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Antibiotic Resistance Profile and association with Integron Type I among *Salmonella* Enterica Isolates in Thailand

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Abstract

***Salmonella* infection is the second most common cause of diarrhea in Thailand; however, the data on antimicrobial resistance is limited. There were137 *Salmonella* strains, isolated from patients and 126 strains isolated from chicken meat, collected from Nonthaburi, Thailand during 2002. The top five serotypes of patients isolates were Enteritidis (22%), Typhimurium (11%), Weltevreden (8.8%), Rissen (8%), and Choleraesuis (6.6%) while the top five serotypes of chicken meat isolates were found as follows: Schwarzengrund (11.91%), Hadar (11.11%), Rissen (8.73%), Amsterdam (7.94%), and Anatum (7.94%). *Salmonella* strains were most resistance to the class of antibiotics that act as inhibitor to nucleic acid synthesis such as antifolates group (Trimethoprim;SXT) and fluoroquinolones (Nalidixic acid; NA, Ciprofloxacin; CIP),while the β lactam antibiotic was more effective, i.e. the 3rd gen cephalosporin (Ceftazidime; CAZ, Cefotaxime ; CTX), Monobactam (Aztreonam; ATM) and carbapenams group (Imipenem; IMP, Meropenem; MEM). The role of class I integron element in transmission of the resistance gene was revealed by detection the gene cassette associated with a class 1 integron in plasmid preparation among 80% of the isolated strains. The gene cassettes containing resistant genes of dhfrA12 (resistant to trimethoprim) and aadA2 (resistant to streptomycin and spectinomycin), were detected more frequently in the resistant strains. These gene cassettes were likely to be transmitted via plasmid, as it could not be detected in genomic DNA.**

Keywords: *Salmonella* Enterica, Antibiotic-resistant, Integron type 1

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INTRODUCTION

Salmonella is a Gram-negative, rod-shaped, facultative anaerobic bacteria that belongs to the family Enterobacteriaceae. These bacteria comprise more than 2,500 serovars and more than 50 serogroup[1]. They are a medically important pathogen for humans and animals [2], and *Salmonella* infections can cause economic losses in animal industries [3]. Salmonellosis is one of the most common foodborne diseases (FBDs) worldwide, accounting for approximately 93.8 million foodborne illnesses and 155,000 deaths per year worldwide [4]. In Southeast Asia, based on data for the year 2010 from the report by FREG group [5], the leading cause of death due to FBDs in the region was *S.* Typhi (more than 32,000 deaths) and nontyphoidal *Salmonella* Enterica were also responsible for deaths due to FBD. That is, nearly 16,000 deaths occurred in the region during this time frame [6].

The primary reservoir of *Salmonella* is the intestinal tract of animals, particularly of poultry and swine. They are also the major reservoirs of non-Typhi serotypes of *Salmonella* Enterica [7,8]. The extensive use of antibiotics during the past 50 years by humans and in livestock production has led to antimicrobial resistance (AMR) among several bacterial strains associated with farm animals, and such resistance has become a global phenomenon [9]. Recent update from Wikipedia; https://en.wikipedia.org/wiki/Category:Antibiotics, was categorized antibiotic into 3 groups. As shown in Table 1, the first group was β-lactam antibiotics, the second group was Antibacteria that act as inhibitor on protein synthesis, and the third was Antibacteria that act as inhibitor on DNA and RNA synthesis. All β-lactam antibiotics bind to PBPs (Penicilin Binding Protein), which are essential for bacterial cell wall synthesis. PBPs are members of a subgroup of enzymes called transpeptidases. β-lactam antibiotics were divided into 6 groups, i.e. Penicillin (Penams), Carbapenems, β-lactam combination, Cephalosporin, Monobactams. Antibiotics in the Penicillin and Cephalosporin group were developed into 4 generations.

The MDR in *Salmonella* has been linked to a higher risk for hospitalization and death, and resistance to fluoroquinolones and extended-spectrum cephalosporins (ESCs) imperil the dispensation and treatment options for other severe infections [10]. The recommended drugs of choice for the treatment of salmonellosis are fluoroquinolones and third-generation ESCs [11].

The major route of acquisition and transmission of a drug-resistance gene is through mobile elements, including plasmids and gene cassettes in integrons [12]. Integrons, first described at the end of the 1980s, are complex mobilome that promote the horizontal transfer of class 1 integrons inside and between bacterial species [13]. Integrons were divided into four classes [14]. The amino acid sequences of IntI integrases have been used as a basis for dividing integrons into "classes," with those carrying intI1 defined as "class 1," intI2 defined as "class 2," and intI3 defined as "class 3." The integrases intI1, intI2, and intI3 were first identified to be associated with mobile genetic elements, whereas IntI4 and others were identified as chromosomal integrons [15]. The class 1 integron is the most prevalent, and it is also clinically important in that it is a broadly distributed integron that is found in 22%–59% of Gram-negative bacteria. These integrons include two conserved segments (5'CS and 3'CS) separated by a variable region that consists of three essential core features: 1) integrase, a member of the tyrosine recombinase family, encoded by *intI*, which catalyzes recombination of captured gene cassettes, 2) a primary integron-associated recombination site, attI, and 3) an integron-associated promoter, Pc, which lies between attI site and *intI* gene [16,17,18]. The promoter (P_c), located upstream of the integration site, which is necessary for the efficient transcription and expression of gene cassettes presented in the integron [13,19]. Open reading frames are specific sites that contain a modular structure called a gene cassette [20]. Gene cassettes contain zero to six genes that associate integrons to multidrug resistance (MDR) [21]. The cassettes are composed of the most common antibiotic resistance genes and an integrase-specific recombination site. They can also exist as either free in a circularized form or integrated at the *attI* site. They are only formally part of an integron when integrated as a cassette [19].

In Thailand, utilization of antimicrobial agents in farm animals as a feed additive is not always carried out under proper veterinarian

supervision. The antibiotic pressure was selected for survival of antibiotic resistant strains, particularly MDR strains of *Salmonella* that have been isolated in the food chain [22, 23 , 24].This process may also increase the resistance of certain bacteria and in the level of antimicrobial residues in animal products. Class 1 integrons may be involved in MDR *S. enterica* that were isolated from chickens, pigs, and their products in the Thailand–Cambodia border provinces [25]. *Salmonella* infection is the second most common cause of diarrhea in Thailand; however, data on Antimicrobial Resistance (AMR) are limited [26]. Many *Salmonella* strains were isolated from fresh

Table 1. List of antimicrobials and the concentration of drugs used to determine inhibition zones

Class of antibiotic			Antibiotic drugs	Abbreviation	Concen. (µg/ml)
1 β-lactam antibiotics bind to PBPs					
1.1 Penicillin (Penams)					
Narrow spectrum	1st gen	β-lactamase sensitive			
	2nd gen	β-lactamase resistant			
Extended spectrum	3rd gen	Aminopenicillins	Ampicillin	AMP	10
	4th gen	Carboxypenicillins			
1.2 Carbapenems			Imipenem	IMP	10
			Meropenem	MEM	10
1.3 β-lactam combination			Amoxicillin–clavulanic acid	AMC	20/10
			Piperacillin/tazobactam	TZP	100/10
1.4 Cephalosporin	1st gen		Cephazolin	KZ	30
	2nd gen				
	3rd gen		Ceftazidime	CAZ	30
			Cefotaxime	CTX	
	4th gen		Cefepime	FEP	30
1.5 Monobactams			Aztreonam	ATM	30
1.6 β-lactamase inhibitors					
2. Antibacterials: protein synthesis inhibitors					
2.1 Binding 30S		Aminoglycosides (initiation inhibitors)	Gentamicin	CN	10
			Amikacin	AK	30
		Tetracycline antibiotics (tRNA binding)	Tetracycline	TE	30
2.2 Binding 50S		Peptidyl transferase (Amphenicols gr.)	Chloramphenicol	C	30
3. Antibacterials: nucleic acid inhibitors					
3.1 Antifolates (inhibits purine metabolism, thereby inhibiting DNA and RNA synthesis)			Trimethoprim DHFR (Dihydrofolate reductase) inhibitor; antifolate	SXT	5
3.2 Fluoroquinolones (inhibits bacterial DNA gyrase and/or topoisomerase)			Norfloxacin	NOR	10
			Nalidixic acid	NA	30
			Ciprofloxacin	CIP	5

chickens bought from the market in Nonthaburi province; in addition, *Salmonella* isolated from patient in Bumrathnaradoon hospital in same province were obtained. The identified serotypes in each population may lead to the prediction whether *Salmonella* serotypes that cause human infection are derived from *Salmonella* serotypes found in chicken-meat or not. The drug-resistance profile among *Salmonella* isolates may support the drugs of choice for medical treatments. This study is attempted to confirm the presence of transferable genetic elements, class I integrons, and try to identify any gene cassette associated with this integron. Any identified resistance genes were likely to be widespreaded among enteric bacteria, and thus increase drug- resistant bacteria to ecosystem.

MATERIALS AND METHODS

Bacterial isolation

There were 137 *Salmonella* strains, isolated from the patients in the routine diagnostic laboratory of Bamratnaradoon Institute in Nonthaburi, Thailand, and were contributed to the research project of Assistant Professor Yuvadee Mahakhunkijcharoen, during the year 2002. In addition, the total of 126 *Salmonella* strains, were isolated from fresh chicken meat bought from the open market in Nonthaburi province in 2002. All of these isolates were kindly donated to this research project by Assistant Professor Yuvadee Mahakhunkijcharoen. *Salmonella* colonies were subcultured from glycerol stock cultures, which were kept at −70 °C. Each strain was reconfirmed as *Salmonella* through a biochemical test in a microbiology lab**.** The pure culture on selective media eg. MacConkey agar was inoculated to the basic enteric panel media as follows, TSI: Triple Sugar Iron Agar, LIA: Lysine Iron Agar, MIO: Motility-Indol-Ornithine Agar, Urea: Urea Agar, Citrate (Simmons): Simmons Citrate Agar. The confirmed culture of *Salmonella* spp were sent to National Institute of Health (NIH), Thailand for serotyping.

Antimicrobial susceptibility testing.

Salmonella isolates were examined in accordance with CLSI criteria for susceptibility to 18 antimicrobial drugs using an agar disk diffusion method [27]. Antimicrobial susceptibility testing was conducted with Müeller-Hinton agar. The following antimicrobial agents were tested, of which the abbreviation name and indicated concentration of µg per disk are in parentheses. The class of available antibiotic drugs were listed and the common drugs included in this study were indicated in Table 1. The first class was β-lactam that could bind to PBPs (Penicilin Binding Protein), which are essential for bacterial cell wall synthesis. Ampicillin (AMP, 10) was the 3rd generation of extended spectrum included in Penicillin group. Imipenem (IMP, 10) and meropenem (MEM, 10) were included in Carbapenems group. Amoxicillin–clavulanic acid (AMC, 20/10), piperacillin/tazobactam (TZP, 100/10) were in β-lactam combination group. Ceftazidime (CAZ, 30) and cefotaxime (CTX, 30) were the third generation of Cephalosporin group, while the fourth generation, cefepime (FEP, 30), was also included in this group. Aztreonam (ATM, 30) was included in Monobactams. The second class was antibacterial agent that inhibit protein synthesis. Gentamicin-aminoglycosides (CN, 10), amikacin (AK, 30) and tetracycline (TE, 30) were bound to 30S ribosomal RNA to inhibit protein synthesis. Chloramphenicol (C, 30) was peptidyltransferase that bound to 50S ribosomal RNA. The third class was antibacterial agent that act as nucleic acid inhibitor, composed of Antifolates and Fluoroquinolones group. SXT (5), trimethoprim / sulfamethoxazole DHFR (Dihydrofolate reductase) was in Antifolates acted as inhibitor of purine metabolism,thereby inhibiting DNA and RNA synthesis. While Norfloxacin (NOR), Nalidixic acid (NA), and Ciprofloxacin (CIP) were included in Fluoroquinolones group.

Escherichia coli ATCC 25922 was used as a control for the susceptible strain. The *Salmonella* isolates with MDR were defined as having acquired non-susceptibility to at least one agent in three or more antimicrobial categories [28, 29].

Plasmid DNA extraction

Salmonella isolates were grown on TSA overnight, and bacterial cells were scraped into 5 ml of Normal Saline Solution (NSS). After centrifugation for 2 min at 10,000 rpm, the pellet was collected for further plasmid DNA extraction. Plasmid DNA was extracted with a Plasmid MiniPrep Kit (ver. 2.0; BioFact, Korea) following the manufacturer's protocol. The purified plasmid DNA was identified with gel electrophoresis in 0.4%

agarose, based on GeneRuler High Range DNA Ladder (Catalog Number SM1351, Fermentas_ ThermoFisherScientific, USA), with the MW marker ranged from 10 to 48 kb, and the obtained DNA was stored at −20 °C for use in further molecular investigations.

Detection of class I integrons and their associated gene cassettes

The specific integrase gene, *intI1*, was identified with primers described by Mazel [30]. The DNA regions of class 1 integrons were screened with PCR using primers of the hep series (Table 1), as described by White [31]. In addition, the primers in the 5' and 3' conserved regions, upstream from the class I integrons, were used as described by Ploy [32]. The PCR product of any gene cassette associated with class I integrons were subjected to DNA extraction from the gel and then subjected to cloning into plasmid vector, in order to determine sequences of the inserted DNGene cloning of PCR products for DNA sequencing

PCR products of the target gene were cloned into pSC-A-amp/kan PCR cloning vectors (StrataClone PCR Cloning Kit, Agilent Technologies, USA, Canada) in accordance with the manufacturer's protocol. Briefly, the inserted PCR product of 2 µl was ligated to 1 µl of the StrataClone Vector Mix and 3 µl of the StrataClone Cloning Buffer. The ligated product was then transformed to StrataClone SoloPack competent cells. The PCR, based on M13 forward and M13 reversed primers, were used to screen colonies with the inserted DNA. The colonies with inserted DNA larger than 1 kb were selected for plasmid preparation, and DNA sequencing was performed using the sequencing primers of the cloning vector.

Data analysis

Microsoft Excel was used to process the descriptive data. The DNA sequence data were assembled as contigs by the SeqMan Program, DNAStar software (Lasergene 8.0, DNAStar Inc., Madison, WI, USA), and Demo Sequencher software version 4.5 (http://www.genecodes.com/sequencher-feature). The nucleotide sequences were compared online using the National Center for Biotechnology's Basic Local Alignment Search Tool (BLAST) software.

RESULTS

***Salmonella* serotypes found in this study**

There were 137 *Salmonella* strains, isolated from the patients in the routine diagnostic laboratory of Bamratnaradoon Institute in Nonthaburi, Thailand, and were contributed for this study. Likewise the 126 *Salmonella* strains were isolated from chicken meat collected from the markets in Nonthaburi province,Thailand during the same period of the year 2002 by Dr. Yuvadee Mahakhunkijcharoen of the Department of Microbiology and Immunology and collected as bacterial stock at the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University. Microbiological techniques identified all *Salmonella* isolates as *S.* Enterica species, whereas serotype identification was conducted by the National Institutes of Health, Thailand. The prevalence of the serotypes among these isolates were included in the 35 serotypes, and two isolates were the non-typable strain from patients meanwhile the 29 serotypes, and one isolate was a non-typable strain from chicken meat. The top five serotypes of patients isolates were found frequently, and their percentages of incidence were as follows: Enteritidis (22%), Typhimurium (11%), Weltevreden (8.8%), Rissen (8%), and Choleraesuis (6.6%) while the top five

Table 2. Primers for the detection of class 1 integrons

Gene	Primer	Oligonucleotide primer sequence 5' to 3'; strand direction (+/-)	Strand	Expected size (bp)	References Mazel, 2006 [24]
Integrase I enzyme	intI1L	ACATGTGATGGCGACGCACGA	+	v (varia-ble)	
	intI1R	ATTTCTGTCCTGGCTGGCGA	-		
Class I integrons	hep58	TCATGGCTTGTTATGACTGT	+	v	[25]
	hep59	GTAGGGCTTATTATGCACGC	-		
	5'CS	GGCATCCAAGCAGCAAG	+	v	Siebor, 2016 [39]
	3'CS	AAAGCAGACTTGACCTGA	-		

serotypes of chicken meat isolates were found as follows: Schwarzengrund (11.91%), Hadar (11.11%), Rissen (8.73%), Amsterdam (7.94%), and Anatum (7.94%). The other serotypes shown in Table 3 were further identified in different percentages ranging from 0.7 - 4.4% in patients

Table 3. Serotypes of Salmonella Enterica isolated from patients and chicken in Thailand during 2002.

No.	Serotypes	Patients		Chicken meat	
		Isolates (137)	(%)	isolates (126)	(%)
1	Enteritidis	30	22	7	5.56
2	Typhimurium	15	11	4	3.18
3	Weltevreden	12	8.8	3	2.38
4	Rissen	11	8	11	8.73
5	Choleraesuis	9	6.6	-	-
6	1,4,12:1:-	6	4.4	1	0.79
7	4,1:i:-	4	2.9	-	-
8	Hadar	4	2.9	14	11.11
9	Panama	4	2.9	3	2.38
10	Albany	3	2.2	5	3.97
11	Brandenrup	3	2.2	-	-
12	Lexington	3	2.2	1	0.79
13	Stanley	3	2.2	5	3.97
14	4,5,12:i:-	2	1.5	-	-
15	Agona	2	1.5	3	2.38
16	Amsterdam	2	1.5	10	7.94
17	Cerro	2	1.5	-	-
18	Schwarzengrund	2	1.5	15	11.91
19	Virchow	2	1.5	7	5.56
20	4,5,12,i:-	1	0.7	-	-
21	4,12:i:-	1	0.7	-	-
22	Anatum	1	0.7	10	7.94
23	Mbandaka	1	0.7	2	1.59
24	Dublin	1	0.7	-	-
25	Heidelberg	1	0.7	-	-
26	Hvittingfoss	1	0.7	-	-
27	Isangi	1	0.7	-	-
28	Javiana	1	0.7	-	-
29	Kentucky	1	0.7	2	1.59
30	Newport	1	0.7	1	0.79
31	Orion	1	0.7	2	1.59
32	Pakistan	1	0.7	-	-
33	Paratyphi B var Java	1	0.7	-	-
34	Saintpaul	1	0.7	3	2.38
35	Thomson	1	0.7	-	-
36	Blockley	-	-	4	3.18
37	Chester	-	-	2	1.59
38	Emek	-	-	2	1.59
39	Give	-	-	2	1.59
40	Senftenberg	-	-	2	1.59
41	Bovismorbificans	-	-	2	1.59
42	Derby	-	-	1	0.79
43	Krefeld	-	-	1	0.79
44	Sandiego	-	-	1	0.79
45	unknown	2	1.5	1	0.79

Table 4. Percentage of Salmonella strains, isolated from human and chicken meat, that resisted to different classes of antibiotic based on disk diffusion assay

Class of antibiotics based on mode of action	cell wall synthesis inhibitord (β-lactam)												Protein Synthesis inhibitor										Nucleic acid inhibitors					
	Penicillin		β-lactam combination				Cephalosporin				Monob-actams		Aminog-lycosides		Tetra cycline		Peptidyl transferase		Antifolates		Fluoroquinolones					
	AMP		AMC		TZP		KZ		CTX		ATM		CN		AK		TE		C		SXT		NOR		NA		CIP	
Source	H	C	H	C	H	C	H	C	H	C	H	C	H	C	H	C	H	C	H	C	H	C	H	C	H	C	H	C
No. of resistant isolates	62	36	3	2	2	0	11	12	1	0	0	2	17	7	0	1	39	64	28	23	41	34	3	0	40	73	6	2
Percentage (%) of incidence	45.3	28.6	2.2	1.6	1.5	0	8	9.5	0.7	0	0	5.6	12.4	5.6	0	0.8	28.5	51	20.4	18.3	30	27	2.2	0	29.2	58	4.4	1.6
%Total for human					57.7											61.3								65.8				
%Total for chicken meat					41.3											75.7								86.6				

Sources H= Human (126 isolates) ; C= Chicken meat (137 isolates)

isolates and 0.79% to 5.56% in chicken meat isolates.

Multi Drug Resistance (MDR) profiles of *Salmonella* isolates

A total of 137 and 126 *Salmonella* strains that, isolated from patients and chickens respectively were analyzed, and their resistance to 18 antibiotics were determined with a disk diffusion assay. Among *Salmonella* isolates from patients, 69.34% were resistant to antibiotics and 10.95% were susceptible to antibiotics, while among chicken meat- isolates, 86.5% were resistant to antibiotics and 13.5% were susceptible to antibiotics. The growth of *Salmonella* strains was partially inhibited by some antibiotic drugs and these cases were assigned as having intermediate effect, of which 19.7% were found in *Salmonella* isolates from patients and 12.7% of *Salmonella* isolates from chicken.

As summarized in Table 4, based on the mode of action of antibiotic, that were divided into 3 classes based on their action as inhibitor on 3 metabolic pathway; cell wall synthesis, protein synthesis and function of nucleic acid. Among *Salmonella* strains isolated from patients**,** 65.8% were mostly resistant to antibiotics that act as nucleic acid inhibitor, i.e. 30% were resisted to antifolates group of trimethoprim (SXT), while among fluoroquinolones group; 29.2% were resistant to nalidixic acid (NA), 4.4% were resistant to ciprofloxacin (CIP) and 2.2% were resistant to norfloxacin (NOR).

The second rank of resistant drugs were included in group of protein synthesis inhibitors, of which 61.3% of *Salmonella* isolates were resistant to the following drugs, i.e. 28.5% were resisted to tetracycline (TE), 20.4% were resisted to peptidyl transferase group of chloramphenicol (C) and 12.4% were resisted to aminoglycosides group of gentamicin (CN) . For the group of cell wall synthesis inhibitors, 57.7% of *Salmonella* strains were resistant to β lactam antibiotic or, including 45.3% were resisted to penicillin group of ampicillin (AMP). For cephalosporin group, 8% were resisted to cefazolin (KZ);1st gen cephalosporin; and 0.7% were resisted to cefotaxime (CTX) of which included in the 3rd gen cephalosporin. The *Samonella* isolates of 2.2% and 1.5% were resisted to amoxicillin–clavulanic acid (AMC) and piperacillin/tazobactam (TZP) of β-lactam combination group.

Among *Salmonella* strains isolated from chicken meat**,** 86.6% of them were mostly resistant to antibiotics that act as nucleic acid inhibitor, i.e. 27% were resisted to antifolates group of SXT (trimethoprim), while among fluoroquinolones group; 58% were resistant to nalidixic acid (NA) and 1.6% were resistant to ciprofloxacin (CIP). The second rank of resistant drugs were included in group of protein synthesis inhibitors, of which 75.7% of *Salmonella* isolates were resistant to the following drugs, i.e. 51% were resisted to tetracycline, and 18.3% were resisted to chloramphenicol (C). In addition, few strains were resistant to aminoglycoside drug, i.e. 0.8% and 5.6% to amikacin (AK) and CN (gentamycin) respectively. There were 41.3% of *Salmonella* strains that resistant to β lactam antibiotic or a group of cell wall synthesis inhibitors, including 28.6% were resisted to ampicillin, 1.6% was resisted to Amoxicillin–clavulanic acid (AMC) of β-lactam combination class, 9.5% were resisted to cephazolin (KZ) of cephalosporin group and 1.6% was resisted to aztreonam (ATM) of monobactams group. All *Salmonella* isolates were susceptible to Ceftazidime (CAZ), of which included in the 3rd gen cephalosporin. In addition, they all susceptible to carbapenems drug, including Imipenem (IMP) and Meropenem (MEM).

In summary, as shown in Table 4, we found that *Salmonella* strains were most resistance to the class of antibiotics that act as nucleic acid inhibitors such as antifolates group (SXT) and fluoroquinolones (NA, CIP), and the moderate resistance was also found among the drug that act as inhibitor to nucleic acid synthesis, while the β lactam antibiotic seem to be more effective in treatment. All *Salmonella* isolates of that time were susceptible to Ceftazidime (CAZ), the 3rd gen cephalosporin and carbapenems drug.

In this study, *Salmonella* strains that were resistant to more than 3 classes of antibiotics demonstrating as multidrug resistance (MDR). For patient and chicken meat isolates, there were 74 (54.02%) and 45 (36%) isolates showed multidrug resistance, of which were varied from 3 to 7 drugs. The MDR pattern can be divided into 47 and 37 profiles in patient and chicken meat – isolates

Table 5. Number of *Salmonella* strains from patients and chicken meat in Thailand resisted to antibiotics

Antibiotics	Patients		Chicken	
	Number of Isolate(s)	Percent (%)	Number of Isolate(s)	Percent (%)
multidrug resistance				
7	2	1.46	1	0.79
6	9	6.57	2	1.59
5	12	8.76	9	7.14
4	19	13.87	14	11.11
3	32	23.36	19	15.1
total	74	54.02	45	35.73
resistance to one or two drugs				
2	13	9.49	35	27.78
1	18	13.14	29	23.02
total	31	22.63	64	50.79
susceptible/intermediate				
total	32	23.36	17	13.49
Total number of isolates	137	100	126	100

respectively, data shown in Supplement Table **1.** The drug resistant profile of 3 drugs and 4 drugs patterns were mostly found in 33 (24.1%) and 19 (14%) of *Salmonella* isolates from patients, while chicken meat were found in 19 (15.1%) and 15 (11.9%) isolates respectively. Furthermore, the MDR profile of 5 and 6 drugs patterns were found in 12 (8.8%) and 9 (6.7%) of *Salmonella* isolates from patient, while in chicken meat were found as 8 (6.4%) and 2 (1.6%) respectively (Table 5). Lastly, the MDR profile of 7 drugs found only in 2 isolates (1.5%) of serotype Schwarzengrund and 4,12:i:- from the patient, while only in 1 isolate (0.8%) of serotype Albany was from chicken meat.

The strains, resistant to 1-2 drugs of different patterns were shown in Supplement Table 1. Among isolates from patients, the resistant profile to 1 antibiotic were detected in 18 isolates (13.14%) and most were presented as resistant to NA (17 isolates) while resistant profile to 2 antibiotics was detected in 13 isolates (9.5%). Among isolates from chicken meat, the resistant profile to 1 antibiotic was detected in 29 isolates (23.02%) and mostly were presented as resistance to NA (17 isolates) as were frequently observed in serovar Amsterdam while resistant profile to 2 antibiotics was detected in 35 isolates (27.78%). The most prevalence was resistance to in NA and TE (22 isolates) and were frequently observed in serovar Hadar.

Identification of integron class 1 from *Salmonella* plasmids

Plasmids were extracted from all *Salmonella* Enterica isolates as DNA templates, and their sizes were larger than 20 kb, based on a high-range DNA marker (Fermantas; Thermofisher, USA). The detection of the integron 1 platform was performed using primers derived from the 5' and 3' conserved regions. A single band or a few bands were observed among the isolates, and the sizes of the PCR products were ranged from 0.5 kb to 3 kb. PCR products could be detected in 102 of the 126 *Salmonella* isolates (80.95%). When the primers that used to detect the integron I part was used to amplify genomic DNA as template, no product was detected, suggesting that the integron I part was presented in plasmid only. Some selected PCR products obtained from integron I units that were larger than 0.8 kb were purified and cloned into TA cloning vectors (Stratagene, USA) to identify any antibiotic resistance genes that could be associated with the integron I unit. The plasmids with the inserted gene were sequenced.

Although PCR product associated with integron type I were detected in most *Salmonella* isolates, the gene cassettes of PCR product larger

Table 6. Gene cassettes associated with integron 1 found in plasmids of Salmonella Enterica, isolated from human and chicken meat

Strains_Serotype	Host	Size (bp)	Resistance - Pattern	Accession no	Identified genes and proposed function
7-14_ Typhimurium	human	1932	AMP, CN, TE, SXT, NA	MT 884062	-DhfrXII ;Dihydrofolate reductase; confer resistance to trimethoprim -AddA2 ;Streptomycin 3' adenyltransferase; confer resistance to streptomycin and spectinomycin
24-7_ Typhimurium	human	1932	AMP, CN, TE, C , SXT, NA	MT 936291	Same profile as strain 7-14
13-3_ Stanley	human	1931	AMP, TE, C, SXT	MT 911987	Same profile as strain 7-14
18-2_ Stanley	human	1932	TE, C ,SXT	MT 911988	Same profile as strain 7-14
36-6_ I,1,4,12:i:-	human	1932	AMP, CN, TE, C , SXT, NA	MT 993752	Same profile as strain 7-14
70-3_ I,1,4,12:i:-	human	1932	AMP, TE, SXT, NA	MT 993753	Same profile as strain 7-14
72-1_ Albany	human	1228	AMP, SXT	MT 993759	blaPSE-1; PSE-1 beta-lactamase
83-1 _ Schwarzengrund	human	1216	AMP, KZ, CN, TE,C, SXT, NOR	MW 008868	blaCARB-2; PSE family carbenicillin-hydrolyzing class A beta-lactamase
96-6_ Rissen	human	1932	SXT, NA	MW 008869	Same profile as strain 7-14
NIH20-9 _Rissen	human	1933	AMP, KZ, SXT, TE, CN	MW 075229	Same profile as strain 7-14
101-5 _Anatum	human	1932	AMP, SXT	MW 013510	Same profile as strain 7-14
108_2_ Agona	human	1934	TE, C, SXT, NA	MW 023089	Same profile as strain 7-14
NIH18-2_ Agona	human	1933	AMP, SXT, KZ, CTX, CAZ, FEP, ATM, TE, CN,C	MW 059029	Same profile as strain 7-14
119-2_ Cholerasuis	human	1932	AMP, TE, NA	MW 030628	Same profile as strain 7-14
90-15_ Senftenberg meat	Chicken	1931	AMP, TE, SXT, NA	MW 075230	Same profile as strain 7-14

than 1.5 kb were selected to clone into PCR cloning kit containing T-tailed plasmid vector. The PCR inserted of 1932 bp was successfully cloned and sequenced from the following serotypes; 2 strains, 7-14 and 24-7 of serotype Typhimurium; strain 13-3 and 18-2 of serotype Stanley; strain 36-6 and 70-3 of serotype I,1,4,12:i:-; strain 96-6 and NIH20-9 of serotype Rissen; strain 108-2 and NIH18-2 of serotype Agona; strain 119-2 of serotype Cholerasuis and strain 72-1 of serotype Albany and lastly, strain 101-5 of serotype Anatum. These gene cassette of 1932 bp insert, contained 2 complete open reading frames; 498 bp of *dhfrXII*; dihydrofolate reductase that confer resistance to trimethoprim (SXT) and 792 bp of streptomycin 3' adenyltransferase that confer rsistance to streptomycin and spectinomycin. The integron cassette from all of these *Salmonella* serovars were

similar to the *Aeromonas hydrophila* strain EG9 with 99%–100% similarity, of which the sequence was deposited in GenBank with accession no. KF442255.1. Most of the *Salmonella* strains, that possessed the gene cassette of dihydrofolate reductase could resist to SXT drug, except for the strain 119-2 of serotype Cholerasuis.

Furthermore, strain no. 72-1 of serotype Albany and strain 83-1 of serotype Schwarzengrund contained insert size of approximately 1200 bp, that was similar to *bla*CARB-2 gene, which codes for carbenicillin-hydrolyzing β-lactamases (also named CARB enzymes) that was first identified from *Pseudomonas aeruginosa* [33]. The *bla*CARB genes are narrow-spectrum class A penicillinases and are known to be widespread among distantly related bacteria, including *Vibrio* spp. and *Salmonella* [34]. Their location on mobile genetic elements as a form of gene cassettes of class 1 integrons, confirmed the widespread of this element.

DISCUSSION

Prevalence of *Salmonella* serovars.

In this study, during 2002, the top five serotypes of patients isolates were found frequently, and their percentages of incidence were as follows: Enteritidis (22%), Typhimuriumb (11%), Weltevreden (8.8%), Rissen (8%), and Choleraesuis (6.6%). The human specimens were derived from varied sources such as feces, urine, blood etc. During 2011-2013 in Siriraj Hospital, Bangkok, Thailand, the available blood isolates were identified as *Salmonella* Choleraesuis (34.2%) was the most common serotype, and *S*. Enteritidis (31.5%) ranked second, accounting for 65.8% of all isolates [35]. *Salmonella* isolates, identified during the year 2002, also had the majority as Enteritidis (22%), although the less number of Cholerasuis (6.6%) were found in that earlier time point.

There were 126 *Salmonella* strains, isolated from chicken meat collected from markets around Nonthaburi province of Thailand in 2002. These *Salmonella* population composed of 26 serovars, of which most common serovars, included Schwarzengrund (11.9%), Hadar (11.1%), Rissen (8.7%), Amsterdam (7.9%), and Anatum (7.9%). Most serovars found in chicken were able to infect human, as they are also found to infect paients in this study. The results are consistent with the study of *Salmonella* prevalence in chicken meat from markets in Bangkok and the central region of Thailand in 2015, which mostly included serovar Schwarzengrund [36]. As with the report from samples collected in five provinces in northeastern Thailand during 2010–2013, the diversity of *Salmonella* isolates from chickens included 17 serovars, and the most common serovars were Schwarzengrund (23.8%) and Rissen (16.3%) [37]. Furthermore, between 1993 and 1994, Boonmar [38] found *S. enteritidis* most frequently, both in samples isolated from frozen chicken in the northeast and from people in Bangkok, Thailand.

Antibiotic resistance of *Salmonella* isolates

This study in 2002, the most resistant drugs were NA (fluoroquinolones); that was found in 58% of the *Salmonella* isolates, TET in 51% and AMP (β lactam) in 28.6% of the *Salmonella* isolates. In Khon Kaen, northeast Thailand, Among *Salmonella* isolated from human patients,pork and chicken meat, the percentages that were resistant to TET were 92.6%, 88.5%, and 100%, respectively [22]. Likewise, during 2003–2005 in Bangkok, the resistance to TET was observed most often in *Salmonella* isolated from swine. Among the isolates from poultry, the highest frequencies of resistance were resistant to AMP and TET [39]. Among the nucleic acid inhibitor drug, *Salmonella* isolates from human, were quite resist to Antifolate drug; SXT (30%) and also resist to Fluoroquinolone drug; NA at 29%, the drug of choice for Fluoroquinolone drug were NOR and CIP that were resistant at 2.2 and 4.4% respectively. Among β-lactam antibiotic, *Salmonella* isolates were quite resistant to Ampicilin (45%), while they were all susceptible to Carbapenems group (IMP, MEM). In β-lactam combination group, *Salmonella* were resistant to AMC and TZP at 2.2 and 1.5% respectively, then could be the drug of choice for treatment. Lastly, Cephalosporin group (CAZ, CTX) were effective drug, as *Salmonella* has less percentage of resistance. In this study, only 0.7% of Salmonella- human isolates were resistant to CTX, while isolates from Siriraj Hospital had 15.2% resistance to CTX [35].

Prevalence of class 1 integrons

In our study, the plasmids that were extracted from 126 *S.* Enterica isolates and the gene cassettes associated with the class 1 integron region were detected in 80.95% of the isolated

strains when analyzed with primer pair 3'CS–5'CS. The integrase 1 (*IntI1*) gene was detected in the genomic DNA of all *Salmonella* isolates, whereas the detection of integron cassettes were only found in plasmid, suggesting the transmission of MDR cassettes via the plasmids. Several reports indicated that class 1 integrons, such as Miko [9], was the most prevalent, and class 1 integrons were detected in 65% of the multidrug-resistant *Salmonella* isolates, comprising 16 different serovars. Van [40] found class 1 integrons in 76% and 43% of the *E. coli* and *Salmonella* isolates, respectively. Similarly, Sinwat [37] found that 41% of the *Salmonella* isolates were positive for *intI1*, of which 60% carried class 1 integrons. These reports emphasize the important role of class 1 integrons in the dissemination of drug-resistant genes.

We mostly focused on *Salmonella* strains that had the size of PCR products in the range of approximately 1 kb to 2 kb, in accordance with previous reports [37, 41-43]. PCR fragments were purified and cloned into plasmid vectors and then sequenced using the primers of the vector. DNA sequences were analyzed with BLAST to find its homologue. In this study, the gene cassettes *dhfrA12* and *aadA2* were identified in the MDR strains of serovars Typhimurium, Stanley, I,1,4,12:i:-, Rissen, Anatum, Agona, Cholerasuis, Senftenberg, while they were also had STX – resistant profile. Strain 96-6 of serovars Rissen and strain 101-5 of serotype Anatum were both possessed the same gene cassettes of dhfrA12 and aadA2, and they were also resistant to STX and another drug. Strain no. 72-1 of serotype Albany and strain 83-1 of serotype Schwarzengrund possessed the PSE-1 beta-lactamase gene associated to integron type I, while they both also were resistant to Ampicilin drug. The presence of integron associated - resistant gene may then influence drug-resistant profile of bacteria.

A few types of drug-resistance genes associated with integron I were reported to exist among the *Salmonella* isolates of Thailand. In Bangkok, during 2003–2005, Khemtong [42] reported that the *intI1* gene was presented in 54 isolates and 33 carried gene cassettes with sizes ranging from 0.7 to 2.3 kb. The sequence analysis showed that resistance genes, including *bla*PSE-1, *dfrA1*, *dfrA12*, *aadA2*, *aadA4a*, and *silB*, were presented and the gene cassette array *dfrA12*- was the most prevalent among the isolates in serovars Kentucky, Anatum, Stanley, Eppendorf, Typhimurium, Rissen, Schwarzengrund, and Weltevreden. In addition, these genes were located in the plasmids and were successfully transferred to other bacterial isolates from Khemtong [42].

In recent decades, class 1 integrons are known to play a major role in the acquisition and dissemination of AMR through horizontal transmission to the spread of antibiotic resistance genes in Gram-negative bacteria [44, 45]. Most *dfr* genes are found to reside in gene cassettes within the variable parts of integrons, which has resulted in the rapid spread of SXT resistance in various bacteria. In Thailand, Sinwat [37] detected the *dfrA12-aadA2* cassette from class 1 integrons in 66.7% of *Salmonella* isolates in pork. Likewise, in this study, *dfrA12* and *aadA2* genes were frequently detected, which suggested that SXT and Str/spectinomycin resistance genes could spread among enteric bacteria. This study reveals that class 1 integrons may play a role in the acquisition and dissemination of AMR through the horizontal transmission and spread of antibiotic resistance genes among *Salmonella* serovars within the food chain. The antibiotic drug; SXT and Str/spectinomycin should not be effective as the resistance genes to these drugs were likely to be widely spread among Gram-negative bacteria.

SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at https://doi.org/10.22207/JPAM.14.4.XX

Additional file: Additional Table S1.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors designed the experiments. YM, PC, and NO performed the experiments. NO and TK analysed the data. NO and TK drafted the manuscript, compiled information from the literature, and designed the figures and tables. All authors read and approved the manuscript.

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ETHICS STATEMENT

Research on human specimens were previously collected, that were non-identifiable was exempted by Faculty of Tropical Medicine, Ethical Committee; TMEC 18-063

DATA AVAILABILITY

The serotype of each isolate and its drug resistant profile was included in Supplement Table 1. The DNA sequences of identified gene associated with integron type I for each isolate was deposited in NCBI of which its accession was included in Table 6.

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