

The molecular Characterization of *Salmonella* from Cattle feces in Chiang Mai, Thailand, its antibiotic susceptibility, and genetic relatedness to clinical isolates

Abstract

Aims: *Salmonella* is the most common pathogenic bacteria causing food-borne and zoonotic diseases in humans, and food-producing animals are considered the primary source of Salmonellosis. Cattle slaughtered for human consumption have been implicated as a source of human infection with antimicrobial-resistant *Salmonella*. The purpose of this study was to determine the prevalence of *Salmonella* in the feces of apparently healthy slaughtered cattle and characterize the circulating non-typhoidal *Salmonella* (NTS) serovars in cattle in Chiang Mai, Thailand as well as determine their antibiotic resistance profiles and genetic relatedness to human isolates.

Methodology: A cross-sectional study was conducted from June to September 2017 at the municipal abattoir in Chiang Mai, Thailand. One hundred and fifty fecal samples were collected from apparently healthy slaughtered cattle for *Salmonella* isolation. One hundred and ten blood samples were also collected from febrile patients at the Chiang Mai provincial hospital prior to any antimicrobial treatment. Circulating *Salmonella* serovars were identified using standard culture techniques and DNA sequencing, antimicrobial sensitivity was tested using a disc diffusion method, and 16S rRNA were used to determine phylogenetic relationships.

Results: The results showed that the prevalence of *Salmonella* from cattle was 22.6%. According to the NCBI blast, three serovars were identified: *S. Typhimurium* (50.0%), *S. Enteritidis* (41.2%), and *S. Weltevreden* (11.8%); these serovars formed distinct clades on the phylogenetic tree. *S. Typhimurium* and *S. Enteritidis* isolates from cattle were closely related to human isolates. All isolates from cattle were susceptible to 10 selected commonly prescribed antimicrobials.

Conclusion: Our study revealed a high prevalence of *Salmonella* in healthy slaughtered cattle in Chiang Mai. *S. Typhimurium* and *S. Enteritidis* isolated from cattle and humans were closely related. The finding suggested that slaughtered cattle is a source of *Salmonella* infection in humans. This poses a major public health threat since slaughtered cattle are a potential source of food-borne salmonellosis. Therefore, the local administration should establish a system for the routine surveillance of infectious sources.

Keywords: *Salmonella*, Antimicrobial susceptibility testing, DNA sequencing, Phylogenetic tree analysis

1. INTRODUCTION

Salmonella is an enteric gram-negative bacterium and a major global food-borne pathogen. It can cause a variety of clinical manifestations from mild gastroenteritis to bacteremia and typhoid fever. The global burden of non-typhoidal *Salmonella* gastroenteritis has been estimated to be 93.8 million cases each year and causes approximately 155,000 deaths [1]. Salmonellosis is an important public health problem in both developing and developed countries, although incidence rates vary by country [2]. In Africa, non-typhoidal salmonellosis (NTS) has been consistently reported as the leading cause of bacteremia among immune-compromised people, and in Thailand, NTS is a common bloodstream isolate in febrile patients [3,4]. However, the sources and transmission routes of *Salmonella* in

developing countries are poorly understood due to the lack of coordinated national epidemiological surveillance systems [5]. Previous studies have found that food-producing animals such as cattle, poultry, and swine are the primary sources of salmonellosis [6]. The pathogen is mainly disseminated by animal trading and uncooked animal food products [7]. Also, fecal waste from infected animals and humans are important sources of bacterial contamination in the environment and the food chain [8]. In

humans, *Salmonella* infections can lead to severe, invasive, and focal infections [9]. Treatment with antimicrobials is crucial for the proper management of bacterial infections, especially in severe human salmonellosis, although the overuse of antimicrobials in treating bacterial infections in humans and animals has led to antimicrobial resistance [10]. Antimicrobial-resistant *Salmonella* is increasing due to the use of antimicrobial agents in food animals at sub-therapeutic levels or prophylactic doses which may promote the on-farm selection of antimicrobial-resistant strains [11,12]. Thus, food-producing animals are considered one of the main routes of transmission of antimicrobial-resistant bacteria, especially pathogenic bacteria, between animal and human populations [13]. Due to the lack of routine surveillance programs in Thailand, as in most developing countries, information on the prevalence of *Salmonella* in food-producing animals is limited. Critically, little is known about the prevalence of antimicrobial-resistant *Salmonella* from food-producing animals and the genetic relatedness of isolates from animal and human salmonellosis. Cattle have been implicated as a source of human infection with antimicrobial-resistant *Salmonella* through direct contact with livestock. Improper conditions in the slaughtering process have also been considered a factor enhancing *Salmonella*'s antimicrobial resistance [14,15]. The purpose of this study was to determine the prevalence, serotype, antibiotic susceptibilities, and genetic relatedness to clinical isolates of *Salmonella* from the feces of healthy slaughtered cattle in Chiang Mai, Thailand.

2. METHOD

2.1 Sample Collection

One hundred and fifty fecal samples collected between June and September 2017 from 150 healthy cattle were obtained from Chiang Mai's municipal slaughterhouse complex. One fecal sample was collected from each cow, and approximately 5 to 10 samples were taken each day. Cattle slaughtered at the Chiang Mai abattoir were bred at various farms across the city. Approximately 50 g of fresh samples were collected in a sterile, airtight stool cup directly from the rectum immediately after the animals were slaughtered. The sample was determined using a prevalence rate of 3.0% from previous studies, with a 99% confidence interval covering an error of 5 percentage points from the true value; the following formula was employed [16,17]:

$$n = \frac{Z^2 P(1 - P)}{d^2}$$

Where n = sample size, Z = Z statistic for a level of confidence, P = expected prevalence or proportion, and d = precision. Based on the above formula, the calculated sample size was 78. One hundred and fifty cattle were randomly selected for this study.

Prior to treatment with antimicrobial agents, 110 blood samples from patients with a fever (axillary temperature > 37°C) or clinical evidence of sepsis admitted to the Chiang Mai provincial hospital were collected between June and September 2017. The samples were then transported in an icebox, cultured within 24 hr and analyzed at the Regional Medical Sciences Center of Chiang Mai's Department of Medical Sciences, Ministry of Public Health, Thailand.

2.2 Isolation of *Salmonella* from Cattle

Isolation was conducted according to the ISO 6579/AMD 1:2007 standard protocol [18] and involved three steps:

- 1) One g of the fecal sample was pre-enriched with 9 ml of buffered peptone water (BPW) (Oxoid, England) and incubated for 24 hr at 37°C.
- 2) A portion (0.1 ml) of the pre-enriched cultured was transferred to 10 ml of selenite cystine (SC) broth (Himedia, India), and another 0.1 ml portion was transferred to 10 ml of

88 Rappaport and Vassiliadis (RV) broth (Merck, Germany); they were incubated at 37°C and
89 42°C for 24 hr, respectively.

90 3) A sample from the selective enrichment media was streaked onto xylose lysine
91 deoxycholate (XLD) (Oxoid, England) agar and incubated at 37°C for 24 hr; the incubation
92 period was prolonged to 48 hr for those that did not show any growth during the initial 24-
93 hr incubation period. Characteristic *Salmonella* colonies, having a slightly transparent zone
94 of reddish color and a black center, were subcultured on nutrient agar (Oxoid, England)
95 and confirmed biochemically using triple sugar iron agar (TSI) (Oxoid, England),
96 Christensen's urea agar (Oxoid, England), lysine iron agar (LIA) (Oxoid, England), Voges
97 Proskauer (VP), methyl red (MR) (Micromaster, India), and Indole tests (Becton Dickinson,
98 USA) [19].

99 **2.3 Isolation of *Salmonella* from Humans**

100 Blood samples of 5 to 10 ml and 2 to 5 ml were collected from adults and children, respectively.
101 Bacteria were isolated from each blood sample following standard procedures and using a BacT/Alert
102 3D automated system (BioMerieux, Inc., Durham, N.C.) according to the manufacturer's instructions.
103 All isolates were identified using standard diagnostic techniques [20].

104 **2.4 Identification of *Salmonella***

105 **2.4.1 Extraction of genomic DNA**

106 Pure colonies of *Salmonella* were cultured in a nutrient broth (Oxoid,UK) and used for DNA extraction
107 with a Nucleospin Tissue Kit (Machrey-Nagel, Germany) according to the manufacturer's instructions.

108 **2.4.2 PCR amplification**

109 A polymerase chain reaction (PCR) procedure was performed as described previously [21], except 25
110 µl of Quick Taq HS DyeMix (TOYOBO, Japan), 18 µl of nuclease free water, 1 µl of each primer, and
111 5 µl of extracted DNA were used. Forward and reverse PCR primers were synthesized by Bio Basic
112 Inc. in Canada.

113 **2.4.3 DNA sequencing**

114 The PCR products obtained were extracted using a QIAquick Gel Extraction Kit (Qiagen, USA). Thirty
115 microliters of purified DNA from each sample was sequenced by Bio Basic Inc. in Canada.

116 **2.4.4 Phylogenetic analysis**

117 The 16S rRNA sequences obtained were compared with known 16S rRNA sequences in the National
118 Center for Biotechnology Information's (NCBI) database using the basic local alignment search tool
119 (BLASTn) [22]. Identification of the sequences at both the genus and the species level was defined as
120 a 16S rRNA sequence similarity of ≥ 99% with that of the prototype strain sequence in GenBank. The
121 obtained sequences and reference sequences derived from Genbank were aligned using Clustal
122 Omega. The probability of their placement on the phylogenetic tree was determined using MEGA
123 software version 6.0 [23].

124 **2.5 *Salmonella* Antimicrobial Susceptibility Tests**

125 Antibiotic susceptibility testing was performed using the Kirby-Bauer disk diffusion method according
126 to the Clinical and Laboratory Standards Institute's (CLSI) guidelines [24]. A total of 10 selected
127 antibiotic disks containing commonly used antibiotics were used: amoxicillin/clavulanic acid (30 µg),
128 ceftazidime (30 µg), ceftriaxone (30 µg), ciprofloxacin (30 µg), colistin (10 µg), imipenem (30 µg),
129 sulbactam/cefoperazone (30 µg), ertapenem (10 µg), piperacillin/tazobactam (10 µg), and tigecycline

(15 µg) (Oxoid, England). Zones of inhibitions were measured to determine whether the bacteria were susceptible, intermediate, or resistant when compared to CLSI critical points.

2.6 Data Analysis

Antimicrobial resistance bacteria data and Phylogenetic analysis tree were described and presented in table 1 and figure 2 respectively. The 16S rRNA sequences of *Salmonella* isolates from human and cattle feces were compared with known 16S rRNA sequences in the National Center for Biotechnology Information's (NCBI) database using the BLASTn algorithm obtained from <https://blast.ncbi.nlm.nih.gov/Blast.cgi>. Identification of the sequences at both the genus and the species level was defined as a 16S rRNA sequence similarity of $\geq 99\%$ with that of the prototype strain sequence in GenBank. The probability of their placement on the phylogenetic tree was determined. The topological robustness of the trees was evaluated by a bootstrap analysis involving 10,000 replications.

3. RESULTS AND DISCUSSION

3.1 *Salmonella* Prevalence and Serotypes

Salmonella was isolated from 34 (22.6%) of the total 150 fecal samples of healthy slaughtered cattle. The bacteria were also isolated from 7 of the 110 blood samples obtained from febrile patients. The PCR product obtained was 572 bp (Fig. 1). BLASTn identified 3 serovars of *Salmonella*: 17 (50%) of the 34 cattle samples and 5 (71.4%) of the 7 human samples were identified as *S. Typhimurium*; 13 (41.2%) of the 34 cattle samples and 2 (28.6%) of the 7 human samples were identified as *S. Enteritidis*; and 4 (11.8%) of the 34 cattle samples were identified as *S. Weltevreden*. No *S. Weltevreden* was isolated from human samples (Table 1).

Table 1 The percentage of *Salmonella*-positive samples isolated from cattle (n = 150) and humans (n = 110) in Chiangmai, Thailand.

<i>Salmonella</i> (S.) serovars	No. of cattle (%)	No. of humans (%)
<i>S. Typhimurium</i>	17 (50)	5 (71.4)
<i>S. Enteritidis</i>	13 (41.2)	2 (28.6)
<i>S. Weltevreden</i>	4 (11.8)	0 (0)
Total	34 (22.6)	7 (6.3)

3.2 Phylogenetic Analysis

S. Typhimurium, *S. Enteritidis*, and *S. Weltevreden* were identified and formed three clades in the phylogenetic tree. *S. Weltevreden* and *S. Enteritidis* clades showed a 58% and 62% probability, respectively, of being from the *S. Typhimurium*. The probability of these isolates belonging to the same serotype ranged from approximately 70% to 100%. *S. Typhimurium* and *S. Enteritidis* from cattle and humans were closely related. The branch lengths of *S. Enteritidis* and *S. Weltevreden* were greater than those of *S. Typhimurium* (Fig. 2).

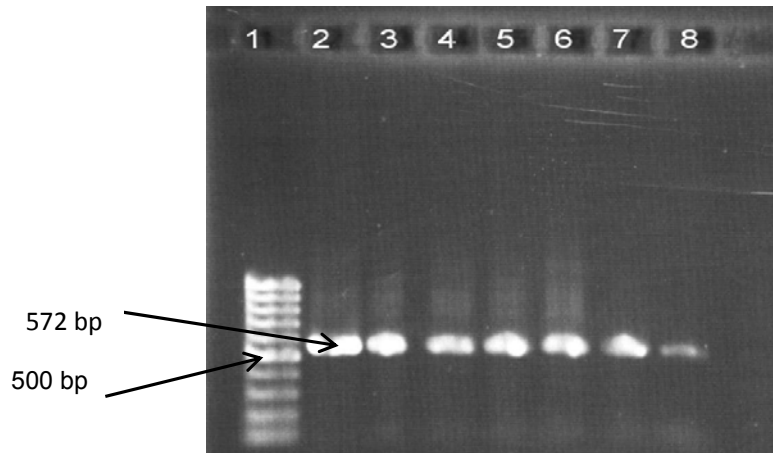


Fig. 1. An agarose gel analysis of 16S rRNA genes of *Salmonella* isolates demonstrates the pattern of positive results. Lane 1: 100 bp, Lane 2: Positive control, Lanes 3, 4: *S. Typhimurium*, Lanes 5, 6: *S. Enteritidis*, and Lanes 7, 8: *S. Weltevreden*.

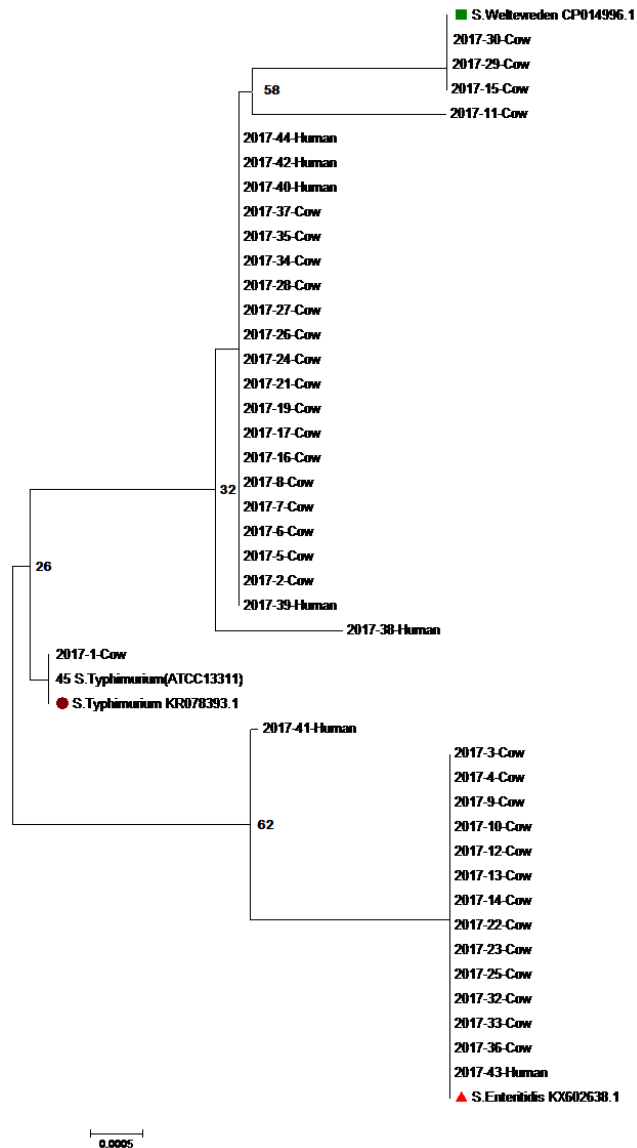


Fig. 2. Phylogenetic tree based on 16S rRNA sequences of *Salmonella* isolates. The phylogeny was inferred by Clustal Omega using MEGA software version 6.0

3.3 Antimicrobial Resistance Profiles of *Salmonella* Isolates

Antibiotic resistance was determined in all the *Salmonella* isolates. All isolates from cattle were susceptible to amoxicillin/clavulanic acid, ceftazidime, ceftriaxone, ciprofloxacin, colistin, imipenem, sulbactam/cefoperazone, ertapenem, tigecycline, and piperacillin/tazobactam. Six (85.7%) of the 7 isolates from humans showed a similar susceptibility pattern. One (14.3%) of the 7 isolates from humans was resistant to amoxicillin/clavulanic acid, ceftazidime ceftriaxone, and ciprofloxacin (Table 2).

Table 2 *Salmonella* isolated from cattle (n = 150) and patients (n = 110) and their antibiotic resistance patterns

<i>Salmonella</i> serovars	Cattle	Antibiotic resistance patterns		Patients	Antibiotic resistance patterns	
		Resistance	Susceptible		Resistance	Susceptible
<i>S. Typhimurium</i>	17	0	17	5	0	5
<i>S. Enteritidis</i>	13	0	13	2	1 (amp, ftz, ftx, cip)	1
<i>S. Weltevreden</i>	4	0	4	0	0	0
Total	34	0	34	7	1	6

Abbreviations: amp, amoxycillin/clavulanic acid; ftz, ceftazidime; ftx, ceftriaxone ; cip, ciprofloxacin

3.4 Discussion

A high prevalence of *Salmonella* may be a sign of considerable threat to human health from seemingly healthy animals slaughtered for human consumption. The prevalence of *Salmonella* in healthy slaughtered cattle reported in this study (22.6%) was higher than rates reported in previous studies conducted in northern Thailand (3.0%) [16] and Australia (14.4%) but lower than in a previous study conducted in Cameroon (27%) [25,26]. This difference may be attributed to the different time periods during which the studies were conducted or the population's cultural traditions or living situation such as housing conditions, feeding habits, types of feed given to the cattle, and the animal management system. For example, in Thailand, people and cattle often live in close proximity; however, in Australia, people's homes and cattle ranches are strictly separate [16, 27].

It is interesting to note that *Salmonella* was detected in the fecal matter of healthy slaughtered cattle even though the animals were inspected by a certified veterinarian who checked each animal prior to slaughter, including its skin, fur, fecal matter, physical appearance, and other clinical parameters for signs of disease. Because slaughterhouses are nearly closed ecosystems, the process of slaughtering animals must be a concern, especially factors that may affect the prevalence and detection of the organism, such as sampling procedures, bacterial isolation, identification methods, and cross-contamination. Therefore, the low prevalence of *Salmonella* in Australian cattle is probably due to the country's strictly regulated animal management systems [26].

In this study, *S. Typhimurium*, *S. Enteritidis*, and *S. Weltevreden* were detected in the feces of healthy cattle in Chiang Mai (northern Thailand) whereas only *S. Brunei* was detected in Sakon Nakorn (northeastern Thailand) [28]. Additionally, previous studies have reported *S. Rissen* and *S. Weltevreden* in Chiang Mai and Lamphung [16]. As observed elsewhere, *S. Anatum*, *S. Weltevreden*, and *S. Lexington* were detected in Vietnam, while *S. Typhimurium* and *S. Newport* were frequently detected in Ethiopia [29, 30]. The results highlight the complexity of *Salmonella*'s global epidemiology as the frequency and occurrence of different serovars change over time in countries and regions. Serotyping is phenotypic characteristics providing useful epidemiological markers, so it may assist in recognizing and tracing newly emerging pathogens [31].

Phylogenetic tree analysis is emerging as an alternative or a complement to established phenotypic identification procedures. For bacteria, 16S rRNA gene sequence analysis is a widely accepted tool for molecular identification [32]. As seen in the phylogenetic tree (Fig. 2), the three serovars *S. Typhimurium*, *S. Enteritidis*, and *S. Weltevreden* formed three distinct clades. This study demonstrated that *S. Typhimurium*, *S. Enteritidis* and *S. Weltevreden* were the most common *Salmonella* serovars observed in healthy cattle whereas *S. Typhimurium* and *S. Enteritidis* were the most common *Salmonella* serovars found in human isolates. Based on the branch length, it appears that more variation has occurred in *S. Enteritidis* and *S. Weltevreden* than in *S. Typhimurium*. *S. Enteritidis* isolated from human blood sample no. 201741 shows bacterial genetic changes in the clade of *S. Enteritidis*. Interestingly, this study reveals the relationship between *Salmonella* from cattle and human Salmonellosis. However, the phenotypic findings are only from an initial investigation, and the 16S rRNA sequences do not provide enough evidence to conclude they are from the same strain.

Thus, more detailed molecular epidemiology studies such as multi-locus sequence typing and pulsed-field gel electrophoresis should be conducted in further studies.

All *Salmonella* isolates from cattle in this study were susceptible to commonly used antimicrobials. A possible reason for this result is the type of feed used for traditional cattle farming in Thailand is naturally growing grass; therefore, the cattle in this study were rarely exposed to antimicrobial agents. However, one of the *Salmonella* isolates from human samples conferred resistance to amoxicillin/clavulanic acid, ceftazidime ceftriaxone, and ciprofloxacin. Thus, the *Salmonella* from human isolates were resistant to commonly used antimicrobials, so this may be a considerable threat in the treatment of future clinical cases. Fluoroquinolones and third-generation cephalosporins are now commonly used for empiric treatment and are listed by the World Health Organization as critically important antimicrobials for human health [33,34]. Antimicrobial resistance is the evolutionary response of bacteria to the strong selective pressure that results from exposure to antimicrobials. The detection of *S. Enteritidis*' resistance to third-generation cephalosporins from human isolate no. 201741 might have been caused by bacterial genetic changes and could be an indicator of the increased use of antimicrobials in Salmonellosis treatment, which may promote the acquired selection of antimicrobial-resistant strains [35]. Although the *Salmonella* isolated from cattle in this study were not resistant to antimicrobials, the cattle were found to be reservoirs of *Salmonella* infection and could serve as a vehicle for its transmission to humans. Therefore, these cattle might be implicated as a source of human *Salmonella* infection.

4. Conclusion

This study provides updated information on the occurrence, serotype distribution, and antibiogram of *Salmonella* in slaughtered cattle in Chiang Mai, Thailand. The prevalence of *Salmonella* from cattle in this study is significantly high and a potential source of food-borne salmonellosis. *S. Typhimurium* and *S. Enteritidis* isolated from cattle and humans were closely related. Thus, the local administration should conduct routine systematic surveillance for the infectious sources.

CONSENT

It is not applicable.

ETHICAL APPROVAL: The study was approved by the Institutional Ethics Board of Chiang Mai provincial hospital, Ministry of public Health, Thailand.

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