62	30	0	2	266	365
2013	0	16	101	101	2	0	520	740
2014	0	3	99	77	0	1	674	854
2015	0	7	52	75	0	0	437	571
Total	0	47	745	346	2	4	4723	5,867



**Figure 1:** Classification of *Salmonella* serovars associated with small turtles imported from the USA to Korea, 2006-2015.

first reported in 2008. In addition, Gr. II and Gr. III isolates were excluded for the variety of host ranges. Therefore, Group IV (Gr. IV) isolates, *S. Sandiego* ( $n = 4$ ) and *S. Pomona* ( $n = 2$ ) serovars, were remaining as possible candidates to link with transmission from turtles; isolates of these serotypes have been reported separately within the last five years, with no more than five isolates annually. However, were able to identify just a single suspected case associated with *S. Pomona*.

### A case of *S. Pomona* infection in Korea

A two-year-old boy began to show symptoms of fever on July, 7, 2013. He visited the emergency room two days later with symptoms of fever, abdominal pain, and diarrhoea. The patient returned home after receiving fluid therapy in combination with antispasmodic, sedative, anti-inflammatory, and analgesic therapies. Soon after, he visited the nearby paediatric and juvenile clinic, displaying the symptoms of fever, abdominal pain, and watery diarrhoea again, along with vomiting, poor oral intake, and weight loss (14.8-16 kg). In the evening, he was re-admitted to the emergency room and received intravenous UBACSIN treatment (Jeilpharm, Korea). He remained febrile and had to be hospitalized. Within two days of hospitalization, treatment was changed to cefotaxime and symptoms improved. In microbiological examination, non-typhoidal *Salmonella* was isolated from patient’s faecal samples and serotyping identified *S. Pomona* (M group, 28:y,1,7). During the epidemiological investigation, the patient’s parents reported that the child had begun to experience symptoms after touching a turtle while visiting a neighbour. Samples from the neighbour’s turtle could not be analyzed as the owner refused to allow testing on the animal, but we purchased two turtles from a nearby traditional market to investigate *Salmonella* infection in turtles in the relevant area.

### Isolation and identification of *Salmonella* spp. from domestic turtles

A total 14 *Salmonella* strains were isolated from five small turtles purchased at randomly chosen commercial markets and two turtles purchased at a traditional market in an area where a patient may have contracted *Salmonella* from a domestic turtle. Ten of the 14 strains belonged to subspecies

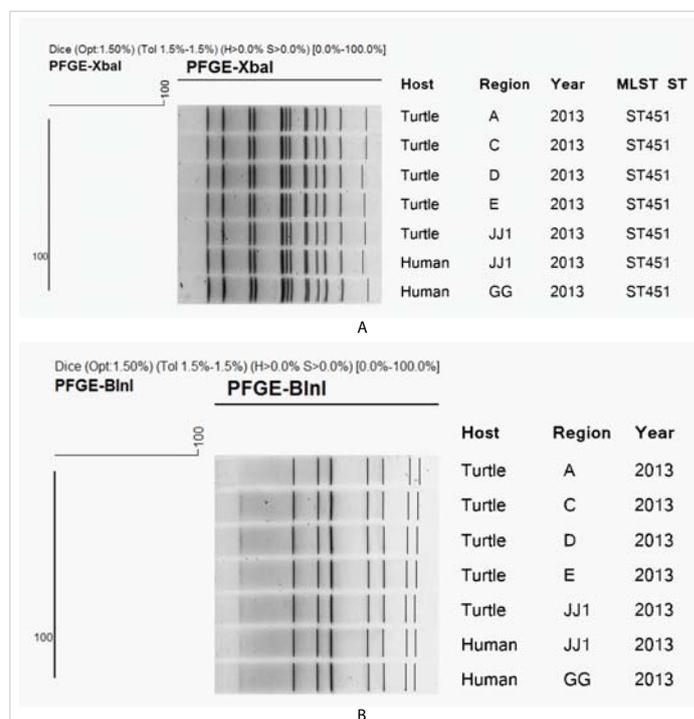
*enterica* and the remaining four strains belonged to subspecies *diarizonae*. Seven serovars were identified, of which the most frequent was *S. Pomona* ( $n = 5$ ), followed by *S. Litchfield* ( $n = 2$ ), *S. Montevideo* ( $n = 2$ ), *S. Sandiego* ( $n = 1$ ), *S. IIIb 50:k:z* ( $n = 1$ ), *S. IIIb 60:[k]:z53* ( $n = 2$ ), and *S. IIIb 61:c:1,5,[7]* ( $n = 1$ ) (Table 2).

### PFGE, MLST, and antimicrobial susceptibility testing

We conducted PFGE and MLST for the characterization of *S. Pomona* and *S. Sandiego* which were commonly identified through risk assessment and bacterial isolation from turtles. For *S. Pomona*, human ( $n = 2$ ) and turtle isolates ( $n = 5$ ) showed 100% homology on PFGE, while MLST showed the same sequence type as the ST451 strain (Figure 2). In contrast, *S. Sandiego* was divided into four clusters as a result of PFGE performed on human ( $n = 4$ ) and turtle isolates ( $n = 1$ ). However, human strains (year 2012) and suspected turtle-

**Table 2:** Distribution of *Salmonella* subspecies and serovar strains isolated in breeding water of small turtles.

<i>Salmonella</i> subsp. Serovar	Samples							No. of isolates
	A	B	C	D	E	JJ1*	JJ2*	
<i>Salmonella enterica</i>								
Litchfield	+	-	+	-	-	-	-	2
Montevideo	+	-	+	-	-	-	-	2
Pomona	+	-	+	+	+	+	-	5
Sandiego	-	-	-	-	-	-	+	1
<i>Salmonella diarizonae</i>								
IIIb 50:k:z	-	-	-	-	+	-	-	1
IIIb 60:[k]:z53	+	-	+	-	-	-	-	2
IIIb 61:c:1,5,[7]	-	-	-	-	-	-	+	1



**Figure 2:** Clustering of (A) *XbaI* and (B) *BlnI*-digested DNA fragments by pulsed-field gel electrophoresis (PFGE) with multilocus sequence typing (MLST) for *Salmonella* *Pomona* strains isolated from turtles and human cases. A, B, C, and D regions are randomized and JJ and GG are abbreviations of Jeju Island and Gyeonggi Province, respectively.

related strains showed 100% homology on PFGE and the same sequence type as the ST20 strain on MLST (Figure 3). All *Salmonella* isolates from turtles and humans were susceptible to the 17 antimicrobial agents tested.

## Discussion

*Salmonella enterica* subspecies *enterica* (I) are mainly isolated from warm-blooded animals; while other subspecies (II, IIIa, IIIb, IV and VI) and *Salmonella bongori* (V) are mainly isolated from cold-blooded animals and from the environment. However, most isolates from turtles in this study were identified as subsp. *enterica*. In recent years, subsp. *enterica* has been identified in a large number of *Salmonella* infection cases associated with reptiles [13-15]. Generally, turtles carry *Salmonella* spp. in their intestinal tracts, and continuously release the bacteria through faeces, causing human infection indirectly. *Salmonella*, which is asymptomatic in reptiles, can cause serious illness in humans. Reptile-associated salmonellosis (RAS) can cause sepsis, meningitis, and bone and joint infections, especially in children [14]. Transmission from turtles accounts for 42% of RAS infections, and children are more affected because they often interact with domestic turtles.

In the United States, at least 473 cases of *Salmonella* infection due to small turtles were reported from March 2012 through October 2013 [16]. The cases were linked to turtles raised on two farms in Louisiana and exported to more than 20 countries. According to statistics from the Korea Customs Service, 28.76 tons of turtles were imported into the Republic of Korea annually over the past sixteen years, of which 20.17% were imported from the United States [17]. It is therefore concerning that turtles can be imported into Korea without quarantine, making it difficult to conduct epidemiologic studies to link turtles to cases of disease because the animals are often imported directly from the United States on a small scale.

No cases of *Salmonella* infection caused by small turtles have been reported in Korea as yet. However several papers reported a possible case of suspected transmission from turtles [18] with cases of rare serovars [19,20]. In the case identified here, it was epidemiologically certain that the patient was infected through contact with a turtle, but it was not confirmed experimentally. Additionally, the identified serovar, *S. Pomona* was genetically identical in isolates from the patient and turtles purchased from a regional traditional market. Moreover, *S. Pomona* strains isolated from additional local patients and further local small turtles were genetically identical to the patient's strain. The case is also similar to two cases previously reported in children younger than 5 years who required treatment [21,22], and we suggest this is the first confirmed case of *Salmonella* infection through turtle in Korea.

Antimicrobial agents are used to reduce *Salmonella* infection in reptiles, which may cause antibiotic resistance [23]. *Salmonella* isolates from reptiles, especially from turtles, were identified to exhibit a variety of multidrug resistance [24]. All isolates in this study were susceptible to the 17 agents used and no antimicrobial resistance genes were detected. However, ampicillin-sulbactam treatment against *S. Pomona* in the identified case did not relieve clinical symptoms, and symptoms only improved after treatment was changed to the cephalosporin, cefotaxime, similar to Kim, et al. [18] who recommended that a third-generation cephalosporin be used in cases of suspected non-typhoidal *Salmonella* bacteraemia with resistance to ampicillin. This may mean experience-based antimicrobial therapy is effective when *Salmonella* is transmitted from small turtle to human.

Small turtles available in the Korean domestic market have *Salmonella* in their intestines. Although *Salmonella* infection does not often cause serious illness, high-risk groups such as the immune-compromised, children under 5 years, and pregnant women are advised to avoid contact with small turtles. In addition, quarantine of exotic pets such as turtles should be strengthened as domestic imports continue to increase.

## Acknowledgement

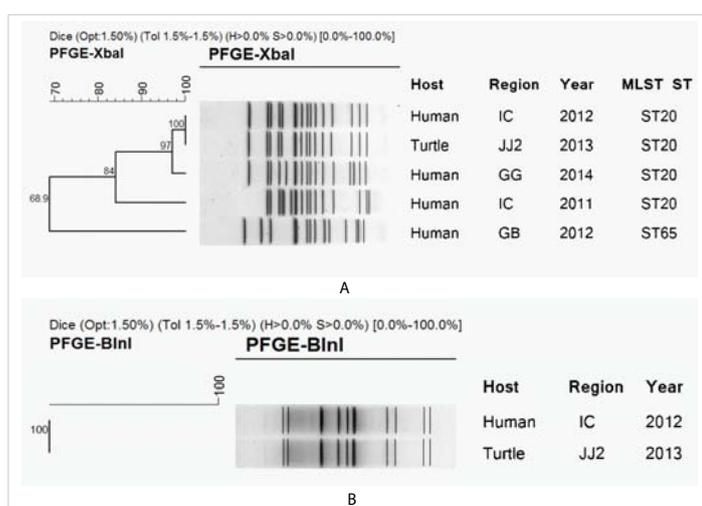
This study was supported by Acute Diarrheal Laboratory Surveillance (EnterNet-Korea) in Korea (4800-4851-304)

## Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required.

## Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.



**Figure 3:** Clustering of (A) *Xba*I and (B) *Bln*I-digested DNA fragments by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) sequence type for *Salmonella* Sandiego strains isolated from turtles and human cases. IC, JJ, and GG are abbreviations of Incheon Metropolitan City, Jeju Island, and Gyeonggi Province, respectively.



## Authors' contributions

Su-Jin Chae was responsible for writing the original draft. Jin-Suk Lim was responsible for the methodology and investigation. Deog-Yong Lee was responsible for the conceptualization and supervision of the study.

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