Original Article

Prevalence, risk factors and antimicrobial resistance of *Salmonella* infection in turtles in Khon Kaen province

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Abstract

Considering the impact of *Salmonella* in turtles and the role of turtles as a source of *Salmonella* outbreaks in humans, knowledge of *Salmonella* status in turtles is important. A cross-sectional study was conducted to investigate the prevalence, risk factors and antimicrobial resistance of *Salmonella* infections in turtles. Cloacal swabs were collected from 378 turtles in Turtle Villages located in Khon Kaen province of northeastern Thailand between January 2018 and April 2019. All samples were examined for *Salmonella* spp. isolation and identification by ISO 6597:2002. The overall prevalence of *Salmonella* in the turtles was 37.57% (142/378). Habitat, season and sex were significantly associated with *Salmonella* positivity (P<0.05) in univariate analysis but only season was significantly associated with *Salmonella* positivity in multivariate analysis. Among *Salmonella* isolates, resistance to ampicillin, tetracycline and sulfamethoxazole/trimethoprim was frequently observed. None of the isolates were resistant to ciprofloxacin or norfloxacin. These results show that turtles are commonly infected with *Salmonella* and present a risk of possible transmission to people who come in close contact with them.

Keywords: *Salmonella*, prevalence, risk factors, turtles

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Received: June 3, 2020.
Accepted: January 19, 2021.
doi: https://doi.org/10.14456/tjvm.2021.26

Introduction

Food-borne zoonotic disease caused by *Salmonella* spp. is an important public health problem in Thailand and worldwide. Nontyphoidal *Salmonella* causes 93.8 million human cases of gastroenteritis and 155,000 deaths annually worldwide (Campos *et al*., 2019). In Thailand, the isolation rate of *Salmonella* from children with acute dysentery was approximately 18% during 1998-2000 (Bodhidatta *et al*, 2002). *Salmonella* was also the most common pathogen found in diarrhea patients in Khon Kaen province, Thailand (Vaeteewootacharn *et al*., 2005). Human cases of salmonellosis are often associated with food consumption, but people frequently become infected by handling infected animals such as infected turtles (Mermin *et al*., 2004.). This disease usually causes abdominal cramps and self-limiting gastroenteritis, but more severe symptoms such as septicemia and meningitis can also occur and can sometimes have a fatal outcome, especially in at-risk patients such as infants, the elderly and immunocompromised adults (CDC, 2003, Van Meervenne *et al*., 2009). Salmonellosis is treated with antimicrobial agents, and *Salmonella* has increased antimicrobial resistance. Antimicrobial resistance causes failure of regular therapy and increases the time and cost of treatment (Angkititrakul *et al*., 2005).

Turtles are well-known reservoirs for *Salmonella* and are an important source of human salmonellosis. For example,Van Meervenne *et al*. (2009) reported a severe case of reptile-associated salmonellosis that caused septicemia and meningitis in a 2-month-old baby girl. *Salmonella* Abony was detected in a sample from the baby as well as in the pet turtle's feces in her home. The *Salmonella* isolate was resistant to ampicillin, tetracycline, streptomycin, sulfonamide and cotrimoxazole. (Van Meervenne *et al*., 2009). Likewise, in 2015, Kuroki *et al*. reported a case of reptile-related salmonellosis, and there were 2 cases of 5-year-old boys with gastroenteritis caused by *Salmonella* Poona and *Salmonella* Abony. A turtle, which was suspected as the source of infection, was kept at the patients' home. The pulsed-field gel electrophoresis (PFGE) analysis suggested that the turtle was the source of infection (Kuroki *et al*., 2015). Until the 1970s, pet turtles represented a major source of salmonellosis in the USA, annually contributing an estimated 14 to 23% of salmonellosis cases among children. For this reason, the epidemiology of pathogenic microorganisms in pet turtles and free-living turtles has been studied. In particular, the results from these studies have shown that the incidence of *Salmonella* in pet turtles ranged from 0% to 72.2% and from 0% to 15.4% in free-living turtles (Nakadai *et al*., 2005, Hidalgo-Vila *et al*., 2008, Sánchez-Jiménez *et al*., 2011, Richards *et al*., 2004, Hidalgo-Vila *et al*., 2007). Despite the adverse impact of *Salmonella* on turtles and on their human owner's health, there is no basic information regarding the epidemiology of *Salmonella* in turtles in Thailand. There have been several reports of *Salmonella* infection in pet turtles and salmonellosis in human related to pet turtles in several countries but not yet in Thailand. In Thailand, especially the Turtles Village, Mancha Khiri District, Khon Kaen province, where turtles live naturally and are raised closely to people. These turtles live under the house, wander there, and eat the foliage or vegetables given by the villagers. This raises research questions whether these turtles are a reservoir of *Salmonella* and are carriers of disease to humans. Therefore, the objective of this study was to study the prevalence and risk factors associated with the infection and antimicrobial resistance of *Salmonella* in turtles in Khon Kaen province, northeastern Thailand. This information might be useful for the planning phase of monitoring activities, for prevention and control the spread of salmonella in animals and humans.

Materials and Methods

Ethical approval: The study was approved by the Office of Animal Ethics Committee of Khon Kaen University (no. 30/2555).

Study area: The Turtle Village is housed in Suan Mon, Mancha Khiri District of Khon Kaen province and is approximately 50 kilometers away from Muang District. In this village, the people and turtles live together in harmony; over 1000 of these charming terrestrial reptiles wander there, grazing the foliage or vegetables given by the locals. During the day, the turtles avoid exposure to direct sunlight, but visitors can still easily see them. The turtles in the village are divided into 2 parts. The first part, called the garden turtles, lives within the garden for tourists to conveniently visit them. The other part, called "the village turtles", includes the turtles that live within the village. This study collected samples from turtles in both parts.

Sample collection: A total of 378 turtle cloacal swab samples were randomly collected from turtles in Khon Kaen province of northeastern Thailand between January 2018 and April 2019. The sample size of this study was calculated using the following formula: $n = 4PQ/ L^2$ (Levy and Lemeshowl., 1991). The population prevalence (P) was estimated from our pilot study (approximately 35%). The margin of error was set at 5%. As a result, a total number of the sample size was 364. Each sample or cloacal cotton swab was placed in Carry-Blair media (Oxoid, UK) and kept chilled in an ice box during transport to the laboratory at the Faculty of Veterinary Medicine, Khon Kaen University, for isolation and identification.

Bacterial isolation and identification: Samples were examined for the presence of *Salmonella* by using ISO 6579:2002 (ISO, 2002). In brief, cloacal cotton swab samples from the turtles were placed in 9 ml aliquots of buffered peptone water (BPW; Merck, Germany) and incubated at 37°C for 24 hours. The suspensions were placed on modified semisolid Rappaport medium (MSRV; Merck, Germany) and incubated at 42°C for 24-48 hours, streaked on xylose-lysine-deoxycholate agar (XLD; Merck, Germany) and on Hektoen enteric agar (HE; Merck, Germany), and incubated at 37°C for 24 hours. The plates were inspected after 24 hours, and positive colonies were transferred onto triple sugar iron agar (TSI; Merck, Germany) and on motility indole-lysine agar (MIL;

Merck, Germany). Colonies, either from XLD or HE, that tested positive on both TSI and MIL were identified as having *Salmonella.*

Antimicrobial susceptibility test: The antimicrobial susceptibility test was performed using the disk diffusion method of the Clinical and Laboratory Standards Institute (CLSI, 2010) using BD Sensidiscs (BD Diagnostics, Sparks, MD) with Mueller-Hinton agar plates. The ATCC strains used in this study was *E.coli* ATCC 25922. The concentrations of the antimicrobial agents were as follows: ampicillin (AMP) 10 μg, amoxicillin/clavulanic acid (AMC) 30 μg, chloramphenicol (C) 30 μg, ciprofloxacin (CIP) 5 μg, cefotaxime (CTX) 30 μg, nalidixic acid (NA) 30 μg, norfloxacin (NOR) 10 μg, gentamycin(GN) 10 μg, sulfamethoxazole/trimethoprim (SXT) 25 μg and tetracycline (TE) 30 μg.

Data analysis: The prevalence was calculated as the number of positive samples divided by the number of total samples. Four risk factors were evaluated for their association with the prevalence. These risk factors were the sex of turtles (male or female), breed of the turtle (Peck (*Indotestudo elongata*) or Ka (*Siebenrockiella crassicollis*)), season (summer, rain or winter), and habitat (village or garden). Univariate logistic regression analysis was used to calculate a crude odds ratio (OR) and its 95% confidence interval (CI). In addition, multivariate logistic regression was used to simultaneously account for several risk factors in a single multivariate logistic regression model. This technique yielded an adjusted OR and its 95% CI. A *P*-value less than 0.05 was considered statistically significant. All statistical analyses were conducted using SPSS program (SPSS Statistics for Windows, Version 22.0, Armonk, NY: IBM Corp)

Results

A total of 378 turtle cloacal swabs were tested; 142 samples were positive for *Salmonella*, and the prevalence in the turtles was 37.57%. The results of univariate and multivariate regression analyses are shown in Table 1. In univariate analysis, the statistical results showed that sex, season and habitat were significantly associated with *Salmonella* positivity ($P<0.05$). *Salmonella* positivity in male turtles (41.53%) was higher than that of female turtles (30.99%) with the crude OR = 1.58 (95% CI: 1.02 to 2.47). The prevalence was higher in winter (41.18%) than in summer (25.00%) with the crude OR = 2.10 (95% CI: 1.12 to 3.94), or in rain (39.29%) than in summer with the crude OR = 1.94 (95% CI: 1.02 to 3.70). The prevalence was higher in the turtles living in village (50.67%) than those living in garden (34.32%) with the crude OR = 1.97 (95% CI: 1.18 to 3.28). However, in multivariate analysis that simultaneously accounted for all four factors in the single model, only season was significantly associated with *Salmonella* positivity. That is, the prevalence was higher in winter than in summer with the adjusted OR = 1.92 (95% CI: 1.01 to 3.68). Additionally, there was more *Salmonella* positivity in the Peck turtles (38.27%) than in the Ka turtles (25.00%). The antimicrobial susceptibility tests indicated that the *Salmonella* isolates were most resistant to tetracycline (49.58%), followed by ampicillin (14.29%), sulfamethoxazole/trimethoprim (9.24%), amoxycillin with clavulanic acid (4.20%), gentamycin (4.20%), nalidixic acid (4.20%), cefotaxime (3.36%), and, finally, chloramphenical (1.68%). None of the isolates were resistant to ciprofloxacin or norfloxacin (Figure 1). There were 12 antimicrobial resistance patterns, many of which have a multidrug resistance. It was found that resistance to 2 or more antimicrobial drugs accounted for 84%. However, it was found that 25.2% of all isolated salmonella were susceptible to all 10 antimicrobial drugs tested. There were not differences of antimicrobial resistance patterns between village and garden turtle communities.

Table 1 Prevalence of and risk factor analysis on *Salmonella* isolation from the turtles

Factors/categories	No. positive/No. total (% prevalence)	Crude OR[a]		Adjusted OR[b]	
		Estimate (95% CI)	*P*-value	Estimate (95% CI)	*P*-value
Sex					
Male	98/236 (41.53)	1.58 (1.02, 2.47)	0.041	1.49 (0.94, 2.37)	0.094
Female	44/142 (30.99)	Reference		Reference	
Breed					
Peck	137/358 (38.27)	1.86 (0.66, 5.23)	0.240	1.31 (0.45, 3.81)	0.617
Ka	5/20 (25.00)	Reference		Reference	
Season					
Winter	70/170 (41.18)	2.10 (1.12, 3.94)	0.021	1.92 (1.01, 3.68)	0.048
Rain	55/140 (39.29)	1.94 (1.02, 3.70)	0.044	1.58 (0.81, 3.09)	0.184
Summer	17/68 (25.00)	Reference		Reference	
Habitat					
In village	38/75 (50.67)	1.97 (1.18, 3.28)	0.010	1.61 (0.94, 2.76)	0.084
In garden	104/303 (34.32)	Reference		Reference	

[a]Crude odds ratio was derived from univariate logistic regression.
[b]Adjusted odds ratio was derived from multivariate logistic regression.
Abbreviations: CI, confidence interval; OR, odds ratio.

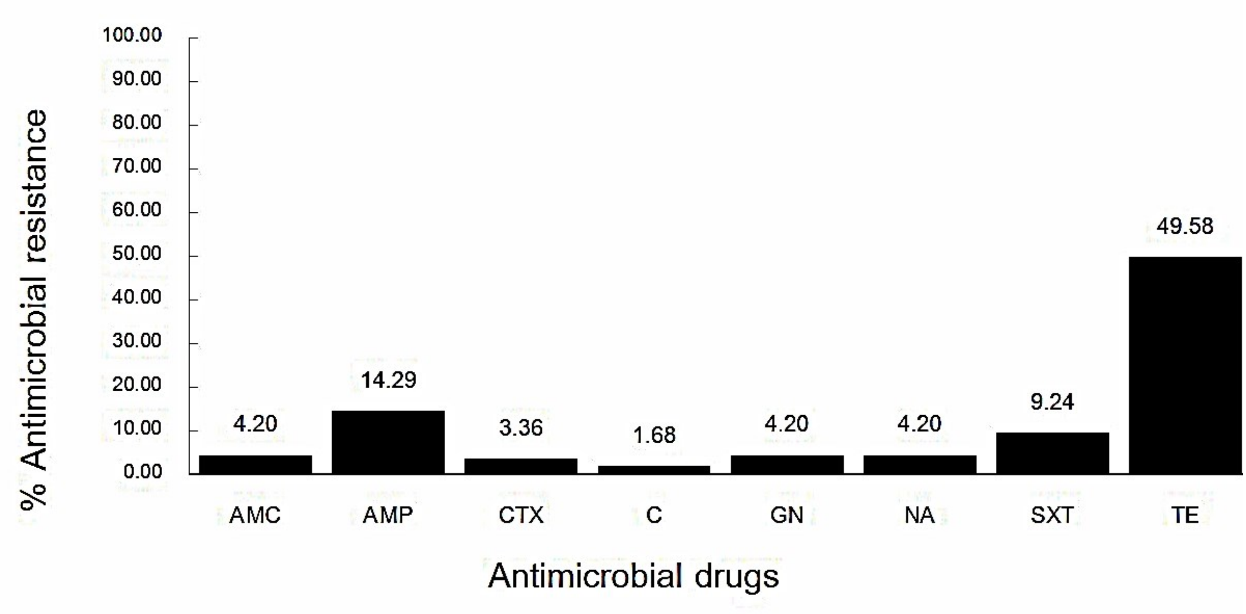

Figure 1 Distribution of antimicrobial resistance in *Salmonella* isolated from turtles. AMP, ampicillin; AMC, amoxycillin/clavulanic acid; C, chloramphenicol; CIP, ciprofloxacin; CTX, cefotaxime; GN, gentamicin; NA, nalidixic acid; NOR, norfloxacin; SXT, sulfamethoxazole-trimethoprim; TE,tetracycline.

Discussion

Different studies have reported the prevalence of *Salmonella* in turtles and its role in reptile-associated salmonellosis in humans. In this study, the prevalence of *Salmonella* spp. in turtles in Khon Kaen province, northeastern Thailand was 37.57% (142/378), and consistent with previous studies in Columbia, where the prevalence of *Salmonella* in turtles at a wildlife protection center was 35% (Sánchez-Jiménez *et al*., 2011). The prevalence we found was lower than that reported in previous studies from pet turtles in Korea (50%) and in turtles from pet stores in Eastern Spain (75%) (Back *et al*., 2016, Martin *et al*., 2016). However, the prevalence we found was higher than that reported in previous studies in free-living turtles from SW Spain (6%) and pet turtles in Taiwan (24.3%) (Hidalgo-Vila *et al*., 2008, Chen *et al*., 2010). Such differences may be due to the variations in the source of the animals sampled, i.e., pet or free-living; additionally, the season that the samples were collected, the method of sampling the feces, i.e., cloacal swab or intestinal content, and the culture methods can all affect the results (Marin *et al*., 2016, Reimschuessel *et al*., 2017).

In risk factor analysis, our study showed different results between univariate and multivariate analysis. In univariate analysis, three factors (sex, season, and habitat) were significantly associated with the prevalence of *Salmonella* infection. However in multivariate analysis, only season was significant. The discrepancy of the results between these two analyses can be explained that the factors may interact with each other in the multivariate model.

Male turtles had a higher prevalence of *Salmonella* infection than female turtles (41.53% versus 30.99%). There was a significant difference (P<0.05) in the prevalence of *Salmonella* infection between the sexes in univariate analysis. No sex difference has been previously reported in terms of the prevalence of *Salmonella* infection in turtles. However, there have been reports of sex differences in other animals. For example, in 2014, it was reported that in Nigerian stray dogs, males had a higher *Salmonella* prevalence (Jajere *et al*., 2014). The reasons for the higher prevalence in male turtles than in female turtles are not clear and might include biological and behavioral determinants (Richards *et al*., 2004, Hidalgo-Vila *et al*., 2007).

Our study showed that the highest prevalence was in the winter, followed by the rain and summer seasons. The greater prevalence estimated in the winter and rain seasons in turtles is consistent with what has been reported in other species, including humans and dogs (Ravel *et al*., 2010, Reimschuessel *et al*., 2017). The reason for this is still not known, but some have speculated that temperature may be a factor. Improper food handling and increased bacterial growth in warm weather were considered potential risk factors that may contribute to the increased caseload. A higher percentage of *Salmonella*-positive dogs was identified at temperatures about 80^{o} F than at other temperatures (Reimschuesse *et al*., 2011). A survey in Canada indicated that the prevalence of human salmonellosis over 4 years (2005 to 2008) increased in June and July (Ravel *et al*., 2010). In one African study, the authors found more cases during early winter; they also found increased rainfall at those times (Venter *et al*., 1988). Other studies in Germany, the Netherlands, England and Wales, Switzerland, Spain, the Czech Republic, Denmark and Australia also indicated a relationship between outdoor temperature and *Salmonella* illness in humans (Kovats *et al*., 2004, Yun *et al*., 2016). The season was statistically significantly associated with *Salmonella* positivity. Our experiments suggest that season is one factor that affected the sampling.

Habitat was found to be significantly associated with *Salmonella* infection in univariate analysis, with the risk of transmission higher in turtles living in the village compared with turtles living in the garden. This finding is consistent with the result of another study in Korea where the prevalence of salmonellosis was shown to be significantly different among provinces (Im *et al*., 2015). This is probably related to the discrepancy in hygienic conditions and the poor hygienic condition of turtles reared in the village. Turtles that live in the village live under the house and travel to various places within the village, thus increasing the opportunity of contact with humans and other animals that are carriers of the disease. Turtles living in the village sustain their life with food scraps from households. This is completely opposite to turtles living in the garden, which provide more hygienic conditions. People entering the garden must soak their feet in an antiseptic, and no other animals enter the garden. There are regular cleaning and sanitizing areas in the garden, and the turtles have regular health checks. The food used to raise the turtles is always selected and cleaned first.

In our study, we did not find a significant difference in *Salmonella* prevalence between animal species. Similarly, a report in the USA indicated that the different turtle species did not impact the presence of *Salmonella* (Geartner *et al*., 2008). However, we found that Peck turtles had a higher *Salmonella* prevalence than Ka turtles. The lower recovery of *Salmonella* from Ka turtles was probably due to an artifact of sampling because almost twenty times more samples from Peck turtles were tested.

Based on the antimicrobial susceptibility test, the highest antimicrobial resistance was to tetracycline followed by ampicillin and sulfamethoxazole/ trimethoprim, which are the most widely used antibiotics for humans and animals. There were no *Salmonella* isolates resistant to ciprofloxacin or norfloxacin. This result is consistent with previous reports in Belgium and Taiwan (Van Meervenne *et al*., 2009; Chen *et al*., 2010). However, the overall resistance percentages studied in Taiwan were lower (for example, antimicrobial resistance to tetracycline (8.7%), ampicillin (7.3%), and sulfamethoxazole/ trimethoprim (6.4%)) than those in this study, which found resistance to tetracycline (49.6%), ampicillin (14.3%), and sulfamethoxazole/trimethoprim (9.2%). The high resistance rates in this study may be due to the widespread application of antibiotics in animals in Thailand. Similarly, these results indicated that no *Salmonella* isolates were resistant to ciprofloxacin or norfloxacin, indicating that fluoroquinolones, which are currently used as the drugs of choice for complicated gastrointestinal infection treatment, can also be used to treat *Salmonella* infection in turtles.

In conclusion, the prevalence of *Salmonella* in turtles is high. This means that there is a high risk of transmission of infection to people in close contact with them, especially in children. Habitat, sex and season were associated with an increased risk of infection in univariate analysis. Therefore, to reduce the possible transmission of *Salmonella* between turtles and humans, hygiene in raising turtles and personal hygiene, especially hand washing after contact with the turtles, are important. In addition, further studies should focus on serotyping and molecular typing to find an association between hosts, environments and time periods as well as the role of turtles on human transmission.

Acknowledgements

The authors would like to thank the Faculty of Veterinary Medicine at Khon Kaen University for the support of a scholarship and Mancha Khiri District Livestock Office and Khon Kaen Provincial Livestock Development Office for assistance in collecting specimens from the turtles.

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