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Serovars and Antimicrobial Resistance of *Salmonella* Isolated from Free-living Turtles in the Turtle Village, Northeastern Thailand: One Health Perspective

Nawarat Rattanadilok na Phuket [2], Juthamas Buahom [2], Ketmanee Senaphan [1], Peerapol Sukon [1], Sarawut Sringam [1], Sunpetch Angkititrakul [1] and Patchanee Sringam [1]*

[1]Faculty of Veterinary Medicine, Khon Kaen University, Thailand, 40002
[2]Regional Medical Sciences Center 4, Saraburi, Thailand, 18180
***Corresponding author:** spatcha@kku.ac.th

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ABSTRACT

Turtles are recognized as reservoirs for a diverse array of *Salmonella* serovars, and understanding their role as potential sources of *Salmonella* outbreaks in humans is crucial. In this cross-sectional study, we aimed to identify the serovars and antimicrobial resistance profiles of *Salmonella* infections in free-living turtles. Out of 325 cloacal swabs obtained from these turtles, 25 distinct *Salmonella* serovars were identified, with the ten most prevalent serovars being *S.* Derby, *S.* Enteritidis, *S.* Weltevreden, *S.* Stanley, *S.* Bredeney, *S.* Cerro, *S.* Brunei, *S.* Mountpleasant, *S.* Risen, and *S.* Agona. Ampicillin, tetracycline, and sulphamethoxazole-trimethoprim resistance was frequently observed among the isolated *Salmonella* strains. However, none of the isolates displayed resistance to ciprofloxacin, enrofloxacin, gentamicin, amikacin, amoxicillin-clavulanate, cefotaxime, cefepime, and meropenem. Importantly, the identified serovars, such as S. Enteritidis, S. Weltevreden, S. Stanley, S. Bredeney, S. Risen, and *S.* Agona, are consistently among the ten most frequently isolated serovars in patients in Thailand. The detection of *Salmonella* in free-living turtles confirms that these species are potential carriers of the bacteria. Additionally, the observed concurrence in serovars and antimicrobial resistance patterns between turtles and humans suggests the circulation of multidrug-resistant *Salmonella* serovars across diverse hosts within the study area.

Key words: Antimicrobial resistance, *Salmonella*, Serovars, Turtles

INTRODUCTION

Salmonellosis represents a significant global public health concern, with non-typhoidal *Salmonella* responsible for 93.8 million cases of gastroenteritis and 155,000 deaths annually worldwide (Campos et al. 2019; Collier et al. 2021). In Thailand, an estimated 120,000 human cases occur each year due to *Salmonella* infections. (Kongsanan et al. 2021; Saechue et al. 2024). Furthermore, *Salmonella* has emerged as the predominant pathogen in cases of diarrhea in Khon Kaen province, Thailand (Vaeteewootacharn et al. 2005). Belonging to the gram-negative bacterial category, the *Salmonella* genus comprises two species: *S. enterica* and *S. bongori*. *S. enterica* further subdivides into six subspecies—*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*. While the digestive tracts of warm-blooded animals serve as the primary reservoir for *Salmonella* subspecies *enterica*, other subspecies are predominantly found in cold-blooded animals. Reptiles, such as turtles, can simultaneously harbor multiple subspecies and serovars (Pedersen et al. 2009; Gay et al. 2014). Human salmonellosis cases are frequently linked to contaminated food; however, infections can also occur through contact with infected animals, particularly turtles (Mermin et al. 2004; Rahman et al. 2021). Although the illness commonly presents with abdominal cramps and self-resolving gastroenteritis, it can also result in more serious conditions like septicemia and meningitis, posing a higher risk to vulnerable populations such as infants, the elderly, or immunocompromised adults (Sodagari et al. 2020). The treatment for salmonellosis involves the use of antimicrobial agents, but the rise in antimicrobial resistance among *Salmonella* strains has become a significant concern. This resistance poses a challenge by leading to the failure of conventional therapeutic approaches, thereby prolonging the duration and escalating the costs associated with treatment. The increasing antimicrobial resistance in

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Salmonella underscores the importance of vigilant surveillance, judicious antimicrobial use and the development of alternative therapeutic strategies to effectively manage and mitigate the impact of this public health issue (Talukder et al. 2023).

Turtles are well-documented as sources of Salmonella and a notable vector for human salmonellosis. A significant case was reported by Van Meervenne et al. (2009) detailing a serious instance of reptile-associated salmonellosis that led to septicemia and meningitis in a 2-month-old infant. The identified *Salmonella* isolate, *S.* Abony, was found in both the baby's sample and the feces of a pet turtle in her home. This particular isolate exhibited resistance to ampicillin, tetracycline, streptomycin, sulfonamide, and co-trimoxazole (Van Meervenne et al. 2009). Similarly, Kuroki et al. (2015) documented two cases of gastroenteritis in 5-year-old boys attributed to *S.* Poona and *S.* Abony, with turtles being the potential origin of the infection. PFGE analysis suggested that a tortoise kept at the patient's home was indeed the origin of the infection. The isolates were sensitive to all antimicrobials tested (Kuroki et al. 2015). In 2012, the United States experienced eight multistate outbreaks resulting in 473 reported illnesses. The identified serovars involved in these outbreaks were *S.* Sandiego, *S.* Poona, *S.* Pomona, *S.* Newport, and *S.* Typhimurium. Among these, the outbreak caused by *S.* Pomona accounted for the highest number of cases and hospitalizations. The analysis of these outbreaks revealed that 8% of the affected individuals had been exposed to small turtles. The transmission of *Salmonella* to humans in the 2012 outbreaks was linked to several high-risk behaviors. These behaviors included activities such as kissing turtles, cleaning turtle habitats in kitchen sinks, and allowing turtles access to kitchen countertops and other areas where food is prepared and consumed (Bosch et al. 2016).

Despite the extensive documentation of *Salmonella's* impact on turtles and its potential consequences for human health, there is a lack of fundamental information about the epidemiology of *Salmonella* in turtles in Thailand. Recently we reported the prevalence and risk factors of *Salmonella* infections in the Turtles Village, Khon Kaen, Thailand (Sringam et al. 2021). This report lacked detail information about the serovars. In the Turtle Village of Mancha Khiri District, Khon Kaen Province, turtles coexist naturally with humans, living under houses and feeding on foliage or vegetables provided by villagers. This close interaction prompts questions about whether these turtles serve as reservoirs for *Salmonella* and pose a risk of disease transmission to humans. Thus, the aim of this study was to investigate the serovars and antimicrobial resistance patterns of *Salmonella* isolated from turtles in the Turtle Village, located in Khon Kaen province, northeastern Thailand. The findings from this research may prove instrumental in the planning and implementation of monitoring activities, as well as in the development of strategies for preventing and controlling the transmission of *Salmonella* among animals and humans.

MATERIALS AND METHODS

Ethical approval

The study received approval from the Office of Animal Ethics Committee at Khon Kaen University, with Record No. IACUC-KKU-52/64.

Study area

The Turtle Village is situated in Suan Mon, Mancha Khiri District of Khon Kaen Province, approximately 50 kilometers from Muang District. In this village, a harmonious coexistence exists between the local residents and over 1000 terrestrial reptiles, mainly turtles. These charming creatures freely roam the area, grazing on foliage or vegetables provided by the villagers. While they tend to avoid direct sunlight during the day, they remain easily observable in their natural habitat.

Sample collection

From January 2018 to December 2019, 325 cloacal swabs were collected from The Turtle Village in Khon Kaen Province, Northeast Thailand. Each sample was carefully collected and placed in Carry-Blair media (Oxoid, UK) to preserve the integrity of the specimens. To maintain optimal conditions during transportation, the samples were stored in an icebox and promptly delivered to the research lab at the Faculty of Veterinary Medicine, Khon Kaen University, for subsequent isolation and identification processes.

Bacterial isolation and identification

To detect the presence of *Salmonella*, samples underwent examination following the ISO 6579:2002 standard. In summary, Cloacal swabs from turtles were placed in 9ml aliquots of buffered peptone water (BPW; Merck, Germany) and incubated at 37°C for 24 hours. The resulting dispersions were then transferred to modified semisolid Rappaport medium (MSRV; Merck) and incubated at 42°C for 24-48 hours. Following this, the samples were streaked onto xylose-lysine-deoxycholate agar (XLD; Merck) and Hektoen enteric agar (HE; Merck) and incubated at 37°C for another 24 hours. After this incubation period, the plates were examined, and colonies presumed positive were moved to triple sugar-iron agar (TSI; Merck) and motility indole-lysine agar (MIL; Merck). Colonies that yielded positive results on both TSI and MIL, derived from either XLD or HE, were confirmed as *Salmonella*. For further characterization, a slide agglutination test with O-antigen (S&A Reagents Lab, Bangkok, Thailand) based on the Kauffman-White Scheme was employed to group *Salmonella* isolates. Subsequently, the isolates were sent to the laboratory at the Department of Medical Science, Ministry of Public Health, Thailand, for additional serovar identification using slide agglutination based on the Kaufman-White scheme, utilizing commercially available antisera (S&A Reagents Lab).

Antimicrobial susceptibility test

The antimicrobial susceptibility test was conducted utilizing the disk diffusion method following the guidelines of the Clinical and Laboratory Standards Institute with Mueller-Hinton agar plates used for the testing. All *Salmonella* isolates underwent susceptibility testing against 12 compounds representing nine antimicrobial classes, including: penicillins (ampicillin), beta-lactams (amoxicillin-clavulanate), cephalosporins (cefotaxime, cefepime), fluoroquinolones (ciprofloxacin, enrofloxacin), phenicol's (chloramphenicol), aminoglycosides (gentamycin, amikacin), folate pathway inhibitors

(sulfamethoxazole-trimethoprim), tetracyclines (tetracycline), and carbapenems (meropenem). *Salmonella* that was resistant to three or more antimicrobial drugs was classified as multidrug-antimicrobial resistance (MDR).

RESULTS

Prevalence and serovars of *Salmonella*

Out of the turtle cloaca swabs collected, 37.54% (n = 122) tested positive for *Salmonella*, revealing the presence of 25 different *Salmonella* serovars. The predominant serovars observed were as follows: *S.* Derby (16), *S.* Enteritidis (14), *S.* Weltevreden (12), *S.* Stanley (10), *S.* Bredeney (9), *S.* Cerro (7), *S.* Brunei (7), *S.* Mountpleasant (7), *S.* Risen (6), *S.* Agona (5), *S.* Hvittingfoss (5), *S.* Poona (4), *S.* Bareilly (3). Other serovars such as *S.* Abony (2), *S.* Corvallis (2), *S.* Saintpaul (2), *S.* Newport (2), *S.* Itami (2), *S.* Urbana (1), *S.* Diarizonae (1), *S.* Powell (1), *S.* Welikade (1), *S.* Singapore (1), *S.* Eastbourne (1), *S.* Rubislaw (1) were also identified. (Table 1)

The antimicrobial susceptibility

The antimicrobial susceptibility test showed that *Salmonella* isolates from turtles were resistant to ampicillin (15.57%) followed by tetracycline (11.48%), sulphamethoxazole-trimethoprim (11.48%), and finally, chloramphenicol (0.82%). All isolates were susceptible to ciprofloxacin, enrofloxacin, gentamicin, amikacin, amoxicillin-clavulanate, cefotaxime, cefepime and meropenem. Among the *Salmonella* serovars, it was observed that *S.* Derby, *S.* Enteritidis, *S.* Risen and *S.* Agona were resistant to a wide range of antimicrobials tested, as compared with resistance exhibited by the other serovars (Table 2).

Table 1: Serovars of *Salmonella* isolated from free-living turtles

Sample type (n)	Group	Serovar (n, %)
Turtles (122)	B	Derby (16, 13.11%), Stanley (10, 8.20%), Bredeney (9, 7.38%), Agona (5, 4.1%), Saintpaul (2, 1.64%), Abony (2, 1.64%)
	C	Rissen (6, 4.92%), Brunei (7, 5.74%), Bareilly (3, 2.46%), Corvallis (2, 1.64%), Newport (2, 1.64%), Singapore (1, 0.82%)
	D	Enteritidis (14, 11.48%), Itami (2, 1.64%), Powell (1, 0.82%), Eastbourne (1, 0.82%)
	E	Weltevreden (12, 9.84%)
	F	Rubislaw (1, 0.82%)
	G	Poona (4, 3.28%)
	I	Hvittingfoss (5, 4.10%), Welikade (1, 0.82%)
	K	Cerro (7, 5.74%)
	N	Urbana (1, 0.82%)
	X	Mountpleasant (7, 5.74%)
	Z	Diarizonae (1, 0.82%)

Table 2: Antimicrobial resistance of *Salmonella* isolated from free-living turtles by *Salmonella* serovars

Serovar (n)	No. (%) of isolates resistant to various antimicrobials											
	ENR	CN	TE	SXT	AMP	AMC	CTX	CIP	FEP	MEM	AK	C
Turtles												
S. Derby (16)			2 (12.50)	3 (18.75)	2 (12.50)							
S. Enteritidis (14)			1 (7.14)		1 (7.14)							
S. Weltevreden (12)												
S. Stanley (10)					3 (30)							
S. Bredeney (9)												1 (11.10)
S. Cerro (7)												
S. Brunei (7)				1 (14.29)								
S. Mountpleasant (7)												
S. Rissen (6)			6 (100)	5 (83.33)	6 (100)							
S. Agona (5)			5 (100)	5 (100)	5 (100)							
S. Hvittingfoss (5)												
S. Poona (4)												
S. Bareilly (3)												
S. Corvallis (2)												
S. Saintpaul (2)					2 (100)							
S. Newport (2)												
S. Abony (2)												
S. Itami (2)												
S. Urbana (1)												
S. Diarizonae (1)												
S. Powell (1)												
S. Welikade (1)												
S. Singapore (1)												
S. Eastbourne (1)												
S. Rubislaw (1)												
Total (%)			14 (11.48)	14 (11.48)	19 (15.57)	0	0	0	0	0	0	1 (0.82)

AMP: ampicillin; AMC: amoxicillin-clavulanate; C: chloramphenicol; CIP: ciprofloxacin; ENR: enrofloxacin; CTX: cefotaxime; CN: gentamicin; AK: amikacin; FEP: cefepime; SXT: sulfamethoxazole-trimethoprim; TE: tetracycline; MEM: meropenem

DISCUSSION

The current investigation revealed a broad spectrum encompassing 25 distinct *Salmonella* serovars. All isolated *Salmonella* strains were identified as belonging to the species enterica, with an overwhelming majority (96%, 24/25) falling under the subspecies *enterica.* This subspecies is predominantly associated with warm-blooded vertebrates, and nearly all instances of *Salmonella* infections in humans are attributed to strains within subspecies *enterica* (Geue and Löschner 2002; Gay et al. 2014). Conversely, only a solitary serovar (4%, 1/25) belonged to the subspecies *diarizonae*, primarily linked to cold-blooded vertebrates (Schröter et al. 2004; Kuroki et al. 2013). Nonetheless, there has been a gradual emergence of reported cases involving *S. enterica* subsp. *diarizonae* in humans, exemplified by an instance of otitis in a 16-year-old immunocompromised boy following lake bathing (Gavrilovici et al. 2017).

The present study underscored a notable diversity in *Salmonella* serovars, with *S.* Derby, *S.* Enteritidis, *S.* Weltevreden, *S.* Rissen, *S.* Stanley, *S.* Bredeney, *S.* Cerro, *S.* Brunei, *S.* Mountpleasant, *S.* Hvittingfoss, *S.* Saintpaul, *S.* Newport, *S.* Poona, *S.* Bareilly, and *S.* Agona being prominent among the isolates. This finding aligns with prior research conducted in different regions. For instance, *S.* Bredeney, *S.* Saintpaul, *S.* Newport, *S.* Poona, and *S.* Bareilly were reported in turtles from Spain, USA, Germany and Taiwan (Geue and Löschner 2002; Gaertner et al. 2008; Hidalgo-Vila et al. 2008; Chen et al. 2010). Zając et al. (2021) documented *S.* Enteritidis and *S.* Typhimurium as the predominant serovars recovered from turtles in Poland, while Sánchez-Jiménez et al. (2011) identified *S.* Enteritidis as the most prevalent serovar in turtles from Colombia.

Several serovars identified in this study have previously been isolated from diverse sources in Thailand, including pets, food-producing animals, and animal products. Notably, the most prevalent serovar, *S.* Derby, along with others such as *S.* Rissen, *S.* Weltevreden, *S.* Stanley, *S.* Saintpaul, *S.* Newport, *S.* Bareilly, and *S.* Enteritidis, have been documented in swine or poultry and their associated products (Sanguankiat et al. 2010; Kongsanan et al. 2021). Concurrently, serovars like *S.* Weltevreden, *S.* Stanley, *S.* Hvittingfoss, and *S.* Eastbourne have been previously reported in dogs and cats (Polpakdee et al. 2012; Srisanga et al. 2017). Indeed, some of the *Salmonella* serovars isolated in the current study align with those reported in various sources, including humans (Sringam et al. 2017; Wu et al. 2020). Furthermore, the serovars identified in free-living turtles in our investigation, such as *S.* Weltevreden, *S.* Enteritidis, *S.* Bredeney, and *S.* Agona, notably feature among the ten most common serovars isolated from human patients in Thailand, as reported by the National Institute of Health. Remarkably, an essential observation from our preliminary study conducted among residents of this village is the identification of four *Salmonella* serovars—namely, *S.* Rissen, *S.* Derby, *S.* Agona, and *S.* Lexington. These exact serovars were also detected in the free-living turtle samples obtained during the study. This suggests a potential link between *Salmonella* serovars in turtles and humans in the study area.

While Thailand has not reported any cases of turtle-associated salmonellosis (TAS), certain serovars identified in our study have been previously associated with such cases in humans in other countries. Notably, instances of pet turtle-associated salmonellosis have been documented in 12 states in the USA, with a predilection for occurrences in children. *S.* Poona was the predominant infectious agent in these cases (Bosch et al. 2016). Similarly, pet turtle-associated *Salmonella* has been implicated in causing gastroenteritis in three infants in Chile, with *S.* Montevideo, *S.* Newport, and *S.* Pomona identified as the causative serovars (Braun et al. 2015). In Japan, two cases of human salmonellosis resulting from exposure to pet turtles were reported, with *S.* Poona and *S.* Abony identified as the causative agents (Kuroki et al. 2013). The detection of *S.* Enteritidis and *S.* Weltevreden in free-living turtles assumes significance due to their pathogenic nature, particularly *S.* Enteritidis, which is known for its high pathogenicity and frequent association with severe infections in human patients in Thailand (Whistler et al. 2018; Buddhasiri et al. 2023). This finding emphasizes the relevance of monitoring and understanding the potential transmission dynamics of these highly pathogenic *Salmonella* serovars from free-living turtles to humans, despite the absence of reported TAS cases in Thailand.

In accordance with the results of the antimicrobial susceptibility test, *Salmonella* isolates obtained from free-living turtles exhibited resistance rates of 15.57% to ampicillin, 11.48% to tetracycline, and 11.48% to sulphamethoxazole-trimethoprim. Notably, these findings align with similar observations in Poland and Taiwan. Comparatively, the resistance percentages in Taiwan and Poland were notably lower than those observed in present study. Specifically, resistance rates to ampicillin, tetracycline, and sulphamethoxazole-trimethoprim were 1.5, 2.6, and 2.4% in Poland (Zając et al. 2021) and 7.3, 8.4, and 6.4% in Taiwan (Chen et al. 2010). One plausible explanation for the higher antimicrobial resistance observed in our study could be attributed to the unique habitat of the turtles. In the studied village, a cohabitation exists between the local community and the turtles, with the latter residing beneath houses and traversing various locations within the village. Their sustenance relies on food scraps from households, exposing them to potential antimicrobial-resistant *Salmonella* from diverse sources, including animal products, contaminated vegetables, water, and human sewage.

The high resistance rates to ampicillin, tetracycline, and sulphamethoxazole-trimethoprim observed in this study are consistent with findings reported in various sources, including food-producing animals, retail meat, and humans in Thailand and other countries (Sirichote et al. 2010; Kumpapong et al. 2013; Wasyl et al. 2014; Sringam et al. 2017). Notably, Vico et al. (2011) documented a predominant resistance pattern, including ampicillin, chloramphenicol, streptomycin, sulphamethoxazole-trimethoprim, and tetracycline, in swine farms in Spain. Similarly, Lim et al. (2009) identified a high frequency of resistance to tetracycline, streptomycin, and sulphamethoxazole-trimethoprim in healthy and diarrheal swine in South Korea. Moreover, these resistant serovars were isolated from chicken meat, with prevalent resistance to ampicillin, chloramphenicol, streptomycin,

sulphamethoxazole-trimethoprim, and tetracycline reported in Vietnam (Thai et al. 2012). The pronounced resistance rates to these antimicrobials could be attributed to their widespread use in animals within this country. Notably, A noteworthy finding from our preliminary study among residents of this village is the identification of resistance patterns in *Salmonella* isolates from humans. Specifically, these isolates demonstrated resistance to tetracycline, ampicillin, sulphamethoxazole-trimethoprim, chloramphenicol, amoxicillin with clavulanic acid, and cefotaxime at rates of 77.78, 44.44, 33.33, 22.22, 11.11, and 11.11%, respectively. This observed higher resistance rate in humans compared to turtles aligns with the findings of Wasyl et al. (2014) supporting the notion that the antimicrobial resistance of *Salmonella* in turtles remains comparatively low when juxtaposed with food-producing animals and humans.

Our investigation revealed variations in resistance rates based on *Salmonella* serovars. Resistant isolates were predominantly associated with the subspecies *S. enterica* subsp. *enterica*, whereas *S. enterica* subsp. *diarizonae* exhibited susceptibility to all tested antimicrobials. Notably, *S.* Derby, *S.* Enteritidis, *S.* Rissen, and *S.* Agona exhibited multidrug resistance, manifesting resistance to ampicillin, chloramphenicol, sulphamethoxazole-trimethoprim and tetracycline—traditional antimicrobial agents clinically utilized in both human and animal settings. This resistance pattern may be attributed to the fact that these serovars are commonly isolated from animal products in Thailand. It is plausible that they have been exposed to various antimicrobials, leading to the development of resistance. Additionally, these serovars might have gained access to turtles through direct and indirect contact, further contributing to their resistance profile. However, it is noteworthy that all identified serovars demonstrated susceptibility to ciprofloxacin, enrofloxacin, gentamicin, amikacin, amoxicillin-clavulanate, cefotaxime, cefepime, and meropenem in our study. This suggests that these antimicrobials, currently recommended as the preferred treatment for complicated gastrointestinal infections may also be effective for treating *Salmonella* infections in turtles in the studied area.

Conclusion

This study underscores the significant role of free-living turtles as a reservoir for a diverse array of *Salmonella* serovars, thereby posing a potential threat to human health. The findings suggest a plausible risk of multidrug-resistant *Salmonella* infections in human populations in close proximity to these turtles. Consequently, it is imperative to direct attention towards the implementation of effective decontamination measures within the environment. The establishment of dedicated disinfection protocols is essential not only for mitigating the risk of turtle-associated salmonellosis but also for preventing the transmission of other zoonotic bacteria commonly found in turtles. Public awareness campaigns are pivotal in promoting preventive measures against zoonotic diseases associated with turtles and encouraging adherence to good hygienic practices. By addressing these concerns, public health can be safeguarded, and potential health risks associated with turtle-human interactions can be effectively minimized.

Conflict of interest

The authors declare no conflict of interest.

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Authors' contributions

Conceptualization, Visualization, Supervision and Methodology; SA, PS*. Methodology and Investigation; NR, JB, SS. Writing, Review and Editing; KS, PS and PS*. All authors have reviewed and approved the final version of the manuscript for publication.

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