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ANTIMICROBIAL RESISTANCE IN *SALMONELLA* SPP. ISOLATED FROM CHICKEN FARMS IN CENTRAL THAILAND

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List of Abbreviations

bp   base pair

CLSI   Clinical and Laboratory Standards Institute

DNA   deoxyribonucleic acid

EFSA   European Food Safety Authority

et al.   *et allii*

ISO   International Standard Organization

$MgCl_2$ magnesium chloride

MIC   minimum inhibitory concentration

ml   millilitre

mM   millimolar

NIAH   National Institute of Animal Health

OIE   Office International des Epizooties

PCR   polymerase chain reaction

UV   ultraviolet

WHO   World Health Organization

^{o}C Degree Celsius

µg   microgram

µl   microliter

µM   micromolar

%   percent

Foreword

This investigation was carried as part of the final internship in the 6th year of the Integrated Master in Veterinary Medicine of Instituto de Ciências Biomédicas de Abel Salazar – Universidade do Porto.

As a final year student, I was involved in two different internships. In the first four months I worked as an intern at the Laboratory of Microbiology in ICBAS. The investigation conducted during this period aimed to identify and study the presence of resistant bacteria in clinical surfaces and medical instruments at the Veterinary Hospital of University of Porto – UPVet. I had the opportunity to collect myself the samples, to culture them, to learn and practice different microbiologic and molecular techniques. The results of the investigation were presented to the staff, including veterinary nurses and doctors. The experience itself was quite enrichment since I obtained good scientific knowledge that allowed to be more confident and proactive for the next stage of my internship.

For the second part of my internship, I was an ERASMUS exchange student sponsored by the Lotus Project (Unversity of Gent) in the Faculty of Veterinary of the University of Kasetsart in Bangkok, Thailand. During this mobility period, I had the privilege of working as an intern in the National Institute of Animal Health (NIAH) in Bangkok, Thailand.

The theme's choice for the present study was due to the high importance of *Salmonella* spp. infections and also its close relation to antimicrobial resistance. Both issues have been extensively discussed nowadays, therefore, the constant necessity of scientific research related to these subjects still represents an important contribute to both animal and human health. Thailand is one of the biggest exporters of poultry meat in the world, thus foodborne infections related to *Salmonella* and the judicious use of antimicrobials are matters of concern. Having the opportunity to develop this thesis in Thailand, allow me a close view of the public policies followed to manage these risks. Also, it enabled to be enrolled in many different activities, which I would like to highlight the oral presentation at the "4th Symposium of Food Safety and Zoonoses for Asia Pacific".

The present work is divided in two sections; the first one is a brief revision of the general situation in Thailand related to *Salmonella* spp. infection, its effects in public health and relation with antimicrobial resistance. The second part refers to the practical work developed at the NIAH and summarizes the techniques, results and conclusions obtained from the tested isolates focusing on future perspectives.

Summary

Poultry production chain is comprised by grandparent and parent stocks, hatcheries and broiler farms. The aim of this study was to determine the minimum inhibitory concentration of 10 antimicrobials for *Salmonella* isolates obtained from environmental samples collected in six poultry farms in central Thailand between 2013 and 2014, and to identify the presence of *int1* in the tested isolates. *Salmonella* isolates (n=100) were firstly tested for serogroup, analyzed for MIC levels through the agar dilution method and amplified by PCR. Following the CLSI 2013 breakpoints, a considerable proportion of the isolates displayed resistance to nalidixic acid (81%), ampicillin (71%), sulfamethoxazole-trimethropim (54%), ceftadizime (38%), tetracycline (38%), enrofloxacin (30%), gentamicin (15%) and ciprofloxacin (6%). All isolates were susceptible to chloramphenicol and cefotaxime. Serogroup B (28%), C (35%), D (10%), E (26%) and G (1%) were identified. Antibiotics exhibiting higher MIC values were nalidixic acid (32 - ≥256 µg/ml), tetracycline (64 - ≥256 µg/ml), ampicillin (32 - ≥256 µg/ml) and sulfamethoxazole-trimpethropim (4/76 – 64/1216 µg/ml). Among the 100 isolates, 36 contained class 1 integron which displayed phenotypic resistances mostly against sulfamethoxazole-trimethropim, ampicillin and enrofloxacin in a rate of 62%, 44% and 43%, respectively. The results of this study show that *Salmonella* isolated from poultry farms in Thailand are still sensitive to the more recent groups of antimicrobials. This information may be useful to compare to other groups of poultry and to further studies of antimicrobial resistant genes distribution.

Acknowledgements

Many people were essential to the development of the present work, in different ways all of them contributed with their time, patience, knowledge and dedication.

Firstly, I would like to thank my advisor in the Faculty of Veterinary in Kasetsart University, Dr. Patamabhorn Amavisit, who was always present and never refused to help during my journey in Bangkok. I appreciate all the effort, ideas and concern about my work. The opportunity of presenting this study at the "4th Symposium of Food Safety and Zoonosis" in Chiang Mai, would not be possible without your support and initiative. For all the work developed, I will always be thankful to my advisor.

I also would like to thank to Professor Doutor Paulo Martins da Costa, my advisor at University of Porto. The patience and the constant availability to help me through the hard times always amazed me and for that I am very grateful. The excellence of your teaching is truly an example to be followed. To all the team at the Laboratory of Microbiology in ICBAS, for making the first part of my internship such a good experience. I am truly grateful for all your patience, dedication and help, to Dr. Ângelo Mendes, Dra. Lucinda Bessa, D. Elisabete Lopes and Sónia Azevedo.

Dr. Pacharee Thongkamkoon and all the NIAH staff were essential for being successful during my laboratory work in Thailand. The concerns about my work and useful advices were significant for this project. The experience of working at the National Institute of Animal Health in Bangkok revealed to be an amazing opportunity.

For being always very responsive and concerned about my permanence in Thailand I would like to thank Professor Mangkorn Rodprapakorn and Mrs Palita from the ISC (International Studies Center) at Kasetsart University. The work you have been developing with exchange students is impressive.

To the Lotus Project (University of Gent) for sponsoring my ERASMUS and enabling me to experience this great adventure in Asia.

Finally, I would like to thank to my family, specially my grandparents, Rui e Luísa for being one of the most important people in my life, and for always being present for me. You were essential in every step that I took in all these years; nothing of this would have been possible without you. Also, to my dear mother Patrícia, who is my biggest confident. Thank you for being so strong, for supporting me always wherever I go and in whatever I chose to do. Your support has been crucial in my success. To my uncles Ricardo and Rodrigo for always sharing with me what they know and for opening my eyes for different worlds, you guys are immensely important to me, I am so grateful for everything you do for me. To my aunt Tita, for having her door always open and for all the time she dedicated to me during

these years, thank you for all your strength. To everyone in my family that always support me and spoiled whenever I go home, having such loving people makes all the effort worthwhile when I am so far away from you.

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To my dear Nicky, he is one of the main reasons that made me realize how important is to take good care of our animals, his unconditional love is overwhelming.

All of you contributed much more than you think, you are part of me and a constant inspiration in my life.

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Literature Review

1. Salmonellosis

Enteric disease transmitted by *Salmonella* spp. is one of most common cause of diarrhea related to foodborne pathogens worldwide. Majowicz *et al.* (2010) estimated that there are around 94 million cases each year which result in 155.000 deaths due to Non-Typhoid *Salmonella* gastroenteritis. According to a report by WHO (2014), the majority of disease burden is in the South-East Asian and in the Western Pacific regions. Akbar *et al.* (2013) reported that the death toll only in South East Asia is around 37.600 per year and the economic burden of the disease estimated by EFSA (2014) is 3 billion EUR each year.

Salmonella spp. is widely distributed due its capability to multiply under various environmental conditions and ability to survive outside its living hosts. Contamination with *Salmonella* can happen through the food chain from livestock feed to food manufacturing, processing and retailing (Pui, 2011). Investigations of outbreaks and sporadic cases indicated that food vehicles were identified as the most common cause of *Salmonella* in humans, being poultry and derived products frequent sources in the transmission of the bacteria (Ibrahim *et al.,* 2013). At the farm context, *Salmonella* spp. can be found in varied founts such as litters, water supplies, feed, and in the hands of farm workers (Boonprasert, 2014). In parent stock farms, the drinking water was considered as the most contaminated source found (Sasipreeyajan *et al.,* 1996). Slaughterhouses and food markets showed to have higher rates of contamination compared to farms (Padungtod, 2006). This fact may be related to high levels of bacterial cross-contamination during the slaughtering process (specially defeathering and water chilling). In addition, stress during transport can increase *Salmonella* excretion before slaughtering (Boonprasert *et al.,* 2014).

The most prevalent *Salmonella* serovar in Thailand isolated from humans between 1999 and 2002 was *S.* Weltevreden (Bangtrakulnonth, 2004). Nevertheless, shifts in the prevalence of serotypes among time and from different sources have been described. In 1995 an increase of human salmonellosis due to *S.* Enteriditis was related to a concurrent higher prevalence of this serovar in Thai poultry (Padungtod, 2006). Variations in the prevalent serovars among the production chain were described in a study by Padungtod (2006), in which the most prevalent serovars in live chickens were *S.* Emek, *S.* Enteriditis and *S.* Rissen. The same study observed that the most common serovar among poultry farm workers were *S.* Weltevreden and *S.* Rissen. Differences in the prevalence of serovars between the north and south regions of Thailand were also encountered. Lertworapreecha *et al.,* (2013) reported that *S.* Albany was the most common serovar in chicken meat in the

south while Angkititrakul *et al.* (2005) found *S*. Anatum as being highly frequent in the northeast. A study by Sirichote *et al.* (2010) in the central region, revealed similar results to the north, where S. Anatum was mostly found in samples from chicken, pork and seafood.

2. Poultry Production Chain

The demands of poultry meat in Thailand grew in the last decades since it is considered the most affordable source of protein in the country. Approximately half of the total chickens in Thailand are raised in the central region due to the easy access to slaughterhouses, feed mills and food processing plants (NaRanong, 2007). Large-size farms with fully vertically integrated systems have become more common in comparison to small business farms due to the high demands of production (OIE, 2007a). The integrated system in poultry farms consists in single companies that own and are responsible for every aspect of the production chain, beginning from the import of stock breeders until the packing of meat for marketing purposes.

Breeders in Thailand are mainly imported from the USA and the UK (FAO, 2008). Imported breeders show faster growth rates, better feed conversion and larger meat yield compared to the native ones (FAO, 2008). Nevertheless, native breeds are considered to be more resistant to diseases and better adapted to environmental conditions which may be convenient to low income smallholder.

Due to the low export taxes and costs of production in the country, Thailand became the fourth largest exporter of poultry products in the world (OIE, 2007). Accordingly to the Thai Broiler Processing Exporters Association, in 2014 Thailand exported 578.886 MT of chicken meat to the main importers including EU (47%) and Japan (43%).

3. Antimicrobial Resistances & Public Health

In Thailand, antimicrobials have been extensively used in food animal production for decades (Chuanchuen et al., 2009). *Salmonella* spp. serovars and antimicrobials resistance rates were found to be similar between human, chicken and pork samples, suggesting that food producing animals may be a major cause of human salmonellosis and spreading of antimicrobial resistances in the country (Angkititrakul et al., 2005). A study by Gebre (2012) in Bangkok's markets, presented antimicrobial resistances in *Salmonella* isolates from chicken meat mainly in ampicillin (75%), amoxicillin (67%), sulfamethoxazole (67%), streptomycin (58%), tetracycline (50%), sulfamethozaxole-trimethropim (42%), kanamycin (33%) and gentamicin (8%).

Additionally, management factors were found to be decisive in the spread of resistance among poultry farms. Persoons *et al.* (2010) identified that hygienic conditions, acidification of drinking water, number of feed changes during the production cycle, hatchery sanitation, breed and litter material were involved. Treatment with amoxicillin was also reported to increase the spread of resistant bacteria in the environment. In parent stock farms, Sasipreeyajan *et al.* (1996) mentioned that the breeders' age should be considered a crucial factor in the dissemination of antimicrobial resistant bacteria among the production chain, being older breeders more problematic compared to younger ones since they are exposed to antimicrobial treatments for longer periods of time.

Public policies related to antimicrobial uses have been an important concern nowadays in Thailand. In 2000, the EU detected nitrofurans and dioxin in Thai broilers consequently leading to stricter importing rules in trade markets (FAO, 2008). After this incident, regulatory organizations such as the Thai Department of Livestock Department and the Thai Food and Drug Administration have required manufactures to submit applications whenever using feed additives with antimicrobials as growth promoters (Chuanchuen, 2009). In more recent years, the Food and Drug Administration in Thailand decided to ban definitely all antibiotics used for growth promoters in food animals due to higher control and biosecurity pressures mainly from the EU markets (Archawakulathep *et al.* 2014).

4. Integron Class I

Class I Integron were reported for the first time in the mid-1960s (Bennett, 1999). They can be located either on the bacterial chromosome or on broad host range plasmids (Dzidic *et al.,* 2008). These mobile genetic elements have the abilities to capture, excise and express genes, being considered an important genetic structure in the dissemination of antimicrobial resistance among gram-negative bacteria (Momtaz *et al.,* 2012). The structure of an integron class I consists of two highly conserved regions (5' CS and 3' CS), intercalated by a variable region that can contain resistance genes. The recombination-site where gene cassettes are inserted is defined by *attI* gene in the 5' conserved segment (Benett, 1999). Gene cassettes in general do not include a promoter, therefore, recombination events are mediated by an integrase enzyme which is encoded by the *int1* gene present in the integron (Recchia and Hall, 1995). Integrons with different combinations of gene cassettes conferring resistance to aminoglycosides, β-lactams, chloramphenicol and trimethoprim have been identified (Bennett, 1999); therefore integrons can combine several genes cassettes resulting in a variety of resistances to different antimicrobial groups.

Several studies (Randall *et al.,* 2004; Peirano *et al.,* 2006; Ribeiro *et al.,* 2011; Mahero *et al.,* 2013; Miko *et al.,* 2005) were conducted in order to identify class I integron in

Salmonella. All mentioned studies confirmed that integron class I was disseminated in resistant isolates. Peirano *et al.* (2006) reported that class I integron was present in 17 different *Salmonella* serovars; Mahero *et al.* (2013), showed that a sizeable proportion of multidrug resistance in *Salmonella* was related to class 1 integron, in which the *aadA1* and *dfra1* genes showed the highest frequency. Also, Miko *et al.* (2005) observed the occurrence and distribution of antibiotic resistance genes related to integron class I in food-borne *Salmonella* isolates and identified that the most prevalent serovar carrying the integron was *S.* Typhimurium. Although all the mentioned studies considered integron I as an important genetic mechanism for antimicrobial resistance in *Salmonella*, they also pointed that most of isolates didn't carry integron class 1. For that reason, other genetic mechanisms such as plasmids, transposons and phages were also responsible for a wide portion of antimicrobial resistance. Mahero *et al.* (2013) observed that up to 51.4% and 70% of multidrug-resistant *Salmonella* isolates from Uganda and North Dakota, respectively, did not have class 1 intregons; Peirano *et al.* (2006) and Randall *et al.* (2004) also showed that most resistance genes in their studies were located outside of the integron structure. Furthermore, integron class I is capable of integrating and expressing resistance genes in *Salmonella*, but it may not be considered as the main source of resistances in this bacteria. In Thailand few studies have been conducted in order to identify class I integron and its relation with *Salmonella* antimicrobial resistance. In 2012, a study by Chaisatit *et al.* in Bangkok markets found that 42.9% of *Salmonella* spp. contaminated chicken meat harbored class I integron genes.

Introduction

Salmonella can be transmitted horizontally and vertically among the poultry production chain, spreading the bacteria from "farm to fork".

From an epidemiological perspective, parent-stock farms are considered a crucial point since they represent the top of the industry chain. Breeders can carry phenotypic and genotypic resistance traits that can easily be transferred to the subsequent levels of the production pyramid. A report by EFSA (2015) indicated that in 2012, *Salmonella* spp. was found in 2.0% of the breeding flocks in EU, where the most commonly reported serovar was *S.*Enteritidis (2.0%).

Antimicrobials have been used in veterinary medicine in the last decades for therapeutic, metaphylatic and prophylactic purposes and as growth promoters (Castiglioni Tessari *et al.,* 2012). The administration of these compounds in poultry starts at the very early stages of the production chain. In addition, the "resident microbiota" of poultry farms is exposed to a selective density due to the simultaneous/successive use of different antimicrobials. This practice creates special conditions for the selection, spread and evolution of resistant strains and the establishment of stable resistance traits (Martins da Costa *et al.,* 2013). Zoonotic organisms, such as *Salmonella*, can be responsible for the contamination and spreading of resistance genes among humans. Infection with multidrug-resistant (MDR) organisms represent a major concern in public health since these bacteria result in higher treatment failures, prolonged or more severe illness, increased hospitalization and mortality (Angulo and Molbak, 2005). Gene cassettes are non-replicating DNA molecules that can move from one genetic site to another (Bennett, 1999) and usually associated with integrons. Three different classes of integrons have been described, being class I commonly related to *Salmonella* spp. The *int1* gene is responsible for promoting the site-recombination of gene cassettes in the integron and it is essential for the expression of resistant genes.

Objective

The aim of this study is to give an overview of the antimicrobial resistance among *Salmonella* spp. isolates recovered from broiler and parent stock farms in Thailand. The minimum inhibitory concentration (MIC) was determined for 10 antimicrobials and PCR amplification for *int1* gene was performed. Both phenotypic and genotypic data gathered are useful to present the resistances profile in poultry farms and to further study the genetic distribution of antimicrobial resistance genes among the poultry farms in Thailand.

Material and Methods

Isolates preparation

Salmonella isolates (n=100) were selected from the Department of Bacteriology at the National Institute of Animal Health (NIAH) in Thailand. Isolates were previously collected by boot swabs from poultry parent-stock farms located in the central region of Thailand during the period between 2013 (n=50) and 2014 (n=50). The isolates were originated from Lop Buri, Saraburi, Singburi, Ang Thon, Chai Nat, Ayutthaya and Pathum Thani provinces at a rate of 61%, 24%, 5%, 5%, 3%, 1% and 1%, respectively. All Salmonella isolates were identified through the ISO-6579:2002 standardized method. The isolates were kept in 10% skim milk at -20°C until being recovered for the study. In order to obtain a pure Salmonella culture, a full loop (10 µL) of the stored solution was subculture in Tryptic Soy Agar (Difco) and grown overnight at 37°C.

Serogroup test

According to the White-Kauffmann-Le Minor scheme (Grimont et al., 2007), an antisera agglutination test was performed to determine serogroup in the Salmonella isolates. A single colony was selected from a fresh culture and mixed with normal saline solution (0.85%) in order to differentiate O (somatic) and H (flagellar) antigens. In case that no agglutination occurred, the isolates were considered as possessing O antigen and test with O multivalent antiserum (OMA and OMB, Oxoid) was executed. When agglutination occurred with OMA the serogroups A, B, D, E and L were tested, while positive reaction to OMB would precede test for C, F, G and H serogroup.

Antimicrobial susceptibility testing

Preparation of antimicrobial agents

Ten antimicrobial agents were selected for MIC test: ampicilin (AMP, Sigma), cefotaxime (CTX, Sigma), ceftodizime (CAZ, Sigma), chloromphenicol (CHL, Sigma), ciprofloxacin (CIP, Sigma), enrofloxacin (ENR, BioChemika), gentamicin (GEN, Sigma), naxilidic acid (NAL, Sigma), sulfamethoxazole-trimpethoprim (SXT, Sigma) and tetracycline (TET, Sigma). All stock solutions were prepared in an initial concentration of 5,120 µg/mL, except SXT which was prepared initially with 10,240 µg/mL. The solvents and diluents for dissolving the working solution were followed according the CLSI (2013) recommendations. The amount of each antimicrobial was weighted for the 100 samples and calculated based on their potency, accordingly to the following formula:

$$Potency\ (\mu g/mL) = (assay\ purity) \times (active\ fraction) \times (1 - water\ content)$$

Solutions containing the antimicrobials were diluted in distilled water in 1:1, 1:4 and 1:8 concentrations, in order to obtain an antimicrobial concentration from 2,560 µg/ml to 1.25 µg/ml. In a log_2 doubling dilution scheme, 2 mL of each stock solution was mixed in 18 mL of liquid Muller-Hinton II agar (1:10) in petri dishes, achieving the final MIC range concentration from 256 µg/ml to 0.125 µg/ml (Table 1, see appendix). Final concentrations in SXT were from 1,280 to 5 µg/ml, and the MIC ranged from 0.5/9.5 µg/ml to 64/1,216 µg/ml (Table 2, see appendix). Petri dishes were left at room temperature until agar solidification.

Preparation of inoculum

Inoculums were prepared from a pure overnight culture. Two to four colonies were selected with a 1 µl loop and added to test tubes containing 2 mL of 0.85% normal saline solution (NSS). Turbidity was adjusted to an equivalent of 0.5 McFarland in order to obtain an approximate suspension of 1 to $2x10^8$ CFU/mL.

Inoculation of plates

Petri dishes containing the different dilutions of antimicrobials were left to dry at 42°C for 1 h before inoculation. In a biological safety cabinet (Telstar), 100 µl of the inoculum was transferred to eppendorf tubes containing 900 µl of 0.85% NSS. An automatic inoculum-replicator device with 27 micropipettes inserted approximately 2 µL of the prepared inoculum into the surface of each plate with the different concentrations of antimicrobial. Inoculation started from the lowest to the highest concentration. Control plates were used before and after the inoculation to discard contamination. Following the inoculation, plates were allowed to dry at room temperature and then incubated at 37°C for 16-20 h. MIC results were interpreted according to the CLSI (2013) guidelines (Table 3) and registered as the lowest concentration without visible growth of the bacteria. Faint hazes, pinpoints colonies and single colonies were not considered as growth bacteria. SXT results were recorded when a growth reduction of 80% was observed. *E. coli* ATCC 25922 served as a quality control strain.

PCR

Preparation of DNA template

Salmonella isolates stored at 4°C were recovered and allowed to grow in Muller-Hinton agar (BBL) in overnight incubation at 37°C. One to two colonies were selected by 1 µl loop and transferred to test tubes containing 100 µl of 0.85% NSS. Centrifugation at 1,200

rpm for 5 min was preceded. The supernatant was discharged and the pellet was suspended with 100 µl of distilled water. Samples were then boiled in a thermo-shaker (Biosan) for 10 min at 100°C. Centrifugation at 1,200 rpm for 5 min was followed. Samples containing the pellet were cooled down and stored at -30°C until PCR amplification.

Int1 gene amplification

PCR reactions were performed in total volume of 20 µl, including 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.7), 200 µM of each dNTP (HotStarTaq), 0.25 µM of each primer and 2 µl of DNA. The intI1-specific primers were intI1-F (5'-AAGGATCGGGCCTTGATGTT-3') and intI1-R (5'-CAGCGCATCAAGCGGTGAGC-3'). A _Pseudomonas aeruginosa_ strain from Thailand (GenBank accession no. AY553333) was used as positive control.

The PCR for _intI1_ was as follows: pre-denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min. Amplification reactions were carried out using a DNA thermo-cycler (TProfessional Basic Gradient, Biometra).

Gel electrophoresis

An amount of 3 µl PCR product was mixed with 1 µl of DNA loading dye (Thermo Scientific) and analyzed by electrophoresis in 1.5% agarose gel stained with 1 µl of ethidium bromide (10 mg/mL). The buffer used for the gel preparation and electrophoresis was 0.5X TBE solution. A molecular weight marker with 100 bp increments (Thermo Scientific) was used as a size standard. The gel ran for 30 min/180 mV in an electrophoresis power supply (Enduro, 300V). DNA fluorescence imaging was processed under UV light in a G:BOX XR5 imaging system (Syngene).

Results

Serogroup test

      Salmonella isolates were serogrouped using specific antisera test, according to the White-Kauffmann-Le Minor scheme (Grimont et al., 2007). All isolates presented a negative reaction with 0.85% NSS implying the presence of somatic (O) antigen. The agglutination test for serogroup A, B, C, D, E, F, G, H and L was preceded and revealed positive results for five different serogroups (Figure 1). Serogroup C had the highest percentage with 35% of samples, followed by B (28%), E (26%), D (10%), and G (1%).
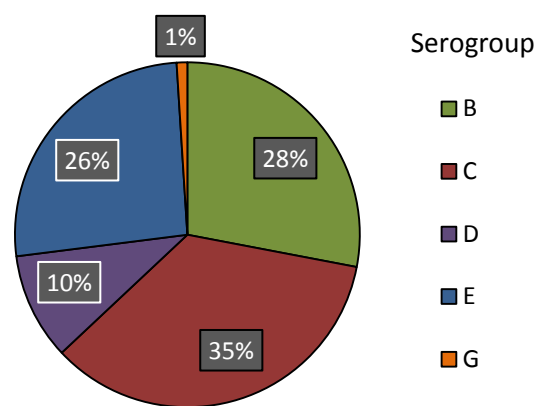
Figure 1. Distribution of five serogroups among the *Salmonella* isolates (n=100) in poultry farms from Thailand in 2013-2014.

Antimicrobial susceptibility

      Many of the isolates displayed resistance to naxilidic acid (80%), ampicillin (72%), sulfomethoxazole-trimethropim (55%), tetracycline and ceftazidime (38%) and enrofloxacin (31%). Resistance was also observed, but to a lesser extent, to gentamicin (15%) and ciprofloxacin (6%). No resistance was found for ceftotaxime and chloramphenicol. Only two isolates demonstrated susceptibility to all classes of antimicrobials. Susceptibility results and MIC range for each antimicrobial tested are shown in Table 4. Maximum levels of MIC ($\geq$256 μg/ml) were registered in ampicillin, nalidixic acid and tetracycline in 66, 47 and 22 isolates, respectively. Also, 16 isolates reached its maximum level of MIC (64/1216 μg/ml) for sulfomethoxazole-trimethropim. A considerable proportion of the isolates exhibited intermediate resistance to enrofloxacin (42%) and ceftadizime (32%).

Table 4. Antimicrobial sensitivity test and MIC range of *Salmonella* spp. against the tested agents.

| Class	Antimicrobial	Number of isolates			MIC range (µg/ml)
		S[1]	I[2]	R[3]	
Aminoglycosides	GEN	83	2	15	<0.125 – 128
ß-lactams	AMP	9	19	72	32 – ≥256
	CTX	97	3	0	<0.125 – 2
	CAZ	30	32	38	1 – 32
Chloramphenicol	CHL	100	0	0	0.5 – 2
	CIP	90	4	6	<0.125 – 8
Quinolones	ENR	27	42	31	<0.125 – 32
	NAL	20	-	80	32 – ≥256
Sulfonamides	SXT	45	-	55	4/76 – 64/1216
Tetracycline	TET	61	1	38	64 – ≥256

[1]=susceptible; [2]=intermediate; [3]=resistant.

GEN=gentamicin; AMP=ampicilin; CTX=cefotaxime; CAZ=ceftadizime; CHL=chloramphenicol; CIP=ciprofloxaxin; ENR=enrofloxacin; GEN=gentamicin; NAL=naxilidic acid; SXT=sulfamethoxazole-trimethropim; TET=tetracycline

Multidrug-resistant (MDR) organisms were considered as being resistant to at least one agent in three or more different antimicrobial categories (Magiorakos *et al.,* 2012). Temporal analysis revealed that MDR strains isolated in 2013 were more frequent than in 2014, with 62% of the total isolates compared to 56%, respectively. The majority of MDR isolates were simultaneously resistant to 3 different antimicrobial categories (Figure 2).

Altogether, different drug resistance profiles were found. Simultaneous resistance to AMP+NAL+SXT was the most common AMR phenotypic profile (9 isolates), followed by AMP+CAZ+NAL+SXT in 6 isolates. AMP+GEN+NAL, AMP+CAZ+SXT+TET and AMP+ENR+NAL+SXT+TET were also typically observed AMR patterns with 4 isolates in each profile. Three isolates depicted simultaneous resistances to 7 of the 10 antimicrobials that were tested: AMP+CAZ+CIP+ENR+NAL+SXT+TET.
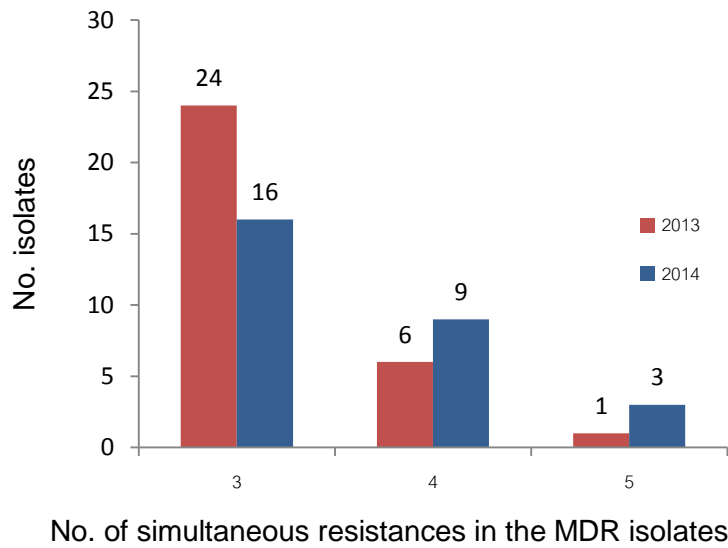
Figure 2. Multi-drug resistant *Salmonella* isolates (n=59) from poultry farms showing simultaneous resistance in 3 to 5 antimicrobial categories.

PCR - Class I integrons

The integrase gene (*intI*) from class I integrons was detected in 36 isolates. PCR results are shown in Figure 3. Isolates harboring the *int1* gene showed to be resistant to SXT, AMP, ENR, CAZ, CIP, GEN, NAL and TE with rates of 62%, 44%, 43% 34%, 16%, 33%, 32% and 37%, respectively. The majority of isolates (86%) which carried the integrase gene were multi-drug resistant organisms. Resistant isolates harboring *int1* gene and their respective MIC range levels are shown in Table 5.
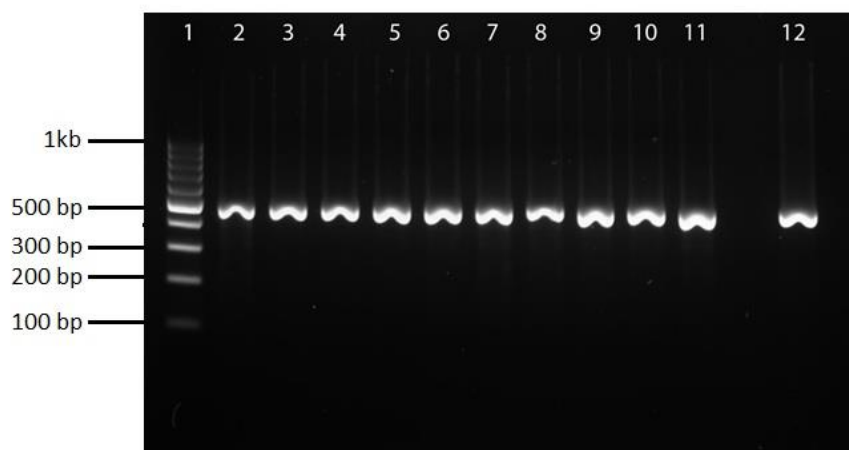
Figure 3. Amplification of *int1* gene in *Salmonella* isolates. Lane 1, 100 bp DNA Ladder. Lane 2-11, isolates carrying *int1* (471 bp). Lane 12, *P. aeruginosa* AY553333, positive control.

Table 5. Number of *Salmonella* spp. isolates positive for *int1* gene within each resistant phenotype

AMR Phenotype (N)	Rates of resistant isolates with *int1* gene (N)	MIC range for organisms with *int1* (µg/mL)
AMP (72)	44% (32)	64 - >256
CAZ (38)	34% (14)	16
CIP (6)	16% (1)	8
ENR (31)	43% (13)	2 - 32
GEN (15)	33% (5)	16 - 64
NAL (80)	32% (27)	32 - >256
SXT (55)	62% (34)	4 – 64
TET (38)	37% (14)	64 - >256

AMP=ampicilin; CAZ=ceftadizime; CTX=cefotaxime; CIP=ciprofloxaxin; ENR=enrofloxacin; GEN=gentamicin; NAL=naxilidic acid; SXT=sulfamethoxazole-trimethropim; TET=tetracycline

Discussion

Selective pressure in *Salmonella* and variations in each serotype may implicate different outcomes of the disease (Jones *et al.,* 2008). Serotyping may be important to understand zoonotic and pathogenic risks posed to humans (Vikash Singh, 2013). In the present study, serotyping was not conducted but we found that the majority of isolates belonged to serogroup C (35%), followed by B (28%), E (26%), D (10%) and G (1%). Previous studies in Thailand (Angkititrakul *et al.,* 2005, Padungtod *et al.,* 2006, Bodhidatta *et al.,* 2013, Lertworapreecha *et al.,* 2013) reported that *S.* Corvalis, *S.* Anatum, *S.* Emek and *S.* Albany were, respectively, the most common serovars isolated amongst chickens. According to the WhiteKauffaman-LeMinor scheme (Grimont *et al.,* 2007) all the mentioned serovars are representative of serogroup C. Also, a study by Chiu *et al.* (2010) found that serogroup B and C were the most frequently isolated among chicken isolates. Additionally, *S.* Weltevreden, was reported to be highly predominant in chicken samples (23/48) and in healthy humans (22/98) in Thailand (Padungtod *et al.,* 2006). This serovar belongs to serogroup E (Grimont *et al.,* 2007) which had also a significant presence in our study. Similarly to our findings, Boodhidatta *et al.* (2013) and Angkititrakul *et al.* (2005) observed that serogroup G had the lower rates in their isolates. Serogroups and serovars prevalence in chickens can be age-related and differ between chicken lines and geographic areas (Chiu *et al.,* 2010). The high rates of serogroup B and C in previous studies taken together with our data, may suggest that these serogroups may be more adapted to chickens in Thailand.

This investigation documents the level of antimicrobial resistance among *Salmonella* spp. isolates obtained in poultry farms in Thailand. High resistance rates to nalidixic acid, ampicillin and sulfamethoxazole-trimethoprim were coherent with the use of antimicrobials in the poultry industry as referred in previous studies (Adeisiji *et al.,* 2014). These antimicrobials are representative of older generation compounds since they were the first to be developed in the quinolones, β-lactams and sulfonamides group, respectively.

Despite the spreading of resistance genes may happen without the direct interaction of antimicrobials by passive or co-selective events, the selective pressure in bacteria due to an active, repeated or intermittently use of antimicrobials in food producing animals may lead to higher prevalence of antimicrobial resistances among animals and humans (Bauer-Garland *et al.,* 2006; Kolár M. *et al.,* 2001). Thus, information such as: daily dose, duration of treatment, number of animals treated and consumption data may be useful to relate the use of antimicrobials to the simultaneous existence of antimicrobial resistances (EFSA, 2015). Unfortunately, in the present study, complementary information regarding antimicrobial usage was not available; therefore it was not possible to associate the observed resistances to an active, passive or co-selective mechanism.

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Resistance to enrofloxacin was observed in 38 isolates in which six of them showed the highest MIC level of 32 mg/ml. Since quinolones have been commonly used for bacterial disease control in poultry farms (Kang *et al.,* 2005), a correlation may be apparent between the use of quinolones such as nalidixic acid and enrofloxacin. In our study, all six resistant isolates to ciprofloxacin showed a MIC of 8 mg/ml and were simultaneously resistant to enrofloxacin and nalidixic acid. Ciprofloxacin is considered a critically important antimicrobial by the WHO (2014) and the OIE (2007b) given its vast potency against gram-negative bacteria. Also, due to the widespread of resistances to chloramphenicol and ampicillin, fluoroquinolones have been commonly used to treat invasive human salmonellosis (Adesiji *et al.,* 2014). Accordingly to Emmerson *et al.* (2003), there is an association between the use of enrofloxacin in animal food additives and the incidence of antimicrobial resistances in ciprofloxacin. Additionally, other studies (Gyles *et al.,* 2013; Poppe *et al.,* 2001) stated that organisms resistant to nalidixic acid usually show reduced susceptibility to ciprofloxacin and are easily converted into fluoroquinolones resistant strains.

Resistances in β-lactams, mostly in third and fourth generation cephalosporins, have been a public health concern in the last few years. In 2007, the European Medicine Agency recommended the use of these substances in food-producing animals only in cases of poorly respond to narrower spectrum antimicrobials (EFSA, 2015). In our study, the number of detected antimicrobial resistant isolates was higher in ceftadizime than in cefotaxime. Cefotaxime is one of the most important antimicrobials for the treatment of *Salmonella* spp. in humans. Children with meningitis caused by invasive *Salmonella* spp. are also treated with cefotaxime (Price et al., 2000). Due to the possible relation between the use of antimicrobials and the existence of antimicrobial resistances, the low amount of resistant isolates to cefotaxime in this study may suggest that this compound is probably not frequently administrated in the tested farms and that the currently used antimicrobials may not co-select for resistances to cefotaxime.

Chloramphenicol administration in food animals was forbidden by the U.S. Food and Drug Administration in 1997 due to its toxicity in humans. Nevertheless, resistant bacteria to chloramphenicol can still be found in food products including poultry. The illegal use of chloramphenicol and remaining residues from past administrations may be responsible for the maintenance of resistant bacteria in farms. Other conditions such as the use of topical medical preparations containing chloramphenicol by farmworkers and the natural existence of the substance in soil and in plants materials may also contribute for the permanence of resistance traits (Berendsen *et al.,* 2010). Also, in 1997, the European Commission approved the ban of avoparcin due to the high increase of vancomycin-resistant enterococci (VRE) organisms in veterinary and human medicine. In spite of the prohibition, prospective studies in Denmark (Heuer *et al.,* 2002) and in Norway (Borgen *et al.,* 2000), after 5 and 3

years, respectively, found that VRE were still extensively present in poultry farms. These studies enhance that antimicrobial resistant organisms may still be disseminated among poultry disregarding the absence of selective pressure. In our study, 100% of the isolates were susceptible to chloramphenicol suggesting that an effective control and monitoring of this compound is probably being conducted in Thailand, and also, that in this case, its interdiction is associated with full susceptibility.

In our study, resistances to tetracycline were lower compared to previous similar studies. Angkititrakul *et al.,* (2005) and Padungtod *et al.,* (2006) conducted their studies in the north of Thailand between 2000-2003 and 2003, respectively, and registered that 100% of *Salmonella* isolates from chicken meat were resistant to tetracycline. However, a more recent study by Lertworapreecha *et al.,* (2013) in south of Thailand showed lower (60%) resistance rates to tetracycline in their isolates. A report by EFSA showed that tetracycline was the most frequent antimicrobial administrated to food-producing animals in Europe with 2,942.6 tonnes of the active ingredient in 2012 (EFSA, 2015). Newly emerged *Salmonella* serovars in Europe such as *S.*Typhimurium DTs 193 / 190 and *S.* Typhimurium DT104 have been observed to be typically of R-type ASSuT and ACSSuT, respectively, showing resistance to ampicillin (A), streptomycin (S), sulfamethoxazole (Su), tetracycline (T) and chloramphenicol (C) (EFSA, 2015; Mandilara *et al.,* 2013). The prevalence of these multi-resistant organisms may be the consequence of a dominant clone that spreads major determinants of a resistance pattern (Gyles, 2008). In Asia, ACSSuT strains are less dispersed than in Europe (Yu *et al.,* 2008), nevertheless, they have been identified in Korea (Yang *et al.,* 2002) and Japan (Sameshima *et al.,* 2000). In Thailand scarce information is available related to the prevalence of ACSSuT phenotype, thus, in the present study, the absence of resistances in chloramphenicol and the low existence of tetracycline resistances may suggest that this R-type may be less prevalent in the country when compared to European countries.

The presence of *int1* gene is related to the existence of integron class I and often considered as responsible for the spreading of MDR organisms. In our study, among MDR isolates, 53% harbored the *int1* gene and showed the same phenotypic common resistance profiles: AMP+NAL+SXT in 6 isolates and AMP+CAZ+NAL+SXT in 4 isolates. A correlation between integron class I and the MDR isolates may be assumed, nevertheless only a fraction of the resistant isolates showed as being linked to integron class I. Other genetic mechanisms and elements such as plasmids and transposons should be considered as involved in the transmission of resistances. Previous studies (Miko et al., 2005; Peirano et al., 2006; Ribeiro et al., 2011 and Mahero et al., 2013) mentioned that integron class I may be responsible for a sizeable portion of *Salmonella* spp. resistances among foodstuffs, animals and humans. Although, the same authors pointed that most resistance genes were

located outside of integrons and that not all MDR isolates carried class I integron which is consistent to our results.

Also, isolates carrying *int1* gene showed as being highly resistant to sulfamethoxazole-trimethoprim (62%). The *sul1*, a sulphonamide-resistance gene, found in most class I integrons on their 3'-conserved segment may be responsible for resistances in sulfamethoxazole-trimethoprim (Recchia and Hall, 1995). A study in Portuguese *Salmonella enterica* strains from clinical and food samples, found that a significant proportion of isolates resistant to sulfonamides carried class 1 integrons in which the presence of *sul1* gene was a consistent marker for sulfonamide resistance (Antunes *et al.,* 2004). In the same study, the authors added that the *sul2* and *sul3* gene can be also present in integron class 1 but with a lower incidence than *sul1*. Additionally, Antunes *et al.* (2004) stated that *sul1* gene creates a selective pressure by sulfonamides that can be useful to the maintenance and spreading of resistances to other antimicrobials.

Conclusions

The frequency of resistances in *Salmonella* spp. isolated in parent stock and broiler farms highlight that both populations can be responsible for the dissemination of MDR *Salmonella* among the poultry industry. Breeders are the top of the pyramid therefore transference of *Salmonella* spp. and resistance genes they harbor to the subsequent levels should be expected. Surveillance, public policies and guidelines in each sector of the production chain should be implemented actively. The spread of resistances among food producing animals should not be considered only as a domestic public health issue, but also as an international one. Complementary data concerning the use of antimicrobials and information related to possible vehicles and sources associated with the antimicrobial resistances in each tested farm would add value to the present study.

In conclusion, even though the tested compounds in this study are considered as classic, awareness should be given to the fact that selective pressure in poultry stock can eventually lead to the integration of genes conferring resistance to antimicrobials of higher generations. Class I integron can be related to the accumulation of resistance genes and to the emergence of MDR in *Salmonella* spp. Further studies of genes cassettes inserted in each integron could be useful to follow the evolution of this mobile genetic element and its role in the dissemination of antimicrobial resistances.

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APPENDIX

Table 1. Preparation of dilutions of agents for agar dilution susceptibility tests. (CLSI, 2012)

Antimicrobial Concentration (mg/L) in stock solution	Volume stock solution (mL)	Volume distilled water (mL)	Antimicrobial concentration obtained (mg/L)	Final concentration in medium after addition of 18mL of agar
5120	3	3	2560	256
5120	1.5	4.5	1280	128
5120	1.5	10.5	640	64
640	3	3	320	32
640	1.5	4.5	160	16
640	1.5	10.5	80	8
80	3	3	40	4
80	1.5	4.5	20	2
80	1.5	10.5	10	1
10	3	3	5	0.5
10	1.5	4.5	2.5	0.25
10	1.5	10.5	1.25	0.125

Agents used with this dilution method: ampilcin, cefotaxime, ceftadizime, ciprofloxacin, chloramphenicol, enrofloxacin, gentamicin, nalidixic acid and tetracycline

Table 2. Preparation of dilutions of Sulfamethoxazole-Trimethropim (SXT) for agar dilution susceptibility test. CLSI (2012)

Antimicrobial Concentration (mg/L) in stock solution	Volume stock solution (mL)	Volume distilled water (mL)	Antimicrobial concentration obtained (mg/L)	Final concentration in medium after addition of 18mL of agar
10240	1.5	10.5	1280	128
1280	3	3	640	64
1280	1.5	4.5	320	32
1280	1.5	10.5	160	16
160	3	3	80	8
160	1.5	4.5	40	4
160	1.5	10.5	20	2
20	3	3	10	1
20	1.5	4.5	5	0.5

Table 3. MIC interpretive standards for *Salmonella* according to CLSI guidelines.

Antimicrobials	Class	MIC Interpretive Criteria (mg / ml)			Remark
		S	I	R	
Ampicillin	Penicillins	≤ 8	16	≥ 32	Human-derived[1]
Cefotaxime	Cephalosporin III	≤ 1	2	≥ 4	Human[2]
Ceftadizime	Cephalosporin III	≤ 4	8	≥ 16	Human[2]
Chloramphenicol	Phenicols	≤ 8	16	≥ 32	Human-derived[1]
Ciprofloxacin	Fluoroquinolone	≤ 1	2	≥ 4	Human[2]
Enrofloxacin	Fluoroquinolone	≤ 0.25	0.5 - 1	≥ 2	Animal[1]
Gentamicin	Aminoglycosides	≤ 4	8	≥ 16	Human-derived[1]
Nalidixic acid	Quinolone	≤ 16	-	≥ 32	Human[2]
Sulfamethoxazole/trimethoprim	Folate inhibitors	$\leq 2 / 38$	-	$\geq 4 / 76$	Human-derived[1]
Tetracycline	Tetracycline	≤ 4	8	≥ 16	Human-derived[1]

1) CLSI (2013) guidelines, VET01-S2 supplement.
2) CLSI (2014) guidelines.

The 4th International Food Safety and Zoonoses Symposium
Professional Learning Community for Human-Animal-Environmental Health

Tuesday 4 August 2015

TIME	ACTIVITIES	PAGE
08:30–09:00	Registration	
09:00–09:30	Creating the Next Generation of Veterinary Public Health and Preventive Medicine Leaders *By Prof.Dr. Trevor R. Ames* *College of Veterinary Medicine, University of Minnesota*	
09:30–09:50	Analysis of Veterinary Workforce in Thailand *By Sukolrat Boonyayatra*	
09:50–10:30	**Panel discussion on** Veterinary Educational Twinning; Implementing OIE day-one competencies from local to global *By Asst.Prof.Dr. Khwanchai Kreausukon, Prof.Dr. Trevor R. Ames,* *Asst.Prof.Dr. Rutch Khattiya, Prof.Dr. Will Hueston, Assoc.Prof.Dr. Armando E. Hoet*	
10:30–11:00	Building a Successful Networking and Collaboration: Lessons learned from international partnerships in FVM-CMU's perspectives *By Asst.Prof.Dr. Khwanchai Kreausukon* *Department of Food Animal Clinics, Faculty of Veterinary Medicine, Chiang Mai University*	
11:00–11:20	Morning Coffee Break	
	Antimicrobial resistance in human and animal session *Chairman: Asst Prof.Dr. Nattawooti Sthitmatee* *Secretary: Asst.Prof.Dr. Duangporn Pichpol*	
11:20–11:50	Implementation of Strategies to Control Antimicrobial Resistance in South East Asian Countries *By Assoc.Prof.Dr. Rungtip Chuanchuen* Research Unit in Microbial Food Safety and Antimicrobial Resistance; Center of Antimicrobial Resistance Monitoring in Foodborne Pathogens (in cooperation with WHO); Global Infections Network; South-East Asia and Western Pacific region, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand	
11:50–12:10	Antimicrobial Resistance Pattern of Bacteria Isolated from Mastitis Dairy Cows in Chiang Mai Province *By Sakulrat Pattamakaew*	
12:10–13:00	Lunch Break	
	Antimicrobial resistance in human and animal session (Cont'd) *Chairman: Asst Prof.Dr. Nattawooti Sthitmatee* *Secretary: Dr. Nattakarn Awaiwanont*	
13:00–13:30	Antimicrobial Resistance in Zoonotic Microorganisms - The Food Safety Perspective *By Prof. Dr. Thomas Alter* *Institute of Food Hygiene, Department of Veterinary Medicine, Freie Universität, Berlin, Germany*	
13:30–13:50	Prevalence of Extended Spectrum Beta-Lactamase Producing *Escherichia coli* in Pig Farms in Nan Province, Thailand *By Penporn Tablerk*	
13:50–14:10	Extended Spectrum Beta Lactamase (ESBL) Producing *Escherichia coli* at Broiler Farms in Jaffna District of Sri Lanka *By Muralithas Mahalingam*	
14:10–14:30	Antimicrobial Resistance of *Escherichia coli* in Broiler in Chiang Mai and Lamphun Provinces, Thailand *By Mohammad Abdullah Al Mamun*	
14:30–14:50	Afternoon Coffee Break	
	Antimicrobial resistance in human and animal session (Cont'd) *Chairman: Prof.Dr. Thomas Alter* *Secretary: Asst.Prof.Dr. Sukolrat Boonyayatra*	
14:50–15:10	Prevalence and Antimicrobial Resistance of *Salmonella* Isolated From Raw Chicken Meat in Retail Outlets from Yangon, Myanmar *By Aung Zaw Moe*	
15:10–15:30	Prevalence and Antimicrobial Resistance of *Salmonella* Isolated from Chicken Production in Chiang Mai and Lamphun Provinces, Thailand *By Kyaw Myo Thant*	
15:30–15:50	Phenotypic and Genotypic Resistances in *Salmonella* Isolates from Broiler in Thailand *By Dujduan Kittiworawat*	
15:50–16:10	Antimicrobial Resistant *Salmonella* from Parent Stock Poultry Farms in Central Thailand, 2013 – 2014 *By Sara Luz Perestrelo*	

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