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Beta-lactamase and integron-associated antibiotic resistance genes of *Klebsiella pneumoniae* isolated from Tilapia fishes (*Oreochromis niloticus*)

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ABSTRACT

Tilapia fishes (*Oreochromis niloticus*) are commonly consumed and exported in Thailand. Bacterial isolation and drug resistance from farmed tilapia fished in Thailand were previously reported. This study was purposed to study on the distribution of human pathogenic bacteria in tilapia fishes, which were collected from Thai farms (n = 180) and fresh markets (n = 160) by identification, antibiotic susceptibility test; and conduct to identify virulence genes by molecular technique. Pathogen isolations were collected from internal organs of fish samples for identification and test of antibiotic susceptibility according to Clinical and Laboratory Standards Institute (CLSI) criteria. bla_{CTX-M} and *Int1* genes detection of antibiotic resistance bacteria was performed by molecular based techniques. *Klebsiella pneumoniae*, *Edwardsiella tarda*, and coagulase-negative *Staphylococci* were most frequent bacteria isolated from farming tilapia fishes, respectively. However, Escherichia *coli*, coagulase-negative *Staphylococci*, and *K. pneumonia* were frequently distributed from tilapia fishes in markets of Bangkok area. *Klebsiella pneumoniae*, *E. coli*, and *Proteus mirabilis* were resisted to penicillin and ampicillin. *Klebsiella pneumonia*e is the most important isolated bacteria due to the distribution in tilapia fishes and positive for bla_{CTX-M} and *Int1* gene detection. However, *E. coli* and *P. mirabilis* were lack of bla_{CTX-M} and *Int1* genes, possibly there may reserve other antibiotic resistance genes.

INTRODUCTION

The aquatic food products are contributed worldwide for humankind consuming and aquatic farming trend to increase in yield and values. Aquaculture production was estimated in most Asian countries as the value of USD 160.2 billion from 1970 to 2008 (Food and Agriculture Organization, 2016). Nile tilapia or tilapia (*Oreochromis niloticus*) is the second most freshwater aquaculture worldwide and this is one of major favorite fish for farming in Thailand (Piumsombun, 2003). Domestic and international consumptions of tilapia fish from Thailand are important by the high amount of products and values, which are 15,496.1 tons and 31.34 million US dollar, respectively (Piumsombun, 2003). Tilapia fish farms are located in all sectors of Thailand, including growth-out pond (well-typed fishponds) and cage (floating baskets) culture, which are commonly fish farming, however, nursery farms are proportionally lower in numbers. Fully standard farming for the export purpose is few in numbers when compared with local farming (Thongkao and Sudjaroen, 2017).

Cultures of Tilapias in open culturing systems are highly vulnerable to stress produced by water quality fluctuations and can easily be infected by naturally occurring microorganisms. Particularly, infestations with *Streptococcus* spp. and other bacteria (e.g., *Aeromonas* spp., *Pseudomonas* spp., and *Vibro* spp.) have been reported to be the main causes of mortality in caged tilapia (Belton *et al*., 2009; Huicab-Pech *et al*., 2016; 2017). Aquatic systems such as rivers are exposed to disposals from different sources, receiving chemical and microbial contaminants of industrial, agricultural, and domestic origins, which can modulate the antibiotic resistome (Tacão *et al*., 2012) and aquatic environments may act as reactors with incubation conditions

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that promote genetic exchanges and contribute to the spread of antibiotic resistance (Lupo *et al*., 2012).

Extended-spectrum beta-lactamases (ESBLs) are capable of antibiotic resistances hydrolyzing penicillin, cephalosporin, and also the monobactam aztreonam. When dealing with bacterial infections caused by ESBL-producers, a multi-resistance phenotype clearly limits the therapeutic options (van Hoek *et al*., 2011). ESBLs and integrons increase in abundance in the receiving rivers, downstream of the wastewater treatment plants (Lu *et al*., 2010; Kristiansson *et al*., 2011). Increasing of antibiotic resistance rate in bacteria is related to waste production and antibiotic resistance genes (ARGs), which are transferred from "bacteria to bacteria" by water environment and also by aquatic animals (Kümmerer, 2009; Marathe *et al*., 2016). In addition, misusing or overusing of antibiotics in fish farms may cause antibiotic resistance of bacteria in fishes and water environment and drug resistance bacteria and can be transmitted from breeding area to farming area or one farm to other farms (Huicab-Pechet al., 2016).

The isolation of bacterial strain from the gut of tilapia fish can survive as a reservoir in environmental pollution, such as urban waste, polluted rivers and lakes, which contained antibiotic-resistant bacteria and carried ARGs. The ARGs bacteria isolates from farming tilapia fish had been reported (Budiati *et al*., 2013; Marathe, *et al*., 2016; Newaj-Fyzul *et al*., 2008). Isolation of ARGs bacteria from tilapia fish farms in Thailand had also reported and may indicate as a zoonotic reservoir (Thongkao and Sudjaroen, 2017). Many potential human bacteria are identifying from Tilapia gut, including *Klebsiella pneumoniae*, Escherichia *coli*, *Serratia marcescens*, *Enterobacter* spp., and *Shigella* spp. The presence of pathogenic bacteria in the Tilapia gut is concern as a reservoir of potential human pathogens in the environment (Marathe, *et al*., 2016; Thongkao and Sudjaroen, 2017) and prevalence of class 1 integrons (*Int1*) and bla_{CTX-M} genes are frequently observed in *Enterobacteriaceae* isolates from tilapia fishes, which are living in polluted rivers (Kristiansson *et al*., 2011; Marathe *et al*., 2016). Previously, we had been reporting the antibiotic resistance of human pathogenic bacteria, which isolate from internal organs of farming tilapia fish (Thongkao and Sudjaroen, 2017). Thus, our present study was purposed to the distribution of human pathogenic bacteria in tilapia fishes, which were collected from farms and fresh markets by identification, antibiotic susceptibility test; and conduct to identify virulence genes of antibiotic resistance bacteria comparing with susceptible bacteria by molecular technique. Our finding may provide information for fish consumers in case of public health suggestion, which is able to prevent and control the mode of bacterial transmission from aquatic animals. In addition, this result is also useful for aquaculture farms in case of appropriate antibiotic used, which supported one procedure of good aquaculture practices (GAP) recommendation.

MATERIALS AND METHODS

Fish identification and sample collection

Tilapia fish (*O. niloticus*) samples were identified according to morphology data from the Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand as follows: 1) Upper and lower lips are of equal size; 2) Cheek with four rows of scales; 3) Lateral line with 9–10 cross stripes, but it is light color when the size is about 25–30 cm. The dorsal fin has only one fin consisting of dorsal spiny fins ray and dorsal soft fins ray; 4) the anal fin is composed of spiny fins ray and soft fins ray as well. The 33 scales along the lateral line in brown green body and the middle dark scales; 5) The cheek bone has dark spots; and 6) The soft fins of the dorsal fins and caudal fins are white and black (National Bureau of Agricultural Commodity and Food Standards, 2010).

Tilapia fishes from farms: Adult tilapia fishes (n = 180) were collected at Surat Thani province, Thailand from six aquacultures (30 fishes/one farm) on January–March 2018. Each sample was collected by swabbing from internal organs of fish, including liver, kidney, and brain and took into on Stuart transport medium (Thermo Fisher scientific). All processes of sample collection were done by sterile technique and icepack-chilled transport medium tubes were sent back to laboratories at Bangkok within 24–48 hours.

Tilapia fishes from markets: adult and fishes (300–400 g/each fish) were collected randomly *20 samples per week* from local markets in Dusit and Bangkok Noi district, Bangkok, Thailand during on February–March 2018 (n = 160). The collection and storage condition are similar to those of farmed tilapia fish.

Bacterial isolation and identification

Bacterial isolation and identification from the fish swabbed samples were carried out at microbiological laboratory unit, Faculty of Science and Technology, Suan Sunandha Rajbhat University. Briefly, swab samples were streaked and cultured with blood, MacConkey, and chocolate agars (Himedia, India) at 37°C, 24 hours; for yeast or *Candida* species was used potato dextrose agar, PDA agar (Sigma-Aldrich). Bacterial isolation and identification were performed by colony morphology, Gram's staining, and biochemical tests, which were explained by Bergey's Manual of Systematic Bacteriology (Brenner *et al*., 2005). The use of biochemical tests, such as catalase (Sigma-Aldrich), oxidase (Himedia, India), coagulase (Himedia, India), TSI (Himedia, India), citrate (Himedia, India), lysine indole motility (Himedia, India), ornithine decarboxylase (Himedia, India), methyl red-Vogeprokauer (Himedia, India), Mannitol (Himedia, India), growth 0% NaCl (Ajax, Australia), growth 6.5% NaCl (Ajax, Australia), and bile esculin test (Himedia, India), which were interpretation for bacterial identification according to Clinical and Laboratory Standards Institute (CLSI) guideline (2016a).

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was also performed by CLSI criteria, which used the agar disk diffusion method (Kirby–Bauer test). Each isolated bacteria was inoculated and cultured in TSB broth at 37°C for 3–4 hours and bacterial cell concentration was approximately 10^8 CFU/ml, 0.5 McFarland turbidity standard or OD = 0.08–0.05 from spectrophotometer (Shimadzu). 0.1 ml of each broth culture was spread on the Mueller-Hinton agar (Himedia, India) plate and the plate was allowed to dry for approximately 5 minutes. Use of an antibiotic disk dispenser to dispense disks containing specific antibiotics onto the plate and incubated at 37°C for 18–24 hours. Ten of antibiotic disks (Difco), including amikacin, ampicillin, ceftazidime, ceftriaxone, chloramphenicol, ciprofloxacin, penicillin, sulfamethoxazole,

tetracycline, and norfloxacin were used in susceptibility testing for each isolated bacteria. Interpretation of this test depended on diameter (mm) of the transparent (clear) zone around the testing disk and compared with the CLSI standard (2016b), which were interpreted to sensitive or susceptible (S), intermediate (I), and resistance (R).

DNA extraction and virulence gene detection (blaCTX-M and Int1 genes)

DNA extraction from antibiotic resistance bacteria were further prepared for virulence gene detection with polymerase chain reaction (PCR) technique. DNA was introduced from the media by using a Presto™ Mini gDNA bacteria kit (Geneaid Biotech, Taiwan) transfer bacterial cells (1×10^9 CFU/ml or about 1 loopful) to a 1.5 ml of Eppendorf microcentrifuge in buffer preparation. The Proteinase K (11 mg/ml) was added and incubated at 60°C for 10 minutes to proceed with lysis step by buffer lysate removal at 70°C for 10 minutes and the mixture was vortexed every 3 minutes for 1 minute. The samples were flowed through place column to transfer mixture passed through the column, the column was washed once with cold 70% ethanol (Hanil Science industrial, Korea) and the centrifuge at 14,000–16,000 g force (g force = relative centrifuge force). The isolated DNA was eluted in 100 µl of pre-heated elution buffer. bla_{CTX-M} gene identification was done by PCR condition: 50-µl of reaction mixtures under the following conditions: 30 *p*mol of each primer, 200 µM concentrations of each deoxynucleoside triphosphate (Vivantis Technologies, Malaysia), 1.5 mM $MgCl_2$, (Vivantis Technologies, Malaysia), and 0.5 U of *Taq* DNA polymerase (Vivantis Technologies, Malaysia). CTX-MF (5′-ATGTGCAGYACCAGTAARGT-3′) and CTX-MR (5′-TGGGTRAARTARGTSACCAGA-3′) primers (Theera trading, Thailand) were used to amplify a 693-bp DNA fragment of *K. pneumonia*e. PCR reaction procedures were run as the following step: initial denaturation of DNA at 94°C for 7 minutes, 35 cycles were run—94°C for 50 seconds, 50°C for 40 seconds, and 68°C for 1 minute—with a 5-minute 68°C extension after the 35 cycles (Pagani *et al*., 2003). In the case of class I integron (*Int1*), Int1F (5′-GGGTCAAGGATCTGGATTCG-3′) and Int1R (5′-ACATGGGTGTAAATCATCGTC-3′) primers (Theera trading, Thailand) were used under the following conditions: 25 pmol of each primer, 200 µM concentrations of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, and 0.5 U of *Taq* DNA polymerase were used to amplify a 493-bp (isolated from local market) and 693-bp (isolated from aquaculture farm) DNA fragment of *K. pneumonia*e. Reactions were run in initial denaturation of DNA at 94°C for 5 minutes, 30 cycles were run—94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 1 minute—with an 8-minute 72°C extension (Mazel *et al*., 2000). Each mixture of PCR product was separated by 1.5% agarose (Vivantis Technologies, Malaysia) gel electrophoresis (BIO-RAD, Thailand); stained with NEOgreen-DNA staining reagent (Everlasting Biotech, Taiwan) and took a photo under BluView Transilluminator MBE 300 (Major science, Saragota).

RESULTS AND DISCUSSION

According to bacterial colony figure, Gram‘s staining, and biochemical characteristic (Brenner *et al*., 2005; Clinical and Laboratory Standards Institute, 2016a), we identified nine types of bacteria (one type of yeast), which were isolated from tilapia fishes in fish farms; and seven of bacteria isolated from tilapia fishes in fresh markets (Tables 1 and 2), respectively. *K. pneumonia*e, *Edwardsiella tarda*, and coagulase-negative *Staphylococci* were most frequently isolated bacteria from farming tilapia fishes, respectively. However, *E. coli*, coagulase-negative *Staphylococci*, and *K. pneumonia* were frequently distributed from tilapia fishes in markets of Bangkok area. *K. pneumoniae*, *E. coli*, and *Proteus mirabilis* were resisted to penicillin and ampicillin (clear zone diameters and susceptibility test of other bacteria were not shown), whereas all isolates of coagulase-negative *Staphylococci* were susceptible for all tested antibiotics (Table 3). Three of antibiotic resistance bacteria were detected for virulence gene detection,

Table 1. Distributions of isolated human pathogens in tilapia fishes from fish farms and fresh markets.

Isolates bacteria	Number of isolates	
	Fish farms (n = 180)	Markets (n = 160)
K. pneumoniae	35	5
E. tarda	8	-
P. mirabilis	4	1
E. coli	2	16
Staphylococcus aureus	2	-
Coagulase-negative *Staphylococci*	8	15
Bacillus spp.	1	4
Streptococcus group D non enterococci	1	1
Enterococcus spp.	-	1
Candida albicans	3	1

Table 2. Frequency of bacteria isolates in tilapia fishes from fresh markets in two areas of Bangkok.

Bacterial isolates/District	Dusit				Bangkok Noi			
Week	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
E. coli	1	1	3	1	3	3	2	2
Coagulase-negative *Staphylococci*	0	0	3	2	3	2	3	2
Enterococcus spp.	0	0	1	0	0	0	0	0
C. albicans	0	0	1	0	0	0	0	0
Bacillus spp.	0	0	1	0	2	1	1	0
Streptococcus viridans	0	0	1	0	0	0	0	0
K. pneumoniae	1	1	1	0	1	1	0	0
P. vulgaris	0	1	0	0	0	0	0	0

Table 3. Interpretation of antibiotic susceptibility of bacteria isolates by agar disc diffusion test*.

Antibiotic disk (dose/disk)	*K. pneumoniae*(*n* = 40)	*E. coli*(*n* = 18)	Coagulase-negative *Staphylococci* (*n* = 38)	*P. mirabilis*(*n* = 1)
Amikacin (30 µg)	S	S	S	S
Ampicillin (10 µg)	**R**	**R**	S	**R**
Cefotaxime (30 µg)	S	S	S	S
Ceftazidime (30 µg)	S	S	S	S
Chloramphenicol (30 µg)	S	S	S	S
Ciprofloxacin (5 µg)	S	S	S	S
Penicillin (10 U)	**R**	**R**	S	**R**
Sulfamethoxazole/Trimetroprim (1.25/23.75 µg)	S	S	S	S
Tetracyclin (30 µg)	S	S	S	S
Norfloxacin (10 µg)	S	S	S	S

*According to Clinical and Laboratory Standards Institute (CLSI) criteria: S = susceptible; R = resistance.

including bla_{CTX-M} and *Int1*genes. Only isolates of *K. pneumonia* were carried bla_{CTX-M} and *Int1* genes (Fig. 1), however, PCR amplification products of *E. coli* and *P. mirabilis* isolates were negative for bla_{CTX-M} and *Int1* genes (data not shown).

The routine microbiological identification of microbial isolates from tilapia fishes in farms and in fresh markets was identified in major bacterial isolates, *K. pneumonia*e, *E. tarda*, and coagulase-negative *Staphylococci*; and *E. coli*, coagulase-negative *Staphylococci*, and *K. pneumonia*, respectively. Further antibiotic susceptibility tests were performed and *K. pneumonia*, *E. coli*, and *P. mirabilis* were antibiotic resistance bacteria, which were against to penicillin and ampicillin. Three of them were extracted for DNA amplification and detected for two virulence genes. The result represented only *K. pneumonia* from both sources of tilapia fishes, which are carried out bla_{CTX-M} and *Int1* genes. As previous study was deduced that growing of pathogens in fishes, especially *K. pneumoniae* may relate to water environment or water qualities of fish farms; and also may get affected by types of fish in well or ponds and seasonal variation (Thongkao and Sudjaroen, 2017), which corresponded to another study that explained environmental condition and temperature variation can cause persistence and spread of *K. pneumoniae* in the soil and other farm produce. Significant up-regulation of genes encoding ribosomal proteins at 20°C and 50°C possibly suggest their role in the survival of *K. pneumoniae* cells under low- and high-temperature stress (Tripathy *et al*., 2014). In addition, *K. pneumoniae* can be antibiotic resistance bacteria and (ARGs) reservoir as corresponded to results from previous studies (Budiati *et al*., 2013; Kristiansson *et al*., 2011; Kümmerer, 2009; Marathe, *et al*., 2016; Newaj-Fyzul *et al*., 2008). The prevalence of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*, including *E. coli*, *K.* pneumoniae, *Enterobacter cloacae*, *Morganella morganii*, *Citrobacter freundii*, and *Proteus vulgaris* among wild fishes had been reported (Brahmi *et al*., 2017). Examples of diseases in aquatic animals, such as hemorrhage and petechiae on the body of *Cyprinus carpi* (Oliveira *et al*., 2013), skin discoloration with an ulcer, and exopthalmia in *Nemipterus japonicus* (Diana and Manjulatha, 2012) and also in Tilapia fishes (Takyi *et al*., 2012).

ESBLs confer resistance in a wide variety of beta-lactam antibiotics in pathogens commonly member in Enterobacteriaceae and they are difficult to treat by normal medication (Naseer and Sundsfjord, 2011; Pagani *et al*., 2003). Various sources of ESBLs producing bacteria, such as environment contaminants

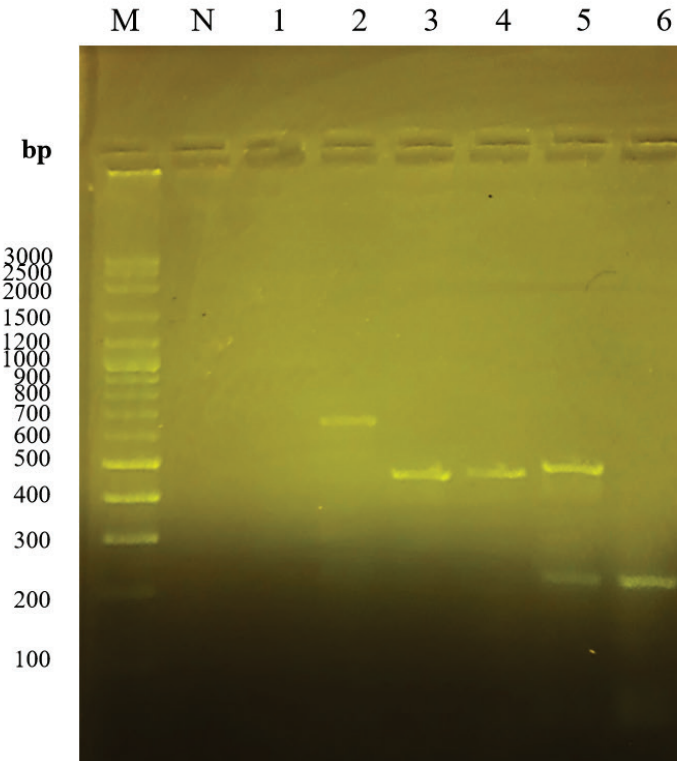

Figure 1. DNA electrophoresis separation of PCR product amplification from different sources of *K. pneumoniae* isolates: Lane M: DNA ladder (100 bp); Land N: negative control; Lane1: negative for bla_{CTX} and *Int1* genes from tilapia fish in nursery farm; Lane 2: positive for bla_{CTX} gene from tilapia fish in fish farm (floating basket type); Lanes 3 and 4: positive for bla_{CTX} gene from tilapia fish in fresh markets, including Dusit and Bangkok Noi districts, Bangkok; Lane 5: positive for bla_{CTX} and *Int1* genes (two bands); and Lane 6: positive for *Int1* gene from well typed fish farm.

from agricultural and domestic origins, which modulate genetic exchanges by aquatic environments, and then antibiotic resistance bacteria can be distributed (Lupo *et al*., 2012; Tacão *et al*., 2012). According to our results, the contribution of antibiotic resistance bacteria may alternatively be reserved and transmitted from environment to human by tilapia fishes as fishes can be transported from farms to markets that implied "environment to human". ESBLs and integrons relating genes are often associated with drug resistance and considered as markers of horizontal gene transfer potential of a bacterial strain (Gillings, 2014; Mazel, 2006).

bla_{CTX-M} is one of the ESBLs genes commonly in aquatic animals (Brahmi *et al*., 2017) and recently reported in tilapia fish (Budiati *et al*., 2013; Newaj-Fyzul *et al*., 2008). Integrons are mobile genetic elements responsible for integration and expression of gene cassettes. Our finding was also confirmed the class 1 integron (*Int1* gene) are more frequent in tilapia fish rather than class 2 integron. However, lack of bla_{CTX-M} and *Int1* genes in *E. coli* and *P. mirabilis* isolates, in this study, may due to there are carried out other antibiotic resistance markers, such as class 2 integron, bla_{CTX-M}, bla_{SHV}, bla_{OXA}, and aac(6')-*Ib*-*cr* genes (Marathe *et al*., 2016), which are often associated with ARGs and considered as markers of horizontal gene transfer potential of a bacterial strain (Gillings, 2014). Horizontal gene transfer in other aquatic animal, such as in white Pacific shrimp (*Litopenaeus vannamei*) is a reservoir of virulence genes and transferring among different *Vibrio* species (Gennari *et al*., 2012; Thongkao and Sudjaroen, 2016).

Hence, we reported *K. pneumoniae* isolated from farmed and marketed tilapia fish, which were carried out bla_{CTX-M} and *Int1* genes, were few reported in Thailand. The previous studies had been reported antibiotic resistances of *K. pneumoniae* in tilapia fish, however, there had been reported the antibiotic resistance bacteria that were isolated from free-living tilapia fishes but not from farming area (Marathe *et al*., 2016; Newaj-Fyzul *et al*., 2008), It may imply that the transmission of antibiotic resistance bacteria by Thai aquatic fishes may occur in both of farming and non-farming sources; and they are reflected to the misusing of antibiotics in Thailand, which is indicated by accumulation of antibiotic resistance bacteria, such as in tilapia fishes that are most consuming in Thailand. Regarding antimicrobial use and antimicrobial resistance in aquaculture, tetracycline use from 25 countries was reported by 18% with tilapia and resistance to tetracycline was reported as "frequent-to-almost always" by 17%. "Frequent-to-almost always" use of quinolone was also reported (Tuševljak *et al*., 2013). The application of antibiotics in tilapia farms along Thai rivers had been reported and commonly used antibiotics, oxytetracycline, enrofloxacin, amoxicillin, and sulfadimethoxine which indicate that aquaculture farms constitute an important source of antibiotic pollution (Rico *et al*., 2014). The repeated antibiotic use is expected to result in the development of antibiotic-resistant bacteria, making antibiotics actually ineffective against the target pathogens (Rico *et al*., 2014). Thus, control of antibiotic use is a need to concern in fish farming and GAP in Thailand is recommended to prevent aquatic diseases and also antibiotic resistance. For local consumers, awareness of proper cooking and good hygiene can help in preventing the zoonotic bacterial transmission from tilapia. Moreover, prevention of bacterial transmission and antibiotic resistance in fish farming environments should be done as public health policy.

CONCLUSION

Tilapia fishes (*O. niloticus*) from farms and fresh markets carried human pathogenic bacteria with antibiotic resistance characteristics. *K. pneumonia*e is the most important isolated bacteria due to the distribution in tilapia fishes and positive for bla_{CTX-M} and *Int1* genes detection. *E. coli* and *P. mirabilis* have contained antibiotic properties, however, they were negative for bla_{CTX-M} and *Int1*gene detection, possibly there may reserve other ARGs.

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CONFLICT OF INTEREST

There is no conflict of interest related to this research study.

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