

Original Research Article

Antimicrobial susceptibility profiles and molecular Characterization of *Salmonella* isolated from fecal matter of Cattle in Chiang Mai, Thailand and their relatedness to human *Salmonella* isolates

Abstract

Background

Salmonella are the most common pathogenic bacteria causing food borne and zoonotic diseases in humans and become an important public health problem worldwide, particularly in the developing countries. The primary sources of Salmonellosis are considered to be food-producing animal. Cattle is also the production animal slaughtered for human consumption which 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 antimicrobial resistance profiles and genetic relatedness to human isolates.

Methodology

A cross-sectional study was conducted from June to September 2017 at the municipal abattoir of Chiang Mai, Thailand. One hundred 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 Chiang Mai provincial hospital, prior to any antimicrobial treatment. The identification of the circulating *Salmonella* serovars using culture techniques and DNA sequencing, antimicrobial sensitivity testing using disc diffusion method of the commonly prescribed antibiotics and phylogenic relationships using 16S rRNA were determined.

Results The result showed that the prevalence of *Salmonella* from healthy slaughtered 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%) and these three serovars formed distinct clades on

the phylogenetic tree. The *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 that the healthy slaughtered cattle in Chiang Mai carried *Salmonella* with a high prevalence. The isolation of *S.Typhimurium* and *S.Enteritidis* from healthy slaughtered cattle and humans were closely related. The finding suggested that slaughtered cattle is a source of human infected *Salmonella*. This poses a major public health threat since slaughtered cattle might be a potential source of food borne salmonellosis. Therefore, the local administration should have regular systematic surveillance for the infectious sources, including effective monitoring programs of Salmonellosis in Thailand accordingly.

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

Introduction

Salmonella is a gram-negative enteric bacteria which is one of the major foodborne pathogens worldwide. 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 of gastroenteritis 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 according to the country (2) . In Africa, Non Typhoidal Salmonellosis(NTS) has been consistently reported as leading cause of bacteremia among immune-compromised people whereas in Thailand, NTS are common bloodstream isolates in febrile patient .(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) From previous study, the primary sources of salmonellosis are considered to be food-producing animals such as cattle, poultry and swine.(6) The pathogen are mainly disseminated by trade in animals and uncooked animal food products.(7) Also the fecal wastes from infected animals and humans are important sources of bacterial contamination of environment and the food chain. (8) In human, *Salmonella* infections can

55 lead to invasive and focal infections that can be severe.(9) Treatment with antimicrobials is crucial for
56 the proper management of severe human salmonellosis. In addition, the overuse of antimicrobials in
57 the treatment of bacterial infection in humans and animals has led to antimicrobial resistance.(10)
58 Particularly, antimicrobial- resistant *Salmonella* are increasing due to the use of antimicrobial agents
59 in food animals at sub-therapeutic level or prophylactic doses which may promote on- farm selection
60 of antimicrobial- resistant strains.(11,12). Thus, food –producing animals are considered one of the
61 main routes of transmission of antimicrobial- resistant bacteria especially pathogenic bacteria between
62 animal and human population. (13) According to the lacking regular surveillance programs in
63 Thailand, as in the most of developing countries, information on the prevalence of *Salmonella* in food –
64 producing animals is limited. Critically, Little is known about the prevalence of *Salmonella*
65 antimicrobial- resistant from food –producing animals and the genetic relatedness of the isolates from
66 animal and human salmonellosis. Cattle have been implicated as a source of human infection with
67 antimicrobial- resistant *Salmonella* through direct contact with livestock and improper condition of
68 slaughtering process was also considered as factor enhancing resistant ability of the organism (14,15)
69 The aims of our study were: first, to determine the prevalence of *Samonella* in the feces of slaughter
70 cattle; second, to characterize the serotype of *Salmonella* isolates; third, to determine the sensitivity
71 of the isolates to the antimicrobial agents; and finally, to access the genetic relatedness of the isolates
72 from the feces of the cattle and from human salmonellosis.

73 Method

74 Salmonella Isolation

75 Approximately 50 g of fresh fecal samples from 150 cattle were collected for this study from
76 June to September 2017. The fecal specimens of cattle were collected in a clean sterile air tight stool
77 cup directly from the rectum. Fifty grams of faces from cattle was collected aseptically from rectum
78 immediately after the animals were slaughtered. Cattle fecal matter was collected at Chiang Mai
79 municipal slaughter house complex. Cattle slaughtered at Chiang Mai abattoir originated from different
80 parts of city. The sample was determined using prevalence rate of 3.0% from previous study, with a

99% confidence interval covering an error of 5 percentage points from the true value and 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, d= precision

Based on the above formula the calculated sample size was 78. A total of 150 Cattle were randomly selected in this study. One hundred and ten blood samples were also collected from febrile patients at Chiang Mai provincial hospital, prior to treatment with any antimicrobial agents during June to September 2017. The samples were transported using an ice box and culture within 24 hours and analyzed at Regional Medical Sciences center, Chiang Mai, Department of Medical Sciences, Ministry of Public Health, Thailand.

Isolation of Salmonella from Cattle

The isolation was conducted according to ISO 6579/AMD 1:2007 standard protocol.(18) The isolation involves three steps; 1 gm of fecal sample was pre-enriched with 9 ml of buffered peptone water (BPW) (Oxoid, Basingstoke, England) and incubated for 24 hrs at 37°C. A portion (0.1 ml) of the pre-enriched cultured was transferred to 10 ml of selenite cysteine(SC) broth (Himedia, Mumbai, India) and another 0.1 ml portion was transferred to 10 ml of Rappaport and Vassilidis (RV) broth (Merck, Darmstadt, Germany) broth and incubated at 37°C and 42°C for 24 hrs respectively. Finally, the sample from the selective enrichment media was inoculated on to Xylose Lysine Deoxycholate (XLD) (Oxoid, Basingstoke, England) agar and incubated at 37°C for 24 hrs and the incubation was prolonged to 48 hrs for those that did not show any growth during the 24 hrs incubation. Characteristic *Salmonella* colonies, having a slightly transparent zone of reddish color and a black center, were sub-cultured on nutrient agar (Oxoid, Basingstoke, England) and confirmed biochemically using triple sugar iron agar (TSI) (Oxoid, Basingstoke, England), Christensen's urea agar (Oxoid, Basingstoke, England), lysine iron agar (LIA) (Oxoid, Basingstoke, England), Voges

106 Proskauer (VP), methyl red (MR) (Micromaster Thane, India), and Indole tests (Becton Dickinson, USA)
107 [19].

108 Isolation of Salmonella from human

109 Blood samples of 5 to 10 ml and 2 to 5 ml were collected from adults and children respectively.
110 Bacteria were isolated from each blood sample following standard procedure by using the BacT/Alert
111 3D automated system (BioMerieux, Inc., Durham, N.C.) according to the manufacturer's instructions.
112 All isolates were identified using standard diagnostic techniques. (20)

113

114 Identification of Salmonella

115 Extraction of Genomic DNA Pure colonies of Salmonella cultured in nutrient broth
116 (Oxoid),UK) was used for DNA extraction using the Nucleospin Tissue (Machrey-Nagel, Germany)
117 according to the manufacturer's instructions.

118 PCR Amplification Forward primer 16SF1 (5'-
119 TGTTGTGGTTAATAACCGCA-3') and reverse primer; 16SIII (5'-
120 CACAAATCCATCTCTGGA-3') of the 16S rRNA gene (Bio Basic Inc.,
121 Canada) were used to amplify the 572 bp PCR product. Amplifications
122 were carried in 50 µl reaction volumes containing; 25 µl of Quick Taq HS
123 DyeMix (TOYOBO, Japan), 18 µl of nuclease free water, 1 µl of each
124 primer and 5 µl of the extracted bacterial DNA. The amplifications were
125 done in 35 cycles with an initial denaturation at 95 °C for 5 min, a
126 denaturation step of 95 °C for 2 min, primer annealing at 55 °C for 30 s
127 and primer extension at 72 ° C for 1 min. Finally, an additional extension
128 was done for 10 min at 72 °C. The PCR products were visualized on 2%
129 agarose gel with ethidium bromide. (21)

130 DNA sequencing The PCR products obtained were extracted using QIAquick gel extraction kit
131 (Qiagen, Valencia CA, USA). Thirty microliters of the purified DNA of each sample was sequenced (Bio
132 Basic Inc., Canada).

133 Phylogenetic analysis

The 16S rRNA sequences obtained were compared with known 16S rRNA sequences at National Center for Biotechnology Information (NCBI) database using BLASTn (Basic Local Alignment Search Tool) algorithm obtained from; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>. Identification of the sequences at both the genus and species level was defined as a 16S rRNA sequence similarity of $\geq 99\%$ with that of the prototype strain sequence in GenBank. The sequences together with reference sequences derived from the Genbank were aligned using CLUSTAL OMEGA. The probability of phylogenetic tree were determined using MEGA software.

Salmonella antimicrobial susceptibility tests

Antibiotic susceptibility testing was tested using the Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. (22) Briefly, pure colonies, bacterial suspension were placed in test tubes and their turbidity adjusted to 0.5 McFarland turbidity standards. The diluted bacterial suspensions were then transferred onto Mueller-Hinton agar plates using a sterile cotton swab and seeded uniformly. Antibiotic impregnated discs were then placed to the plate surfaces using sterile forceps. The plates were incubated aerobically at 37 °C for 24 h and *Escherichia coli* ATCC 25922 was used as a quality control organism for the antimicrobial susceptibility test . A total of 10 selected antibiotic disks were used which contained the commonly used antibiotics namely; amoxycillin/clavulanic acid(30 µg) , ceftazidime (30 µg) , ceftriaxone, (30 µg) ciprofloxacin(30 µg), colistin(10 µg), imipenem (30 µg), sulbactam/cefoperazone(30 µg), ertapenem(10 µg), piperacillin/tazobactam(10 µg) and tigecycline (15 µg) (Oxoid, Basingstoke, England). Zones of inhibitions were measured to determine whether the bacteria were susceptible, intermediate or resistant in comparison to CLSI critical points.

Data Analysis

Data was described and presented in tables and figure in antimicrobial resistance bacteria and Phylogenetic analysis tree. The 16S rRNA sequences of *Salmonella* isolates from human and cattle's feces were compared with known 16S rRNA sequences at National Center for Biotechnology Information (NCBI) database using BLASTn (Basic Local Alignment Search Tool) algorithm obtained from; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>. Identification of the sequences at both the genus and

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 phylogenetic tree was determined. The topological robustness of the trees was evaluated by a bootstrap analysis involving 10,000 replications.

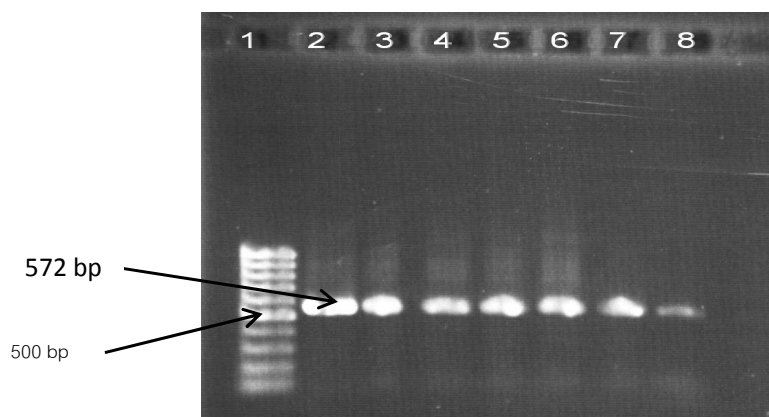
Result

Salmonella prevalence and serotypes

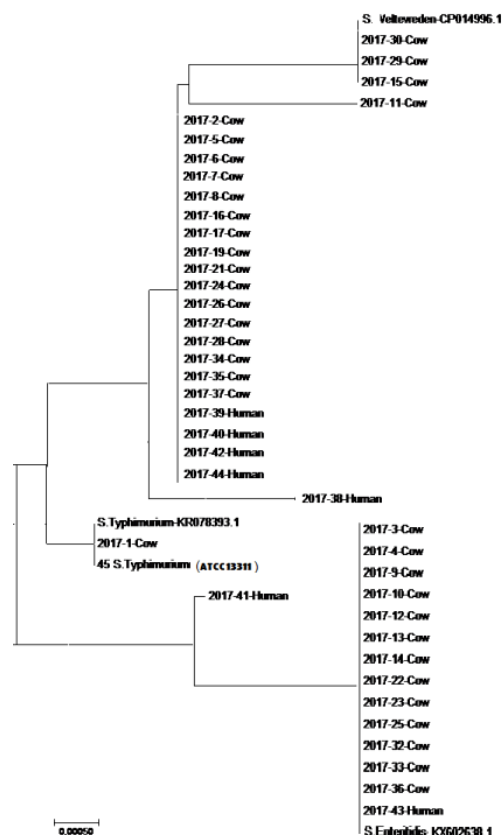
Salmonella was isolated in 34 (22.6%) out of the total 150 fecal samples of healthy slaughtered cattle. Seven of 110 blood samples obtained from febrile patients were also isolated. The PCR product obtained was 572 bp (Fig.1). According to the NCBI blast, 3 serovars of *Salmonella* were identified : 17 (50%) out of 34 of cattle samples and 5 (71.4%) out of 7 of human samples were identified to be *S.Typhimurium*. Thirteen out of 34 (41.2%) of cattle samples and 2 out of 7 (28.6%) of human samples were identified to be *S.Enteritidis*. Four out of 34 (11.8%) of cattle samples were identified to be *S.Weltevreden*. None of *S.Weltevreden* was identified from human samples.

Phylogenetic analysis

S.Typhimurium and *S.Enteritidis* and *S.Weltevreden* were identified and formed three clades in the phylogenetic tree. *S.Typhimurium* and *S.Enteritidis* from cattle and humans were closely related whereas *S.Weltevreden* isolates from cattle had no relationship to human. The branch length of *S.Enteritidis* and *S.Weltevreden* were more than *S.Typhimurium* (Fig. 2)



197 Fig. 1 Agarose gel analysis of PCR (572 bp) of *Salmonella* isolates. Lane 1: 1000 bp, Lane 2:
198 Positive control Lane 3,4 : S.Typhimurium Lane 5,6 : S. Enteritidis Lane 7,8 : S. S.Weltevreden
199



200
201 Fig. 2 phylogenetic tree based on 16S rRNA sequences of *Salmonella* isolates. The phylogeny was inferred by
202 CLUSTAL OMEGA using MEGA software.

203

204 Antimicrobial resistance profiles of *Salmonella* isolates

205 Antimicrobial resistance was identified in all of *Salmonella* isolates. All isolates from cattles
206 were susceptible to amoxycillin/clavulanic acid, ceftazidime, ceftriaxone, ciprofloxacin, colistin,
207 imipenem, sulbactam/cefoperazone, ertapenem, tigecycline and piperacillin/tazobactam. Six of
208 7(85.7%) isolates from human showed susceptible pattern the same as cattle. One of 7(14.3%)
209 isolates from human was resistant to amoxycillin/clavulanic acid, ceftazidime, ceftriaxone and
210 ciprofloxacin. (Table 1)

211 **Table 1** *Salmonella* Isolated from Cattles(n=150) and patients(n=110) and their antimicrobial
212 resistance patterns

Salmonella serovars	Cattle	Antimicrobial resistance patterns		Patients	Antimicrobial resistance patterns	
		Resistance	Susceptible		Resistance	Susceptible
		-	-		-	
<i>S.Typhimurium</i>	17	-	17	5	-	5
<i>S.Enteritidis</i>	13	-	13	2	1	1
<i>S.Weltevreden</i>	4	-	4	-	-	-

213

214 Discussion

215 The *Salmonella* prevalence of healthy Slaughter cattle reported in this study was higher than that
216 reported in a previous study in northern Thailand (3.0%). (16) In addition, the prevalence of *Salmonella*
217 found in this study was higher than a study in Australia (14.4%) but lower than a previous study in
218 Cameroon (27%). (23, 24) This difference may be attributed to the difference in time of study,
219 traditional of living condition, like housing conditions, feeding habits, types of feed given for the cattle
220 including animal management system ; for example, in Thailand the humans and cattle often live in
221 close vicinity whereas in Australia the human house and the ranch are strictly separate.(16, 25) It is
222 interesting to note that *Salmonella* was detected in the fecal matter of healthy slaughtered cattle
223 although the animals are slaughtered a certified veterinarian inspects the health status of the animal
224 by checking the skin fur, fecal matter, physical appearance and other clinical parameters. According
225 to nearly closed ecosystem of slaughterhouse, the process of slaughtering animals must be a
226 concern. In addition, factor that may affect prevalence that affect detection of the organism, such as
227 sampling procedures, bacterial isolation, identification methods and cross contamination. The low
228 prevalence of *Salmonella* from Australian cattle probably, therefore, represents a result of strict
229 regulation of animal management systems.(24) Thus, the high prevalence of *Salmonella* may be a
230 sign of considerable threat in the human health from seeming healthy animal slaughtered for human
231 consumption.

232 In this study, *S. Typhimurium*, *S. Enteritidis* and *S.Weltevreden*, were detected from healthy
233 cattle in Chiang Mai (northern Thailand) whereas only *S.Brunei* was currently reported in Sakon
234 Nakorn (north-Eastern Thailand). (26) In addition, previous studies have reported *S. Rissen* and *S.*
235 *Weltevreden* in Chiang Mai and Lamphung. (16) As observed elsewhere, *S. Anatum*, *S. Weltevreden*

236 and *S. Lexington* were detected in Vietnam while *S. Typhimurium* and *S. Newport* were the frequently
 237 isolates in Ethiopia. (27,28) The results highlight the complexity of global epidemiology of *Salmonella*
 238 as the frequency and occurrence of different serovars changes overtime in countries and regions.
 239 According to, *Serotyping* is phenotypic characteristics providing useful epidemiological markers so it
 240 may assist in the recognition and tracing of new emerging pathogens.(29)

241 Phylogenetic tree analysis is emerging as an alternative or complement to established
 242 phenotypic identification procedures. For bacteria, 16S rRNA gene sequence analysis is a widely
 243 accepted tool for molecular identification (30). From the phylogenetic tree (Fig. 2) the three serovars:
 244 *S. Typhimurium* , *S. Enteritidis* and *S. Weltevreden* formed three distinct clades. This study
 245 demonstrated that *S. Typhimurium*, *S. Enteritidis* and *S. Weltevreden* were the most common observed
 246 in healthy cattle whereas *S. Typhimurium* and *S. Enteritidis* were the most common *Salmonella*
 247 serovars found from human isolates. According to the branch length, it appears that more variation
 248 has occurred in *S. Enteritidis* and *S. Weltevreden* than the *S. Typhimurium*. Particularly, *S. Enteritidis*
 249 isolated from human blood sample no. 201741 shows bacterial genetic change in the clade of *S.*
 250 *Enteritidis*. Interestingly, this study reveals the relationship of *Salmonella* from cattle to human
 251 *Salmonellosis*.

252 . All *Salmonella* isolates from cattle in this study were susceptible to the commonly used
 253 antimicrobials whereas one of *Salmonella* isolates from human was multi-resistant to the common
 254 antimicrobials. Antimicrobial resistance was observed in *S. Enteritidis* isolated from human which
 255 conferred resistance to amoxycillin/clavulinic acid, ceftazidime ceftriaxone and ciprofloxacin. As the
 256 *Salmonella* from human isolates were resistant to commonly used antimicrobials so this may be a
 257 considerable threat in the treatment of clinical cases in future. Especially, fluroquinolones and third
 258 generation cephalosporins are now commonly used for empiric treatment and are listed by the World
 259 health Organization as critically important antimicrobials for human health.(31,32) The antimicrobial
 260 resistance is the evolutionary response by bacteria to the strong selective pressure that results from
 261 exposure to antimicrobials, the detection of resistance of *S. Enteritidis* to third generation
 262 cephalosporins from human isolate no. 201741 might cause by bacterial genetic change and could
 263 be an indicator of the increased use of the antimicrobials in *Salmonellosis* treatment which may
 264 promote on acquired selection of antimicrobial resistant strains. (33). Even though, the *Salmonella*
 265 isolated from cattle in this study were not antimicrobial resistance strain , the cattle was found to be
 266 reservoirs of *Salmonella* infection and could serve as vehicle for its transmission to human. Therefore,

these cattle might be implicated as a source of human *Salmonella* infection. However, phenotypic finding was provided only primary investigation and the 16S rRNA sequences are not enough to conclude into the same strain. Thus, studies of molecular epidemiology such as Multi Locus Sequence typing and Pulse Field Gel Electrophoresis should be conducted in further studies.

Conclusion

The present study helped to update the information on the occurrence, serotype distribution and antibiogram of *Salmonella* in slaughter cattle in Chiang Mai, Thailand. This study was carried out molecular characteristic of *Salmonella spp.* which isolated from healthy Slaughter cattle and human. The prevalence of *Salmonella* from cattle in this study is significantly high to be a potential source of food borne salmonellosis. *S. Typhimurium* and *S. Enteritidis* isolated from cattle and human were closely related. Even though, all *Salmonella spp.* from cattle isolates were susceptible to commonly used antimicrobials but *Salmonella* isolate from human was multidrug resistance that are common used in public health set up. This may pose a considerable threat in human health especially difficulties in treatment of human clinical cases in future.

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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