Prevalence and antimicrobial resistance of non–typhoid *Salmonella* in military personnel, 1988–2013

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**Article info**

**Article history:**
Received 29 December 2017
Revision 13 February 2018
Accepted 18 February 2018
Available online 20 June 2018

**Keywords:**
Non-typhoid *Salmonella*
Antimicrobial resistance
Azithromycin
Deployed military
Public health
Thailand

**Abstract**

**Objective:** To describe the spanning 25 years data for the occurrence, magnitude, and trends regarding antimicrobial resistance of non-typhoidal *Salmonella* (NTS) isolated from non-immune travelers to Thailand participating in joint military operations. **Methods:** A total of 355 NTS isolates, obtained from 2,052 fecal samples from US soldiers deployed for military maneuvers in Thailand during 1988-2013, were examined for NTS serogroup/serotypes and tested for antimicrobial susceptibility by disk diffusion to these 10 antibiotics: ampicillin, azithromycin (AZM), ciprofloxacin, colistin, gentamicin, kanamycin, nalidixic acid, streptomycin (STR), tetracycline (TET), and trimethoprim/sulfamethoxazole. Identified AZM-resistant NTS isolates were further evaluated for their minimal inhibitory concentration by the E-test method. **Results:** NTS infections accounted for 17.3% (355/2,052), including 11 serogroups and 50 different serotypes. The most prevalent serogroup was *Salmonella* group C2-C3 (35.8%, 127/355) followed by groups B (21.1%, 75/355) and C1 (18.6%, 66/355). Identified serotypes included *Salmonella hadar* (n=60), *Salmonella rissen* (n=45), and *Salmonella blockley* (n=34). Among the predominate serogroups, antimicrobial resistance was consistently high against TET (76.9%, 273/355) followed by STR (40.8%, 145/355). One *Salmonella senftenberg* isolate demonstrated decreased ciprofloxacin susceptibility. Most isolates (94.6%) were resistant to one or more antimicrobials, and the most common multidrug resistance was TET-STR-nalidixic acid (11.5%, 41/355). **Conclusions:** The prevalence of NTS serotypes and the growing magnitude of antibiotic resistant bacteria isolated from deployed US military in Thailand are documented from 1988-2013. This study demonstrates the antibiotic resistance profiles, highlighting the effectiveness of AZM that is a first-line treatment for travelers to Southeast Asia. AZM-resistant NTS isolates are periodically observed over a 25-year period. Hence, the ongoing surveillance and prevalence efforts are required to monitor NTS resistant strains causing further treatment failure.

1. Introduction

Infectious diarrhea remains the most common problem facing travelers to developing countries, including US military and civilian personnel deployed overseas. Thailand is one of the developing countries in the Pacific region in which the US has a functional security alliance. Since 1982 the largest Asian-Pacific US military exercise, Cobra Gold, has been held annually in Thailand, and with each cycle military and support personnel experienced diarrheal disease from *Campylobacter, Shigella* and non-typhoidal *Salmonella* spp. (NTS) pathogens. Analyses from previous Cobra Gold exercises indicated that US military personnel suffered diarrheal attack rates ranging from 6.5% to 36.0%\(^{(1,2)}\). In accordance with these findings, the prevalence and patterns of antimicrobial resistance of non-typhoidal *Salmonella* isolated from deployed US military personnel participating in joint military operations in Thailand, 1988-2013, were examined for NTS serogroup/serotypes and tested for antimicrobial susceptibility by disk diffusion to these 10 antibiotics: ampicillin, azithromycin (AZM), ciprofloxacin, colistin, gentamicin, kanamycin, nalidixic acid, streptomycin (STR), tetracycline (TET), and trimethoprim/sulfamethoxazole. Identified AZM-resistant NTS isolates were further evaluated for their minimal inhibitory concentration by the E-test method.
with the travelers’ diarrhea categories, Southeast Asia including Thailand was considered ‘moderately risky’ compared with the reference destination[3]. Nonetheless, there was limitations about the published data of NTS-associated diarrheal disease in Southeast Asia travelers[4].

Salmonella enterica, the etiological agent of salmonellosis, is a global endemic food-borne pathogen associated with diverse clinical manifestations[5], such as acute gastroenteritis, fever, abdominal pain, nausea, and/or death[6]. NTS has a broader host-range that includes poultry and cattle and is the leading cause of 2.8 billion diarrheal cases each year worldwide[7] as well as NTS-induced gastroenteritis statistically results in morbidity and mortality, causing 155 000 deaths each year[8].

In the past decade, human infections with antimicrobial resistance in food-borne pathogens were recognized as an increasing problem in many countries and counting challenge to treatment recommendations[9]. The development of antimicrobial resistance NTS strains possibly resulted from selective pressures such as improper antibiotic use in human medicine, veterinary medicine and animal husbandry, as well as for agricultural aspects. Most antimicrobial resistance NTS infections were acquired from the consumption of contaminated foods of animal origin[10], and the transmission of multidrug-resistant NTS strains were frequently reported worldwide[11-13]. The increasing prevalence of multidrug resistance among NTS, was not only against the first-line antibiotics like ampicillin, chloramphenicol and trimethoprim/sulfamethoxazole (SXT), but also against clinically important antimicrobial agents such as fluoroquinolones, and third-generation cephalosporins presents a serious threat to global public health[14-17]. Antimicrobial resistance surveillance data demonstrated that NTS serotypes increased two fold from 20%-30% in the 1990s to 70% in some countries in 2000[14]. The main objective of this study is to document the serotype and serogroup prevalence of NTS isolated from US Military personnel deployed in Thailand, and to determine their antibiotic resistance patterns, particularly to azithromycin (AZM).

2. Materials and methods

2.1. Source of isolates

A total of 355 NTS isolates were obtained from 2 052 fecal samples (1 975 enteritis human cases and 77 from non-diarrhea persons) collected from U.S. soldiers on military maneuvers involved in numerous community-acquired diarrheal studies in Thailand between 1988 and 2013. These studies were conducted under a protocol with informed consent by the Department of Enteric Diseases, Armed Forces Research Institute of Medical Sciences in Thailand. These isolates were initially examined for common causes of enteritis, and Shigella, NTS, Vibrio, Campylobacter, ETEC as well as viral pathogens were included before focusing upon NTS identification and characterization.

2.2. Microbiology

Enteric pathogens were cultured and identified by standard methods as previously described[18]. From 2003-2013, the Modified Semisolid Rappaport-Vassiliadis medium (Oxoid, UK) and Buffer Peptone Water enrichment broth (BD, USA) were incorporated into conventional microbiology procedures for improving NTS isolation[19]. Serological grouping of NTS was performed based on O-antigens using the slide agglutination test (Denka Seiken Co, Ltd., Tokyo, Japan). Serological typing was performed at the WHO National Salmonella and Shigella Center at Regional Medical Sciences Center 5, Samut Songkhram, Department of Medical Sciences, Ministry of Public Health, Thailand, and serotypes were assigned according to the Kauffmann-White scheme[20].

Antimicrobial susceptibility testing was performed with the standard Kirby-Bauer disk diffusion method by using Mueller Hinton II Agar (BBL[20]) and the following nine commercial available antimicrobial disks (Becton-Dickinson, New Jersey, US): ampicillin (AMP) 10 μg, ciprofloxacin (CIP) 5 μg, colistin (CL) 10 μg, gentamicin (GEN) 10 μg, kanamycin (KAN) 30 μg, nalidixic acid (NAL) 30 μg, streptomycin (STR) 10 μg, tetracycline (TET) 30 μg, SXT 1.25 μg/23.75 μg. Among 355 isolates, 316 isolates were also assessed for antimicrobial susceptibility testing to AZM 15 μg (Becton-Dickinson, New Jersey, US), by using disk diffusion procedure as previously described. Inhibition zones were recorded and interpreted following the breakpoints interpretation of the concomitant Clinical and Laboratory Standards Institute (CLSI), as well as the M2 and M100 documents in the year in which the NTS were isolated. For isolates placed into the ‘intermediate’ category, these were deemed ‘reduced susceptibility’ for purpose of this study. Concomitant resistance to at least 3 of 10 antimicrobials qualified an isolate as multidrug resistance. For quality control purposes, the Escherichia coli (E. coli) ATCC strain 25922 was processed in parallel with study isolates, according to CLSI guidelines. The zone diameter interpretive criteria for AZM are undefined by CLSI or the European Committee on Antimicrobial Susceptibility Testing to Salmonella spp. and other Enterobacteriaceae; therefore, interpretive criteria for Staphylococcus spp. were utilized for this study[18]. Consequently, the Staphylococcus aureus ATCC strain 25923 was also included for quality control purposes for AZM evaluation.

Twenty-seven NTS isolates (from 34 isolates) with an AZM inhibition zone diameter ≤13 mm by disk diffusion test, or AZM resistant, were subcultured and their MICs were determined by the E-test method, as described by the manufacturer (AB Biodisk, Solna, Sweden). Briefly, an inoculum equivalent to 0.5 McFarland turbidity standards was prepared from each fresh isolate and inoculated onto Mueller-Hinton agar. An AZM E-test strip was applied onto the agar within 15 min after inoculation, followed by incubation in 35 °C. Plates were observed and interpreted after 16-20 h. The MIC values of all isolates were recorded.

2.3. Statistical analysis

Data from Cobra Gold Salmonella isolates obtained between 1988 and 2013 were compared regarding their antibiotic resistance profiles with odds ratios (ORs) and 95% confidence intervals (CIs), using the initial data obtained in 1988 as a reference point.

3. Results

A total of 355 NTS isolates were identified from the 2 052 stool samples, accounting for 17.3% of enteric bacterial pathogens detected. Percentages of NTS isolates from various locations during
Cobra Gold exercises were listed in Table 1, and the distribution of NTS serogroups by year was presented in Figure 1. For NTS isolates obtained over this 25 year period, the serogroup distribution data indicated that serogroup B, C1, and C2-C3 were the top three serogroups identified (Figure 1). A total of 11 serogroups (B, C1, C2-C3, E1, E4, D1, F, G, I, K, and Q) and 50 serotypes were identified among these isolates. The leading serogroup was serogroup C2-C3 (35.8%, 127/355), followed by B (21.1%, 75/355), C1 (18.6%, 66/355), D1 (7.9%, 28/355), E1 (7.6%, 27/355) and E4 (5.9%, 21/355). Of the 50 Salmonella serotypes detected, the predominant serotypes were Salmonella hadar (S. hadar) (n=60), Salmonella rissen (S. rissen) (n=45), Salmonella blockley (S. blockley) (n=34), Salmonella stanley (n=23), Salmonella derby (n=19) and Salmonella krefeld (n=17), respectively.

Regardng the antimicrobial susceptibility testing results, 5.4% (19/355) of the isolates were susceptible to all 10 of the antimicrobials tested, whereas the highest resistance was to TET, accounting for 76.9% (273/355). Additional antimicrobial susceptibility testing profiles include resistance to STR 40.8% (145/355), NAL 23.0% (82/355), SXT 19.2% (68/355), AMP 18.0% (64/355), KAN 17.5% (62/355), and GEN 4.2% (15/355), respectively. None were resistant to CL and CIP; however, a decreased susceptibility to CIP (intermediate category) was observed in one serogroup E, serotype Salmonella senftenberg. Furthermore, the multidrug resistance profiles were TET-STR-NAL (11.5%, 41/355), TET-STR-AMP (7.9%, 28/355), and TET-STR-SXT (7.0%, 25/355), respectively. Since AZM is the treatment drug choice for traveler’s diarrhea, 316 NTS isolates were tested for resistance to AZM by disk diffusion (Table 1) and the result indicated a decreased susceptibility (‘!’ category) to AZM of 45.9% (145/316), whereas resistance was 10.8% (34/316). Additional characterization of these AZM-resistant NTS isolates identified the following serotypes: S. blockley 82.4% (28/34), S. rissen 17.6% (6/34), Salmonella enek 2.9% (1/34), Salmonella agona (S. agona) 2.9% (1/34), S. hadar 2.9% (1/34), and Salmonella brunei 3% (1/34), respectively. Among 34 AZM-resistant NTS isolates, 27 isolates were further subcultured to determine MIC values by the E-test method (Table 2). AZM-MIC values of S. blockley isolated from 1988 to 2002 ranged from 32 μg/mL to 128 μg/mL, and S. rissen isolates from 2002 and 2004 were 64 μg/mL. The most resistant isolate was a S. agona isolate from 2009, with an AZM-MIC ≥256 μg/mL. None of AZM resistant isolates were obtained during 2010 to 2013 (Table 2). For the S. blockley isolates, 35% (12/34) exhibited a multidrug resistance profile with up to five antimicrobial agents (KAN-TR-TET-NAL-AZM).

Table 1
Number of samples and percentage of NTS-positive isolates by year and location in Thailand during Cobra Gold exercises.

| Year     | Location          | No. of NTS (%) | No. of isolate tested | No. of isolate tested with AZM |
|----------|-------------------|----------------|-----------------------|--------------------------------|
| 1988     | Nakhon Ratchasima | 19 (5.4)       | 19                    | 19                             |
| 1990     | Chonburi          | 37 (10.4)      | 37                    | 37                             |
| 1993     | Ubhon, Chonburi, Pitsanulok | 17 (4.8) | 17                    | 17                             |
| 1994     | Chonburi          | 12 (3.4)       | 12                    | 12                             |
| 1995     | Chonburi, Pitsanulok, Nakhon Ratchasima | 36 (10.1) | 36 | 36                             |
| 1996     | Chonburi          | 1 (0.3)        | 1                     | 0                              |
| 1997     | Pitsanulok        | 10 (2.8)       | 10                    | 10                             |
| 1998     | Chonburi, Kanchanaburi | 42 (11.8) | 42                    | 42                             |
| 1999     | Nakhon Ratchasima | 38 (10.7)      | 38                    | 38                             |
| 2000     | Nakhon Si Thammarat | 49 (13.8) | 49                     | 49                             |
| 2001     | Pitsanulok        | 20 (5.6)       | 20                    | 20                             |
| 2002     | Sa Kao            | 27 (7.6)       | 27                    | 27                             |
| 2003     | Prachuap Khiri khan | 6 (1.7)      | 6                     | 6                              |
| 2004     | Nakhon Ratchasima | 34 (9.6)       | 34                    | 34                             |
| 2009     | Chonburi, Pitsanulok | 5 (1.4)     | 5                     | 5                              |
| 2010     | Chonburi          | 1 (0.3)        | 1                     | 1                              |
| 2011     | Chonburi, Lopburi | 1 (0.3)        | 1                     | 1                              |
| 2012     | Chonburi, Nakhon Ratchasima | 0 (0.0) | 0 | 0                              |
| 2013     | Chonburi, Pitsanulok | 0 (0.0)     | 0                     | 0                              |
| Total    |                   | 355 (100.0)    | 355                   | 316                            |

*Tested by disk diffusion method to all nine antimicrobial agents including AMP, CIP, CL, GEN, KAN, NAL, STR, TET, SXT.

Figure 1. Number of NTS isolates collected by year, and serogroup distribution.

Regardless of the antimicrobial susceptibility testing results, 5.4% (19/355) of the isolates were susceptible to all 10 of the antimicrobials tested, whereas the highest resistance was to
Regarding to the highest resistance rate of NTS to TET, this association along with the year isolated and the NTS serogroup identified were analyzed accordingly. The statistical analysis revealed that TET resistance increased during 1988-1997, dramatically increased again from 1999-2002, and then decreased from 2003 forward (Table 3). The majority of NTS serogroups, including serogroup B, C1, and C2-C3, exhibited dynamic changes during this time period, hence there was no association between the TET resistance and a specific serogroup observed in this study. Furthermore, the association between other antimicrobial susceptibility testing profiles including AZM, the year of isolation, and NTS serogroups were not notably observed in this study (Table 3).

### Table 3

| Year found | Resistance (95%CI) | OR (95%CI) | Resistance (95%CI) | OR (95%CI) |
|------------|--------------------|------------|--------------------|------------|
| 1988       | 1                  | 36.84(16.29-61.64) | 1                |
| 1990       | 37                 | 4.20(0.48-36.98) | 1000(0.22-10000) | NA         |
| 1993       | 15                 | 0.00(0.00-18.10) | NA                | 88.24(63.96-85.4) | 128(22.4-736.64) |
| 1994       | 12                 | 83.3(21.38-48.14) | 164(0.09-29.69) | 75.0(42.8-94.51) | 514(10.02-25.60) |
| 1997       | 10                 | 1000(0.25-44.50) | 200(0.11-35.81) | 90.0(55.99-97.95) | 15.43 (1.60-148.83) |
| 1998       | 42                 | 0.00(0.00-6.88) | NA                | 45.24(29.85-63.13) | 1.42(0.47-4.31) |
| 1999       | 38                 | 0.00(0.00-21.80) | 212(0.22-23.99) | 89.47(73.97-97.06) | 14.57(3.62-58.72) |
| 2000       | 49                 | 4.08(0.30-13.98) | 0.77(0.07-8.89) | 97.96(89.15-99.95) | 82.29 (9.22-734.31) |
| 2001       | 20                 | 300(11.89-54.28) | 77.1(0.83-71.69) | 80.0(56.34-94.27) | 68.6(16.3-280.00) |
| 2002       | 27                 | 11.11(2.35-20.16) | 225(0.22-23.46) | 74.07(53.78-88.89) | 45.0(13.47-147.42) |
| 2003       | 6                  | 0.00(0.00-30.30) | NA                | 66.67(22.89-95.67) | 34.3(0.49-23.78) |
| 2004       | 34                 | 2.94(0.07-15.33) | 0.55(0.09-9.25) | 58.82(40.70-75.35) | 245(77.77-77.78) |
| 2009       | 5                  | 200(0.51-71.64) | 450.0(23.82-88.25) | 1000(548.0-1000.0) | NA |
| 2010       | 1                  | 0.00(0.00-95.00) | NA                | 0.00(0.00-95.00) | NA |
| 2011       | 1                  | 0.00(0.00-95.00) | NA                | 0.00(0.00-95.00) | NA |

The AZM resistant rate was calculated from the confirmed MICs data. The significant data referred to the 95% CI presenting a lower limit < 1 or upper limit 1 when compared to the year 1988. (NA = not applicable; no resistant isolates).

### 4. Discussion

NTS infection is a leading cause of gastroenteritis and is a growing public health concern. In this study, serogroup C2-C3 (35.8%) was the most frequent and prevalent serogroup, followed by serogroup B (21.1%), serogroup C1 (18.6%) and serogroup D (7.9%), respectively. In contrast to a study in Saudi Arabia, Taiwan, and the US, the incidence of NTS serogroup D was more prevalent than serogroup B and other serogroups[21,22]. Regarding the high prevalence of serotype S. hada, this was more commonly isolated in European countries[23,24] and the US[25], associated with NTS isolates from US military populations. The second most prevalent serotype, S. rissen, was generally isolated from pork and chicken meat samples, and it was the most common non-human serotype found in Asia[24]. In this present study, S. blockley is the third most prevalent serotype, but these isolates possess the highest multidrug resistance attributes, including AZM resistance. The first documented S. blockley outbreak was from frozen, unpasteurized egg yolks used in the preparation of ice cream[26]; however, recent studies have reported that chicken is a major source of S. blockley and that it can be globally isolated from contaminated pigs[27-29]. In Thailand, the first isolation of S. blockley was reported in 1989, obtained from animal feed and chicken feather[30]. S. blockley was also reported in other countries, such as Europe and US. This suggests that the NTS distribution is attributed to international travel and global agricultural commerce.

Regarding to the World Health Organization report, the frequency of antimicrobial susceptibility testing and number of NTS isolates have risen markedly[21], and several studies worldwide have reported increased morbidity and mortality in patients infected with resistant NTS[31,32]. Evidence suggests that increasing antimicrobial resistance in NTS was significantly related to overuse and misuse of antimicrobial agents in animal feeds as growth promotion supplements[14,15]. Antibiotics can be freely purchased in veterinary drug stores, and farmers intensively use antibiotics as prophylactics for their animals[33]. The highest antimicrobial resistance observed in this study was TET, which is one of the most widely antibiotics used in human and veterinary medicine practices. This finding is in harmony with TET resistance patterns previously reported from Vietnam and Thailand[28]. Currently the drugs of choice for empiric treatment of acute infections diarrhea, in which Salmonella spp. are etiologically implicated fluoroquinolones in adults and third-generation cephalosporins in children. Alternative treatments may use AZM and imipenem in life-threatening, systemic Salmonella infections. Aminoglycosides are considered ineffective in gastrointestinal salmonellosis[34], which is further supported by our results documenting resistance to KAN, GEN, and STR over a 25 year span. This study expands those antimicrobial resistance findings to now including macrolide and multi-drug resistance profiles in isolates obtained from Southeast Asia travellers. These findings potentially influence the selection of empiric or therapeutic antimicrobial agents to treat enteric pathogens, especially in Thailand where Campylobacter isolates possess high rates of CIP resistance[35]. Additionally, S. blockley, Salmonella derby, S. rissen, and Salmonella stanley isolates demonstrated AZM and other antimicrobial agent resistant patterns. These strains may be more successfully adapting to the selective pressures in the environment, in contrast to the other serotypes with only one or two multidrug resistance representatives identified. Our results further suggest that NTS resistance was the highest against TET, SXT, and STR, as previously reported in other studies conducted in Thailand[36].

The antimicrobial susceptibility testing patterns observed and reported here are important findings for the treatment of bacterial diarrhea with fluoroquinolone and macrolide antibiotics. A unique
finding decreased susceptibility to AZM, which was important since AZM is typically utilized for the treatment of traveler’s diarrhea. In accordance with the MICs values, the broad efficacy against common bacterial pathogens associated with travelers’ diarrhea, such as enterotoxigenic E. coli, enteraggregative E. coli, multi-drug resistant Shigella species, and CIP-resistant Campylobacter species. It was unclear whether the AZM resistance was correlated between NTS isolates and resistant Campylobacter spp.; however, AZM-resistant Campylobacter in Thailand has been noted frequently.

Due to the limited number of NTS isolates from each Cobra Gold exercise, it is difficult to assess the relationship to other low prevalent NTS serotypes and multidrug resistant patterns. Nonetheless, these findings may guide further research studies regarding antimicrobial resistance mechanisms, and develop better strategies to diminish the spread of resistant NTS. Systematic surveillance and timely reporting of antibiotic resistance patterns among enteric pathogens from different regions of the world will remain a high priority until an effective vaccine or other effective prophylactics become available, particularly in areas where resistance prevalence and under reporting remain, such as Southeast Asia.

Conflicts of interest statement

The authors declare that there is no conflict of interests.

Disclaimers of authors

Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense.

Acknowledgements

The study is supported by the Armed Forces Health Surveillance Branch and its Global Emerging Infectious Disease Surveillance and Response Section. We thank Dr. Forrest Littlebird, and Ms. Chittima Pitarangsi and Armed Forces Research Institute of Medical Sciences Enteric Diseases Department Staff, Bangkok, Thailand, for their assistance and kind support. We also acknowledge the WHO Collaborating Centre for Reference and Research of Salmonella, the Ministry of Public Health, Bangkok, Thailand. Funding of this project was partially provided by the U.S. Army Medical and Material Command.

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