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Original Article

The first report of antimicrobial residues in cultured tilapia fillets in three provinces of Lao PDR

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Abstract

The objective of this study was to investigate antimicrobial residues in the fillets of Nile tilapia fish (n=180) from 3 provinces (Vientiane, Savannakhet Province and Champasak Province) in Lao PDR in the summer, rainy and winter seasons. Clean meat test (CMT) was used for screening the antimicrobial residue samples. Then, the antimicrobial residue groups were examined and identified by the european six-plate test (ESPT), and the concentration of each positive antimicrobial residue from the ESPT was further assessed by high-performance liquid chromatography (HPLC). The evaluation results of the three antimicrobial detection methods were 19.44% (35/180) for the CMT, 22.86% (8/35) for the ESPT and 75% (6/8) for the HPLC. The levels of enrofloxacin and ciprofloxacin residues detected by HPLC did not exceed the maximum residue limits according to the European Union standard. The highest occurrence of positive CMT results among three provinces was in Champasak Province at 26.67%. The occurrence of antimicrobial residues in the summer season was significantly higher than the rainy season ($P<0.05$), implying that the seasonal climate is an important factor influencing the use of antimicrobials as well as the occurrence of antimicrobial residues in the cultured tilapia fillets in Lao PDR.

Keywords: Antimicrobial, culture, Lao PDR, residue, season, tilapia

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Introduction

Global aquaculture systems have been growing and responding to an increase in aquaculture product consumption (The World Bank, 2013; SEAFDEC, 2018). Over the past five decades, the rate of global fish production has increased with an average annual growth of 3.2% (FAO, 2014). The fishery contributes significantly to the total agricultural gross domestic product on the forestry, aquaculture and fisheries (Government of the Lao People's Democratic Republic, 2014; FAO, 2016).

Lao PDR contains various aquatic animal resources, and fish is one of the major food products that provides quality protein for Laotians (LARReC, 2001; Garaway, 2005; Phonvisay, 2013). Due to aquatic ecosystem problems not only along the Mekong River but also inland reservoirs, a significant amount of the natural fish populations have decreased for a decade. Presently, tilapia fish cultures are increased substantially to decrease the demand pressures on the reduction of natural fish resource (Celik, 2012). Tilapia culture contributes not only to Laos economic growth but also as to Laotian food protein security. To address the high fish demand in the aquaculture industry, intensive caged and farmed tilapia cultures are being promoted, and inevitably inducing physiological stress which usually causes the occurrence and spread of several pathogens leading to fish morbidity and mortality (Suanyuk *et al*., 2008; Amrullah *et al*., 2018; Jansen *et al*., 2018). The well-known and major outbreak diseases in cultured Nile tilapia *Oreochromis niloticus* in Lao PDR as well as several countries worldwide are *Streptococcus agalactiae*, *Aeromonas hydrophila*, *Edwardsiella tarda* and *Flavobacterium* spp. (Suanyuk *et al*., 2008; Wamala *et al*., 2018) To treat detrimental bacterial diseases, antimicrobials are chemically applied as the first line of curing fish diseases as well as for animal welfare reason. Inappropriate uses of antimicrobials in caged and farmed fish cultures, particularly over dose or continuous treatment or irresponsible harvest before the end of the antimicrobial withdrawal period could cause certain amounts of antimicrobial residues to remain in the fish muscle, and after consumption, the antimicrobial drugs accumulated in the tissues can adversely affect to human health, including carcinogenicity, allergy, teratogenicity as well as antimicrobial resistance (Kemper, 2008; Budiati *et al*., 2013; Beyene, 2016; Nguyen *et al*., 2016; WHO, 2016). To date, antimicrobial residues carry a risk for food safety and consequently also challenge in international trade in animals and their products.

Many techniques are established to determine the antimicrobial residues in animal tissues, including microbiological screening and confirmation methods such as clean meat test, microbiological plate test and the high-performance liquid chromatography (Kirbis, 2007; Tajik *et al*., 2010; Ramatla *et al*., 2017; Chaisowwong *et al*., 2018) in order to ensure consumer food protection. These techniques have been used to determine antimicrobial residues in the tissues of the fish, chicken, cattle and pig (Myllyniemi *et al*., 2001; Guidi *et al*., 2017; Ramatla et al., 2017). Among food contaminants, antimicrobial residues in fish in terms of disease treatment purposes are in social concern in Lao PDR. Therefore, quality control and assurance of human food safety, particularly for fish products, are in line with Lao government as well as public to monitoring and arranging the products to be as safe as international standards (Moffitt and Cajas-Cano, 2014; Okocha *et al*., 2018). However, baseline data related to antimicrobial residues in foodstuffs consumed in Lao PDR are very limited. Therefore, this first survey study aimed to investigate the antimicrobial residues from the Nile tilapia fillets in three provinces for three seasons. In case of the occurrence of the antimicrobial residue in fish fillets, the Lao PDR will devise action plans for monitoring, establishing and managing policies, regulations as well as laws related to fish food protection and safety for the well-being of Lao people in the future.

Materials and Methods

Sample collection: Total of 180 tilapia fillet samples were randomly and equally collected from 20 fish at each collection from local markets and farms in 3 provinces (Vientiane, Savannakhet Province and Champasak Province) of Lao PDR as in geographical locations (Figure 1; Table 1) for three seasons (summer, rainy and winter seasons) from March 2018 to February 2019. Each fish fillet with a weight of approximately 120 g was dissected, sealed in plastic bags, and stored at -20°C for laboratory assessments.

Clean meat test (CMT): The tilapia fillet samples were primarily screened for antimicrobial residues by CMT (Grand Siam Company, Bangkok, Thailand) as an antimicrobial rapid test. Twenty grams of each tilapia fillet was smashed by an electrical hammer box at 300 times/min for 2 min. Then, 3 drops of extracted fillet juice were dropped onto a 6 mm diameter paper disk placed on the top surface of the CMT tube. The negative control, positive control and unknown CMT test tubes with 3 replicates were incubated in a water bath at 65°C for 3 h. The results were photographed and analyzed statistically. The positive antimicrobial residue changed the color of the CMT media from purple to yellow.

European Six Plate Test (ESPT): The positive samples from the CMT results were further identified for antimicrobial residue groups following the ESPT methodological guidelines of the Laboratory of Veterinary Research and Development Center in the Upper Northeastern Region of Thailand. ESPT was prepared with 4 bacterial strains as *Bacillus subtilis*, *B. cereus*, *Krebsiella rhizophila* and *Escherichia coli* at different pH (plate 1: *B. subtilis* + basic agar pH 6.0; plate 2: *B. subtilis* + basic agar pH 7.2; plate 3: *B. subtilis* + basic agar pH 8.0; plate 4: *B. cereus* + basic agar pH 6.0; plate 5: *K. rhizophila* + basic agar pH 8.0, and plate 6: *E. coli* + basic agar pH 8.0). The bacteria were purified on blood agar at 37°C for 24 h, then the pure colonies were cultured in 5 ml Mueller Hinton Broth at 37°C for 4 h, and the bacteria were adjusted to a density of 0.5 McFarland standard (1.0×10^8 CFU/ml). The antimicrobial residue groups were examined based on the diameter of the fillet sample inhibition zone (clear zone) after being cultured at 37°C for 24 h. The diameter of sample inhibition zone exceeded or equaled 2 mm was reported as positive antimicrobial residue, and the diameter less than 2 mm was reported as negative antimicrobial residue. The ESPT of each positive CMT sample was conducted in 5 replicates.

High Performance Liquid Chromatography (HPLC)

Clean up: The positive contaminated residues in the fish samples screened by CMT and ESPT were later measured for the levels of antimicrobial residues by HPLC (Ferdous *et al*., 2019; Zhang *et al*., 2020) as follows. The fish fillet a weight of 10 g was added with 1 ml phosphate buffer saline PBS (pH 7.2), homogenized using Ultra Turrax T25 (IKA, Staufen, Germany) and centrifuged at 1400*g* for 10 min. Subsequently, the supernatant was added to 3 ml trichloroacetic acid (15%), vortexed for 2 min and centrifuged at 1400*g* for 10 min. The supernatant was subjected to solid phase extraction.

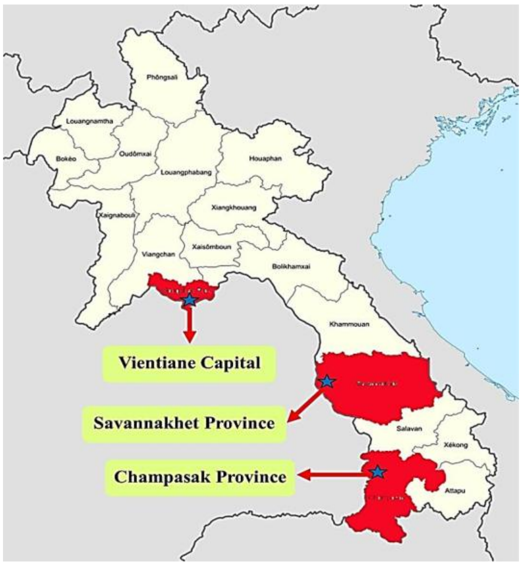

Figure 1 Geographical locations of the Nile tilapia fillets collected from three provinces in Lao PDR.

Table 1 Geographical locations of the Nile tilapia fillets collected from three provinces in Lao PDR.

Location	GPS N	GPS E	Note
Vientiane Province			
• Xaythany district			
○ Ka sed	18°07'33.6576"	102°47'24.6768"	Market
○ Luk 10 (Nongsonghong)	18°09'44.4060"	102°46'0.4728"	Market
○ Dong mark khaiy	18°04'53.6268"	102°40'13.7964"	Market
○ Tha ngon	18°07'45.1956"	102°37'36.0660"	Market
○ Ban hai	18°09'33.3342"	102°36'47.1024"	Farm
• Xaysetha district			
○ Hua khua	17°58'31.7316"	102°39'04.4532"	Market
• Hatxayfong district			
○ Sa la kham	17°52'52.3452"	102°39'00.3816"	Market
○ Luk 8	17°53'54.0672"	102°38'05.5284"	Market
○ Hard dork keo	17°51'48.1356"	102°35'54.1536"	Farm
• Sikhottabong district			
○ Sikhai	17°58'37.5096"	102°33'25.7040"	Market
○ Nong neiw	18°00'07.5060"	102°32'35.6820"	Market
○ Suan mone	17°55'06.5568"	102°37'14.8440"	Market
Savannakhet Province			
• Kaysone Phomvihane district			
○ Savanhxai	16°34'41.0628"	104°44'58.8948"	Market
○ Ban xok	16°36'29.7816"	104°50'23.5644"	Market
○ Luk 8	16°36'02.1348"	104°47'05.0028"	Market
○ Samukkhy xai	16°32'45.7944"	104°45'59.3676"	Market
○ Pak bor 1	16°37'51.1788"	104°44'59.6976"	Farm
○ Na kea	16°37'06.8340"	104°44'55.5540"	Farm
○ Pak bor 2	16°39'29.3400"	104°45'27.5076"	Farm
• Outhoumphone district			
○ Seno	16°40'35.0364"	104°57'51.4296"	Market
Champasak Province			
• Pakse district			
○ Dao hueang	15°06'55.6272"	105°48'53.2008"	Market
○ Ban non sa wang	15°05'28.5576"	105°49'54.4548"	Farm

GPS = Global positioning system

Solid Phase Extraction (SPE): An Empore SPE cartridge analytical column (10 mm x 0.75 mm, 50 µl) was prepared and maintained at 25°C. Mobile phase A consisting of 0.15% formic acid in water and mobile phase B consisting of methanol were prepared. The injection volume was 20 µl, and the autosampler temperature was set at 10°C. The extractions of drug residues were passed through the cartridge. The columns were washed with distilled water, dried by N_2 stream and washed with methanol. The eluted samples were dried under a slow stream of N_2 and dissolved in the mobile phase for HPLC-UV analysis.

HPLC-UV analysis: Prior to analysis, the eluent samples from the cartridges were evaporated until dry under a stream of nitrogen and redissolved with an appropriate volume of mobile phase. The sample injection volume was set at 15 µl in an Empore SPE Cartridge analytical column (10 mm x 0.75 mm, 50 µl). The mobile phase consisted of a mixture of water and methanol at a ratio of 60:40 (v/v). The flow rate was set at 1.0 ml/min. The residues were detected by means of a UV detector at the wavelength 276 nm. The preparation and analysis of the samples were conducted in 3 replicates.

Enrofloxacin and ciprofloxacin were analyzed using a core-shell silica particle size 5 µm as stationary phase, and 0.002 M phosphoric acid/acetonitrile (83:17, v/v) as mobile phase. Excitation and emission wavelengths for determination of enrofloxacin and ciprofloxacin were set at 277 nm and 418 nm, respectively. Limits of quantification for enrofloxacin and ciprofloxacin were 2 µM and 3 µM, respectively.

Statistical analysis: The occurrences of positive CMT samples from three provinces in Lao PDR (n=60/province) for three seasons were analyzed with Pearson's chi-square test. Odds ratios for the occurrences of positive CMT samples were calculated to compare the risk of the subcategories within provinces or seasons. The subcategory with the lowest occurrence was used as a reference for the comparison. The significant level was defined as $P<0.05$ under SPSS version 20 (IBM Corp., Armonk, New York, USA).

Results

Clean Meat Test (CMT): Thirty-five of the total of 180 samples tilapia fillet samples (19.44%) were positively screened by the CMT. The occurrences of the antimicrobial residues from the Vientiane, Savannakhet Province and Champasak Province were 15%, 16.67% and 26.67%, respectively (Figure 2; Table 2), and they were not significantly different in term of the location aspect ($P>0.05$) (Table 2). The occurrences of the antimicrobial residues in the summer, rainy and winter seasons were 25%, 10%, and 23.33%, respectively (Figure 3), and the occurrences in the summer season were significantly higher than those in the rainy season ($P<0.05$) (Table 3).

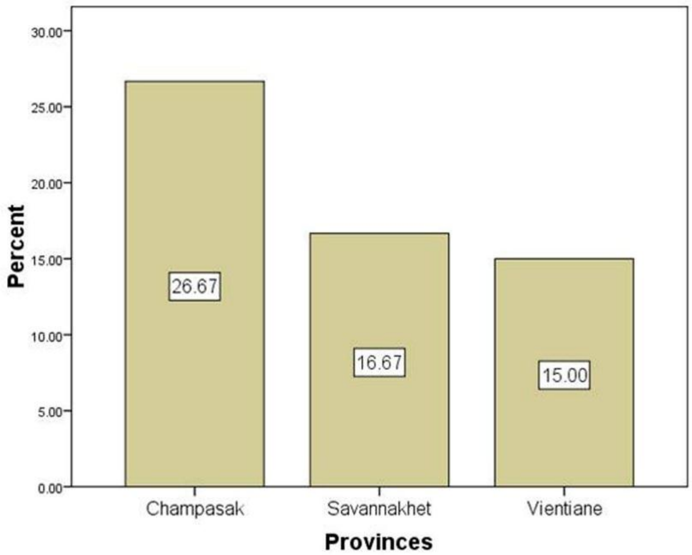

Figure 2 Percentage of positive CMT for the Nile tilapia fillets collected from three provinces in Lao PDR.

Table 2 Number and percentage of positive antimicrobial residues screened by the clean meat test for the Nile tilapia fillets collected from three provinces in Lao PDR for three seasons.

Location	Season			Total
	Summer (%)	Rainy (%)	Winter (%)	(%)
Vientiane	6/20 (30%)	1/20 (5%)	2/20 (10%)	9/60 (15%)
Savannakhet	1/20 (5%)	3/20 (15%)	6/20 (30%)	10/60 (16.67%)
Champasak	8/20 (40%)	2/20 (10%)	6/20 (30%)	16/60 (26.67%)
Total	15/60 (25%)	6/60 (10%)	14/60 (23.33%)	35/180 (19.44%)

Summer season (from March to May); rainy season (from June to October); winter season (from November to February).

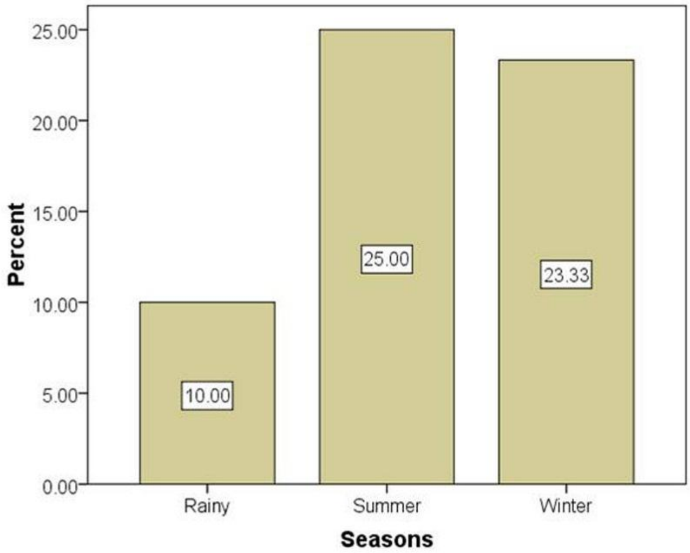

Figure 3 Percentage of positive CMT for the Nile tilapia fillets collected in Lao PDR for three seasons.

Table 3 Risk factor analysis for antimicrobial residues in the Nile tilapia fillets in Lao PDR.

Factors	No. positive/No. sample (%)	OR (95% CI)	P-value (2-tails)
Season			
Rainy	6/60 (10)	Ref.	Ref.
Summer	15/60 (25)	2.97 (1.08-8.97)	0.034
Winter	14/60 (23.33)	2.72 (0.98-8.25)	0.055
Location			
Vientiane	9/60 (15)	Ref.	Ref.
Savannakhet	10/60 (16.67)	1.13 (0.42-3.12)	0.809
Champasak	16/60 (26.67)	2.05 (0.83-5.30)	0.124

Summer season (from March to May); rainy season (from June to October); winter season (from November to February); OR = odds ratio; CI = confidence interval; Ref. = reference

Odds ratio is a measure of the association between an exposure and an outcome. From the results in Table 3, the odds ratios of the summer and winter seasons as compared with the rainy season were greater than 1, implying the increased odds of having positive antimicrobial residue among seasons as a risk factor. Additionally, the odds ratios of the Savannakhet Province and Champasak Province compared with Vientiane were greater than 1, implying the increased odds of having positive antimicrobial residue among the sample collection locations as a risk factor.

European Six Plate Test: A total of 35 positive CMT samples were further tested by the ESPT for antimicrobial residue group identification. Eight of the 35 samples (22.86%) (3 samples from Vientiane and 5 samples from Champasak Province) or 8 of the 180 fillet samples (4.44%) were positively screened. The 8 ESPT-positive samples were identified as the quinolone group (Table 4).

High-Performance Liquid Chromatography: Only 6 of 8 (75%) positive ESPT samples or 6 of the 180 fillet samples (3.33%) were positive for enrofloxacin and ciprofloxacin based on HPLC, with the average and maximum values of 31.50 ± 28.09 and 69.02 ppb, and 2.30 ± 1.12 and 3.44 ppb, respectively (Table 4-5). The levels of enrofloxacin and ciprofloxacin residues in the tilapia fillets did not exceed the maximum residue limits (MRLs) according to the European Union (EU) standard.

Table 4 Antimicrobial residue evaluation of the positive fish fillet samples by the European six plate test and high-performance liquid chromatography

Sample number	European Six Plate Test (+/-)			Quinolone residue concentration by HPLC (ppb)	
	Penicillin	Sulfamethazine	Quinolone	Enrofloxacin	Ciprofloxacin
1	-	-	+	-	-
2	-	-	+	7.86	-
3	-	-	+	7.46	-
4	-	-	+	-	-
5	-	-	+	19.93	1.17
6	-	-	+	65.08	3.06
7	-	-	+	19.69	1.51
8	-	-	+	69.02	3.44

HPLC = High-performance liquid chromatography

Table 5 Validation concentrations of quinolone residues (mean ± standard deviation) of the Nile tilapia fillet by high-performance liquid chromatography

Sample (n)	MRLs (ppb)	Concentration (ppb)			
		Enrofloxacin		Ciprofloxacin	
		Mean±SD	Range	Mean±SD	Range
6	100	31.50 ± 28.09	7.46-69.02	2.30±1.12	1.17-3.44

MRLs = Maximum residual limits according to the European Union standard

Discussion

The occurrences of the antimicrobial residues in this study revealed the potential to apply the CMT for detection of residues in the cages or farms fish before being sent to slaughter or market. Furthermore, Chalermchaikit *et al*. (2002) and Chaisowwong *et al*. (2018) reported the detection limit of the CMT for several veterinary drug administrations. Detection limits for beta-lactams (penicillin, ampicillin, amoxicillin and cloxacillin) at 0.005-0.02 ppm were below the maximum residue limits (MRLs); for oxytetracycline and sulfonamides (sulfamethazine, sulfathiazole, sulfadiazine and trimethoprim) at 0.15-0.8 were close to the MRLs; for chlortetracycline, kanamycin and erythromycin at 0.8-2 ppm were close to the MRLs; and for enrofloxacin at 7 ppm was higher than the MRLs which set it at 0.1 ppm. In addition, the validity results for the CMT performed on meat samples were 87% sensitivity and 100 % specificity. The above laboratory reports support and ensure that the positive antimicrobial residue results in the fish from three provinces in Lao PDR were reliable and suitable for screening as a rapid microbial assay for antimicrobials. However, further laboratory validation and sensitivity of the CMT compared with Charm II test, HPLC and other methods for the drug residues in fish fillets should be conducted and evaluated.

Comparing with other studies using microbiological plate tests, the most commonly detected antimicrobials in fishery products in Korea were quinolone and tetracycline groups (Kang *et al*., 2018), whereas the cultured fish around Tai Lake in China was sulfonamide (Song *et al*., 2017). Drug residues in the tilapia fish (*O. niloticus*) in the state of Minas Gerais in Brazil and in Bangladesh were quinolone and oxytetracycline, respectively (Barman *et al*., 2018; Guidi et al., 2018). Furthermore, the antimicrobial residues in the cultured bream fish (*Megalobrama amblycephala*) around Tai Lake in Southeast China was sulfonamides (Song *et al*., 2017), whereas the shrimp and fish samples from regional markets in Vietnam near the Mekong River Delta and the Red River Delta were quinolone and tetracycline residues, respectively (Pham *et al*., 2015).

The levels of enrofloxacin and ciprofloxacin residues in the tilapia fillets did not exceed the maximum residue limits (MRLs) according to the European Union (EU) standard. This result was in accordance with the results of several reports on residues of veterinary drugs in fishery products. Kang *et al*. (2018) demonstrated the detection rate of veterinary drugs in fish samples at 22.7% (217 of a total of 958 samples) from markets in Korea. Enrofloxacin and oxytetracycline were mostly detected in the products, and 1.3% of the fish products exceeded the MRLs. Barman *et al*. (2018) found oxytetracycline residues in 5 of 24 (20.85%) tilapia fish (*O. niloticus*) samples from Bangladesh with an average of 38.88 ± 2.99 ppb, and the detected oxytetracycline residues did not exceed the MRLs of 100 ppb recommended by the European Commission. Guidi *et al*., (2017) found that 29 of 193 fish samples (15%) were positive for enrofloxacin in tilapia and trout from Brazil, and only one sample was higher than 50 ppb. Guidi *et al*. (2018) reported that 14% of the enrofloxacin residue in 24 Nile tilapia (*O. niloticus*) from Brazil was above the limit of quantification (12.53-19.01 ppb), but below the MRLs (100 ppb). Pham *et al*. (2015) detected that 28 of 104 (26.9%) of shrimp and fish samples contained fluoroquinolone and tetracycline residues in Vietnam at less than 66 ppb and less than 17 ppb, respectively.

From the Odds ratios and p-values of the risk factor analysis results, summer and winter seasons as well as Savannaket and Champasak provinces demonstrated the relative high values implying that these 2 seasons and 2 provinces possibly involve in fish disease occurrences and the uses of oral antimicrobial drugs for disease prevention and treatment in the Lao PDR. Positive antimicrobial use in the cultured Nile tilapia samples from Lao PDR markets and farms in the three provinces suggests a regulated or responsible use of antimicrobials in the tilapia production; however, human health and food safety still pose concerns in Lao PDR as well as most developing countries. Consequently, from these first survey results, Lao PDR needs to continue action plans for monitoring, establishing and managing policies, regulations as well as laws related to fish food safety for the well-being and sustainable public health of the Lao people at present and in the future.

Generally, antimicrobials have been used to treat fish disease in aquaculture, and to ensure of the risks of drug use to humans, responsible use of antimicrobials requires strong collaboration among stakeholders from farm to table by providing suitable label directions or dosage; proper and certified equipment in antimicrobial production; proper handling and distribution and strictly veterinary supervision of drug administration to farmers with recommended withdrawal periods before slaughter. Animal species and their physiology, water qualities and environmental conditions also affect to antimicrobial residues in fish bodies (Canadian Food Inspection Agency, 2014; Speksnijder, 2017). Future work to determine the pharmacokinetics of antimicrobials on site (caged and farmed cultures), environments and other factors, such as climate and water quality should be conducted.

In summary, this is the first report on the antimicrobial residues in the Nile tilapia fillet collected from three provinces of Lao PDR for three seasons. Although the occurrence of the positive CMT results was not significantly different among the three provinces, the occurrence was highest for Champasak

Province. In addition, the occurrence in the summer season was significantly higher than that in the rainy season.

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