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Species diversity, tetracycline resistance and virulence factor gene profile of pathogenic *Aeromonas* spp. isolated from Nile tilapia seed farms in southern Thailand

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Abstract The pathogenic bacteria causing motile *Aeromonas* septicemia (MAS) in Nile tilapia seeds cultured in southern Thailand between 2016 and 2020 were identified and tested for its antibiotic resistance ability against tetracycline drugs. In addition, tetracycline resistance genes and virulence genes were determined in the tetracycline-resistant *Aeromonas* spp. Our results indicated that almost 70% (172/250 isolates) was tetracycline resistance. According to the biochemical test, 250 isolates were assigned to 7 different platforms. Five isolates from each biochemical platform were identified by *16S rRNA* gene sequence and phylogenetic reconstruction analysis. *A. veronii* biovar *veronii*, *A. veronii* biovar *sobria*, *A. hydrophila*, *A. caviae*, and *A. jandaei* were identified of which *A. veronii* biovar *veronii* was a dominant species. Efflux antibiotic genes (*tet*A, *tet*B, *tet*C, *tet*D, and *tet*E) were only found in the tetracycline-resistant *Aeromonas* spp. The predominant tetracycline resistance gene detected was *tet*A. Multiple *tet* genes were found in the tetracycline-resistant *Aeromonas* spp. In addition, 9 different virulence factor gene profiles of *lipase*, *elastase*, *enolase*, *aerolysin* (*aerA*), and *heat-labile cytotonic enterotoxin* (*alt*) were established. Twelve of 35 isolates (34.29%) had 3 virulence genes; 6 (17.14%) for *lipase/enolase/alt*, 3 (8.57%) for *lipase/enolase/aerA*, 2 (5.71%) for *elastase/enolase/aerA*, and 1 (2.86%) for *elastase/enolase/alt*. Our results suggested that there is a variation in isolated *Aeromonas* spp. with different phenotypes of tetracycline resistance and genotypes of tetracycline resistance genes and virulence factor genes.

Keywords: *Aeromonas* spp., Nile tilapia, Tetracycline resistance, *Tet* genes, Virulence factor gene

Introduction

Nile tilapia (*Oreochromis niloticus*) is the third high-production species of total finfish production in world aquaculture (FAO, 2020). In Thailand, this fish is an important cultured freshwater fish due to its high production and consumer demands. Its production has been increased with approximately 200,000 tons per year (Srisapoome and Areechon, 2017). Many tilapia farms

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have been organized in southern part of Thailand including Nakhon Si Thammarat, Surat Thani, Phattalung, Songkhla, and Satun.

The mortality especially due to bacterial diseases can be occurred in every life stages from seed to grow-out fish of cultured tilapia. High stocking density and improper farm managements and practices considerably increase the risks of opportunistic pathogens such as *Aeromonas* infection (Bebak *et al*., 2015; Reyes, 2018). *Aeromonas* spp. have been reported as causative agents of motile *Aeromonas* septicemia (MAS), an acute or chronic infectious disease, that can infect a wide range of host including Nile tilapia. The frequent isolated species are *A. hydrophila*, *A. veronii*, *A. veronii* biovar *sobria*, *A. jandaei* and *A. shuberti* (Dong *et al*., 2017; Chirapongsatonkul *et al*., 2018; 2019; Liu *et al*., 2018; Raj *et al*., 2019). We previously reported that *A veronii* or *A. veronii* biovar *veronii* is a predominant pathogenic species isolated from juvenile tilapia (U-taynapun *et al*., 2020). However, the prevalent *Aeromonas* species in MAS-expressing tilapia seeds is not well investigated.

Tetracyclines are common antibiotics used in aquaculture practically through medicated feed or immersion baths. Excessive use as medicated feed considerably leads to a liberation of antibiotics into the environment with the consequence being an induction of resistant bacteria and an increase in the transfer of antibiotic-resistant genes (Higuera-Liantón *et al*., 2018). In addition, the virulence and pathogenicity of *Aeromonas* spp. rely on diverse virulence factors associated with primary and secondary metabolites such as antigen, membrane protein (S-layer), enterotoxins, cytotoxins, exoenzymes and type III secretion system which are able to adhere to and invade host cell (Oliverira *et al*., 2012). Therefore, the molecular characterization and prevalence of tetracycline resistance (*tet*) genes and virulence factor genes are currently focused to get insight into the pathogen and to develop effective disease control practices.

The aim of this study was to investigate the *Aeromonas* spp. diversity and characterize the tetracycline-resistant bacteria isolated from MAS-expressing Nile tilapia seeds cultured in southern Thailand between 2016 and 2020. Tetracycline resistance determinants and virulence factor genes were determined in tetracycline-resistant *Aeromonas* spp.

Materials and methods

Fish samples and Aeromonas isolation

The Nile tilapia seeds (0.3-3.0 g) those exhibit the clinical signs and symptoms of MAS disease were collected from 5 provinces in southern

Thailand including Surat Thani, Nakhon Si Thammarat, Phatthalung, Songkhla, and Satun between 2016 and 2020. Fish samples were aseptically dissected then bacteria were collected from the brain and liver. *Aeromonas* spp. were selectively isolated using *Aeromonas* selective medium (BSIBG agar, Himedia). The primarily screened *Aeromonas* were further purified and morphological checked by Gram stain method and direct examination under a light microscope. The pure *Aeromonas* isolates were stocked in Tryptic Soy Broth (TSB, Difco) containing 15% (v/v) glycerol and kept at -80^{o}C until use.

Biochemical characterization and molecular identification of Aeromonas spp.

The pured 250 *Aeromonas* isolates were randomly selected from a large collection from all incidences; however, the bacterial samples were equally chosen from all 5 provinces. The biochemical test of each isolate was performed using API 20E kit (BioMérieux) according to the manufacturer's instruction. Afterthat, the bacterial stains sampled from each platform regarding the biochemical results were species identified using the *16S rRNA* sequences. Briefly, DNA of the test *Aeromonas* isolates was extracted using Bacterial DNA Isolation Kit (Geneaid) then the extracted DNA was checked for its concentration and purity by measuring an absorbance at 260 nm (A_{260}) and a ratio of $A_{260}/_{A280}$ and A_{260}/A_{230}, respectively. The obtained DNA was specifically amplified for *16S rRNA* gene using a set of universal primer, 20F/1500R (Table 1) by PCR using EmeraldAmp® GT PCR Master Mix (Takara). The PCR reactions were carried out in a T100™ Thermal Cycler (Bio-Rad) under the following conditions: 95^{o}C for 5 min, 35 cycles of 95^{o}C for 45 s, 60^{o}C for 30 s, 72^{o}C for 1 min and final extension at 72^{o}C for 5 min. The PCR products were purified using Gel/PCR DNA Fragments Kit (Geneaid) and each purified DNA fragment was cloned into pGEM-T® Easy Vector (Promega). All *16S rRNA* gene of the test *Aeromonas* spp**.** were analyzed compared to those of other *Aeromonas* spp**.** submitted in the GenBank using nucleotide BLAST tool (Altschul *et al*., 1990). The phylogenetic trees were reconstructed using MEGA X **(**Kumar *et al*., 2018). Maximum likelihood (ML) method with Kimura 2-parameter model was conducted to infer phylogenetic relationships among the *Aeromonas* spp. and its sister group. The bootstrap analysis was perfromed with 1,000 replicates and values below 50% were excluded as non-significance. The *16S rRNA* of *Lactobacillus delbrueckii* (EF015468.1), *Lactobacillus plantarum* (D79210.1), *Vibrio alginolyticus* (MG645335.1), *Vibrio cholerae* (MK615861.1), and 3 sequences of *Vibrio parahaemolyticus* (MF372386.1, FJ594056.1 and EU624428.1) were used as out-group species in phylogenetic analysis.

Antibiotic (tetracycline) resistance

The antibiotic resistance against the tetracycline drugs of each *Aeromonas* isolates was determined by disc diffusion method (U-taynapun *et al*., 2018). *Aeromonas* strains were streked on Mueller-Hinton (MH, Difco) agar, then the antibiotic discs of doxytetracycline (DO, 30 μg), tetracycline (TE, 30 μg) and minocycline (MN, 30 μg) (Oxoid) were placed under aseptic technique. After 24 h of incubation at 35^{o}C, the inhibition zones were measured and compared to the manufacturer's instruction referred to CLSI document M100-S23 (CLSI, 2013).

Determination of tetracycline resistance genes and virulence factor genes

PCR assays were performed to amplify 12 tetracycline resistance genes including *tet*A, *tet*B, *tet*C, *tet*D, *tet*E, *tet*G, *tet*K, *tet*L, *tet*M, *tet*O, *tet*S, and *tet*34 from the tetracycline-resistant *Aeromonas* isolates. In addition, the virulence factor genes, *lipase*, *elastase*, *enolase*, *aerolysin* (*aerA*), and *heat-labile cytotonic enterotoxin* (*alt*), were also determined. Primers used for the amplification of the tetracycline resistance genes and virulence factor genes are listed in Table 1. The PCR reactions were performed in the T100^{TM} Thermal Cycler (Bio-Rad) according to amplification conditions described by the references. All obtained PCR products and a 100-bp marker (100 bp Plus DNA Ladder, Vivantis) were separated on 2% (w/v) agarose gel to analyse the appearance of the tetracycline resistance genes and virulence factor genes. Positive result (+) refers to the visualized band at the desired amplicon sizes whereas (–) refers to no band or not detected.

Results

Prevalence and occurrence of tetracycline-resistant Aeromonas spp.

In the present study, Nile tilapia seeds exhibiting clinical signs of external signs of eroded fins, epidermal ulcers and hemorrhagic ulcers were collected between 2016 and 2020 for *Aeromonas* isolation using *Aeromonas* selective medium. The single colony of green translucent colony was cultured and morphological characterization based on Gram-stain. Approximately 420 bacterial isolates exhibiting typical *Aeromonas* morphological characteristics such as Gram-negative with shot-rod shape were sub-cultured and collected as a bacterial stock. Two hundred and fifty bacterial isolates were randomly selected for further biochemical characterized using API 20E kit. The results showed that there were 7 different platforms among the test 250 *Aeromonas* isolates as shown in Table 2. Based on API 20E result, 17 isolates (6.80%)

were classified as *A. hydrophila* and grouped as Bio-Gr. I while 23 isolates (9.20%) were classified as *A. jandaei* and grouped as Bio-Gr. VII. Fifty-eight isolates, classified *A. hydrophila/caviae/sobria* 1, were catagorized into 2 small groups of Bio-Gr. II (28 isolates, 11.20%) and Bio-Gr. III (30 isolates, 12.00%). In this study, the largest group of *Aeromonas* classified as *A. hydrophila/caviae/sobria* 2 (152 isolates, 60.80%) was divided into 3 groups; Bio-Gr. IV (74 isolates, 29.60%), Bio-Gr. V (37 isolates, 14.80%), and Bio-Gr. VI (41 isolates, 16.60%).

Table 1. Primer used in this study

Gene	Primer name	Sequence	Product size (bp)	References
16S rRNA	20F	5´-AGAGTTTGATCATGGCTCAG-3´	~1,500	Weisburg *et al*. (1991)
	1500R	5´-CGGTTACCTTGTTACGACTT-3´		
*tet*A	tetA-F	5´-GCTACATCCTGCTTGCCTTC-3´	211	Nawaz *et al*. (2006)
	tetA-R	5´-GCATAGATCGCCGTGAAGAG-3´		
*tet*B	tetB-F	5´-TCATTGCCGATACCACCTCAG -3´	391	Nawaz *et al*. (2006)
	tetB-R	5´-CCAACCATCATGCTATTCCATCC-3´		
*tet*C	tetC-F	5´-CTGCTCGCTTCGCTACTTG-3´	897	Nawaz *et al*. (2006)
	tetC-R	5´-GCCTACAATCCATGCCAACC-3´		
*tet*D	tetD-F	5´-TGTGCTGTGGATGTTGTATCT-3´	844	Nawaz *et al*. (2006)
	tetD-R	5´-CAGTGCCGTGCCAATCAG-3´		
*tet*E	tetE-F	5´-ATGAACCGCACTGTGATGATG-3´	744	Nawaz *et al*. (2006)
	tetE-R	5´-ACCGACCATTACGCCATCC-3´		
*tet*G	tetG-F	5´-CAGCTTTCGGATTCTTACGG-3´	844	Lin *et al*. (2021)
	tetG-R	5´-GATTGGTGAGGCTCGTTAGC-3´		
*tet*K	tetK-F	5´-TTAGGTGAAGGGTTAGGTCC-3´	697	Aarestrup *et al*. (2000)
	tetK-R	5´-GCAAACTCATTCCAGAAGCA-3´		
*tet*L	tetL-F	5´-CATTTGGTCTTATTGGATCG-3´	456	Aarestrup *et al*. (2000)
	tetL-R	5´-ATTACACTTCCGATTTCGG-3´		
*tet*M	tetM-F	5´-GTGGACAAAGGTACAACGAG-3´	406	Ng *et al*. (2001)
	tetM-R	5´-CGGTAAAGTTCGTCACACAC-3´		
*tet*O	tetO-F	5´-AACTTAGGCATTCTGGCTCAC-3´	515	Ng *et al*. (2001)
	tetO-R	5´-TCCCACTGTTCCATATCGTCA-3´		
*tet*S	tetS-F	5´-ATCAAGATATTAAGGAC-3´	573	Gevers *et al*. (2000)
	tetS-R	5´-TTCTCTATGTGGTAATC-3´		
*tet*34	tet34-F	5´-ATGAAAACGAACGCTAATTAACCA-3´	270	Miranda *et al*. (2003)
	tet34-R	5´-ACATAGAGATCGATGCTAGTACTA-3´		
lipase	Lip-F	5´-GACTCCCTCAAGGACAGCAG-3´	594	Chirapongsatonkul *et al*. (2018)
	Lip-R	5´-AGAGGCTTTCAGGGCATTG-3´		
elastase	Elas-F	5´-CGACATGTACCGCAACTGGTA-3´	466	Chirapongsatonkul *et al*. (2018)
	Elas-R	5´-GGTGTTGGCCAGCAGGTA-3´		
enolase	Enol-F	5´-CGACGGTACCGAGAACAAA-3´	212	Chirapongsatonkul *et al*. (2018)
	Enol-R	5´-CTTGGATGTCGACGTTGTTG-3´		
alt	Alt-F1	5´-AGGATGCCCTCAACACCATC-3´	272	Chirapongsatonkul *et al*. (2018)
	Alt-R1	5´-GCTCTGTTTCAGGTTGTCGC-3´		
aerA	aerA-F	5´-GAAGGTGACCACCAAGAACAA-3´	409	U-taynapun *et al*. (2020)
	aerA-R	5´-CCAGTCCCACCACTTCACTT-3´		

The representative 250 *Aeromonas* isolates were also determined for the tetracycline-resistant phenotype. The results showed that 172 of 250 (around 70%) isolates were tetracycline-resistant *Aeromonas* spp. In accordance with biochemical characteristics, 5 of these isoates were tentatively classified as *A. hydrophila* (Bio-Gr. I), 33 as *A. hydrophila/caviae/sobria* 1 (16 as Bio-Gr. II and 17 as Bio-Gr. III), 118 as *A. hydrophila/caviae/sobria* 2 (58 as Bio-Gr. IV, 28 as Bio-Gr. V and 32 as Bio-Gr. VI), and 16 as *A. jandaei* (Bio-Gr. VII) (Table 2).

Table 2. Prevalence, biochemical characterization and tetracycline resistance of the test *Aeromonas* spp.

Platform	API 20E classification	Prevalence	Occurrence of tetracycline resistance
Bio-Gr. I	*A. hydrophila*	17/250 (6.80%)	5/17 (47.06%)
Bio-Gr. II	*A. hydrophila/caviae/sobria* 1	28/250 (11.20%)	16/28 (57.14%)
Bio-Gr. III	*A. hydrophila/caviae/sobria* 1	30/250 (12.00%)	17/30 (56.67%)
Bio-Gr. IV	*A. hydrophila/caviae/sobria* 2	74/250 (29.60%)	58/74 (78.38%)
Bio-Gr. V	*A. hydrophila/caviae/sobria* 2	37/250 (14.80%)	28/37 (70.27%)
Bio-Gr. VI	*A. hydrophila/caviae/sobria* 2	41/250 (16.40%)	32/41 (78.05%)
Bio-Gr. VII	*A. jandaei*	23/250 (9.20%)	16/23 (69.57%)

Phylogenetic analysis

Since the species classification using biochemical descriptions indicated the percent probabilities of the identification in a range of 89-99%, molecular aspect was further analysed for the species of 35 isolates of *Aeromonas* spp. (5 isolates from each biochemical classification platform**).** Phylogenetic analysis was reconstructed via *16S rRNA* gene using the ML algorithm resulted in a single most parsimonious tree (Figure 1). Sequence alignment revealed that there were 4 clades of *Aeromonas* that could fit with *16S rRNA* gene of the 35 test *Aeromonas***.** Bio-GR**.** I and Bio-Gr**.** III, a total of 47 isolates (18.80%), was classified in *A. hydrophila* clade**.** Bio-Gr**.** II, 28 isolates (11.20%), was found in *A. caviae* clade while Bio-Gr-VI, 41 isolates (16.40%) was categorized in *A. veronii* biovar *sobria***.** Comparable to the biochemical characterization, Bio-Gr**.**VII (23 isolates, 9**.**20%) was in the clade of *A. jandaei***.** A majority of *Aeromonas* in this study was *A. veronii* biovar *veronii* with a total of 78 isolates (31.10%) that consisted of 2 groups, Bio-Gr**.** IV and Bio-Gr. IV.

Tetracycline resistance gene and virulence factor gene profiles

The appearance of 3 groups of tetracycle resistance genes; tetracycline efflux genes (*tet*A, *tet*B, *tet*C, *tet*D, *tet*E, *tet*G, *tet*K and *tet*L), ribosomal protection genes (*tet*M, *tet*O and *tet*S) and enzyme activation gene (*tet*34) were examined in 35 tetracycline-resistant *Aeromonas* isolates (Table 3). Only tetracycline efflux genes, *tet*A (25, 71.43%), *tet*B (10, 28.57%), *tet*C (20, 57.14%), *tet*D (22, 62.86%), and *tet*E (4, 11.43%), could be detected. Our results indicated that *tet*A was the predominant tetracycline resistance gene, followed by *tet*D, *tet*C, *tet*B, and *tet*E, respectively.

For the *tet* gene profile, single *tet* gene was detected as *tet*A (1 of 35 isolates, 2.86%). In the test tetracycline-resistant *Aeromonas*, many isolates contained multiple *tet* genes simaultameously (Table 4). Two *tet* genes observed namely, *tet*A along with *tet*B and *tet*C in 4 (11.43%) and 8 (22.86%), respectively, while *tet*D along with *tet*E and *tet*C in 4 (11.43%) and 6 (17.14%), respectively. Moreover, 12 isolates (34.29%) had 3 *tet* genes, *tet*A, *tet*C and *tet*D.

Table 3. Genetic detection of tetracycline resistance genes and virulence genes in *Aeromonas* spp. isolated from MAS-expressing Nile tilapia seeds

Gene	*A. hydrophila*		*A. caviae*	*A. veronii* biovar *veronii*		*A. veronii* biovar *sobria*	*A. jandaei*	Total (%)
	Bio-Gr. I (n=5)	Bio-Gr. III (n=5)	Bio-Gr. II (n=5)	Bio-Gr. IV (n=5)	Bio-Gr. V (n=5)	Bio-Gr. VI (n=5)	Bio-Gr. VII (n=5)	
*tet*A	3	3	4	5	5	3	2	25 (71.43%)
*tet*B	–	2	2	2	3	1	–	10 (28.57%)
*tet*C	2	3	3	4	4	3	1	20 (57.14%)
*tet*D	3	2	4	3	3	4	3	22 (62.86%)
*tet*E	1	–	–	1	1	2	–	4 (11.43%)
*tet*G	–	–	–	–	–	–	–	–
*tet*K	–	–	–	–	–	–	–	–
*tet*L	–	–	–	–	–	–	–	–
*tet*M	–	–	–	–	–	–	–	–
*tet*O	–	–	–	–	–	–	–	–
*tet*S	–	–	–	–	–	–	–	–
*tet*34	–	–	–	–	–	–	–	–
lipase	2	1	2	3	3	2	1	14 (40%)
elastase	2	1	1	3	2	2	–	11 (31.43%)
enolase	5	5	5	5	5	5	5	35 (100%)
alt	1	1	2	2	2	2	1	11 (31.43%)
aerA	1	3	2	1	1	2	–	10 (28.57%)

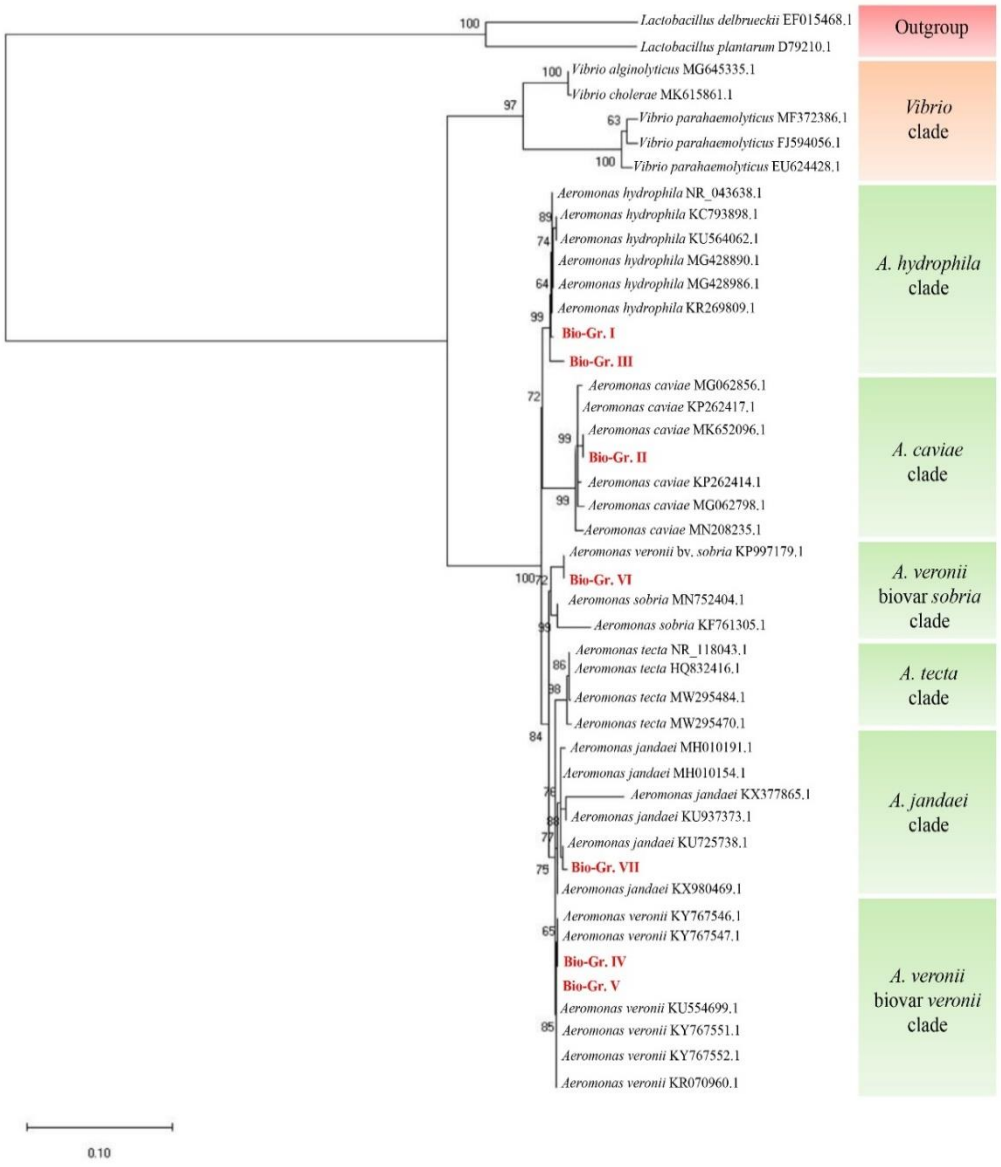

Figure 1. Phylogenetic analysis by Maximum Likelihood (ML). The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018)

Table 4. Tetracycline resistance gene profiles in tetracycline-resistant *Aeromonas* spp.

Tetracycline resistance gene profile	Number and percentage of *Aeromonas* isolates	Tetracycline efflux gene								Ribosomal protection gene			Enzyme activation gene
		*tet*A	*tet*B	*tet*C	*tet*D	*tet*E	*tet*G	*tet*K	*tet*L	*tet*M	*tet*O	*tet*S	*tet*34
1	1 (2.86%)	+	–	–	–	–	–	–	–	–	–	–	–
2	4 (11.43%)	+	+	–	–	–	–	–	–	–	–	–	–
3	8 (22.86%)	+	–	+	–	–	–	–	–	–	–	–	–
4	12 (34.29%)	+	–	+	+	–	–	–	–	–	–	–	–
5	4 (11.43%)	–	–	–	+	+	–	–	–	–	–	–	–
6	6 (17.14%)	–	+	–	+	–	–	–	–	–	–	–	–

Virulence gene *enolase* was predominantly observed in the test isolates (35, 100%), followed by *lipase* (14, 40%), *elastase* and *alt* (11, 31.43%) and *aerA* (10, 28.57%), respectively (Table 3). Among 35 tetracycline-resistant *Aeromonas* isolates, 9 virulence genotypes could be detected (Table 5). Most of the isolates (22, 62.86%) contained two-virulence genes simultaneously; however, diverse profiles were noticed. Twelve isolates (34.29%) had 3 virulence genes; 6 (17.14%) for *lipase/enolase/alt*, 3 (8.57%) for *lipase/enolase/aerA*, 2 (5.71%) for *elastase/enolase/aerA*, and 1 (2.86%) for *elastase/enolase/alt*.

Table 5. Virulence factor gene profiles in tetracycline-resistant *Aeromonas* spp.

Virulence factor gene profile	Number and percentage of *Aeromonas* isolates	*lipase*	*elastase*	*enolase*	*alt*	*aerA*
1	1 (2.86%)	–	–	+	–	–
2	5 (14.92%)	+	–	+	–	–
3	8 (22.86%)	–	+	+	–	–
4	4 (11.43%)	–	–	+	+	–
5	6 (17.14%)	+	–	+	+	–
6	5 (14.29%)	–	–	+	–	+
7	3 (8.57%)	+	–	+	–	+
8	2 (5.71%)	–	+	+	–	+
9	1 (2.86%)	–	+	+	+	–

Discussion

Aeromonas spp. have been reported for decades as the common causative agents of aquatic animals especially freshwater organisms. A few earlier studies have shown that *A. veronii* is the predominant species isolated from the collected aquatic animals. Esteve *et al.* (2015) has reported that *A. veronii* are accounted as a largest species isolated from river, fish and clinical samples in

Spain. In addition, this species is also found as a large proportion pathogens isolated from shellfish imported from 6 aquaculture bases in Jinlin Province, China (Xu *et al*., 2021). Our previous study has demonstrated that *A. veronii* or *A. veronii* biovar *veronii* is the predominant pathogenic species found in MAS-expressing Nile tilapia cultured in Thailand (U-taynapun *et al*., 2020). Correspondingly, the results in this study indicated that *A. veronii* biovar *veronii* is the dominant species, 111 of 250 isolates or approximately 44%, isolated from the MAS disease tilapia seeds in southern Thailand. Other detected species comprised *A. veronii* biovar *sobria*, *A. hydrophila*, *A caviae*, and *A. jandaei*.

Among these 250 *Aeromonas* isolates, a high prevalence of antibiotic resistant phenotype against the tetracycline drugs, TE, DO and MN, was detected (172 isolates or almost 70%). Xu *et al.* (2021) also reported that most of *A. veronii* isolated from shellfish resist against TE and DO while Yang *et al*. (2017) demonstrated that most of *A. veronii* isolated from channel catfish in China were drug resistant to penicillin, oxaline and DO. Therefore, the appearance of tetracycline resistance (*tet*) genes were also determined in the 35 isolates randomly selected, 10 isolates from *A. veronii* biovar *veronii* and *A. hydrophila*, while 5 isolates from *A. veronii* biovar *sobria*, *A caviae* and *A. jandaei*. Three groups of *tet* genes coding proeins with diverse functions including efflux genes (*tet*A, *tet*B, *tet*C, *tet*D, *tet*E, *tet*G, *tet*K, and *tet*L), ribosomal protection genes (*tet*M, *tet*O and *tet*S) and enzymatic inactivation gene (*tet*34) were analysed. Only *tet* genes associated with the drug efflux, *tet*A, *tet*B, *tet*C, *tet*D, and *tet*E, were detected while none of ribosomal protection genes and enzymatic inactivation gene was observed in the tetracycline-resistant *Aeromonas* spp. Similarly, there have been reported that *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetH*, *tetG* and *tetM* mostly found in *Aeromonas* spp. isolated from water, sediments and diseased fish in aquaculture systems (Schmidt *et al*., 2001; Nawaz *et al*., 2006; Akinbowale *et al*., 2006; Verner-Jeffreyes *et al*., 2009). Many studies indicate that tetracycline resistance is plasmid-encoded and the spread of *tet* genes is facilitated by their location on mobile genetic element transposons (DePaola and Roberts, 1995; Schmidt *et al*., 2001). Our results showed that a high prevalence of *tet* genes is of *tet*A corresponding to the study of Nawaz *et al.* (2006) and Kim *et al*. (2011). In contast, *tet*E is reported as the predominant *tet* gene in oxytetracycine-sulfonamide/trimethoprim-resistant aeromonids characterized in the rainbow trout in Danish farms (Schmidt *et al*., 2001). Interestingly, the obtained results revealed the appearance of multiple *tet* genes simultaneously in one *Aeromonas* isolate. There has been noticed that the strength of antibiotic resistance in bacteria could be correlated with the multiple resistance determinants making

them torelant in certain conditions saturated with antibiotics (Palleroni, 2010; Miranda *et al*., 2013; Olivares *et al*., 2013).

A variety of virulence factors of *Aeromonas* spp. have been studied and reported for the correlation between these factors and the bacterial pathogenicity (Sadique *et al*., 2021). In this study, the prevalence of virulence factor genes including *lipase*, *elastase*, *enolase*, *alt* and *aerA* were determined in 35 isolates of tetracycline-resistant *Aeromonas* spp. The results suggested that all isolates had *enolase* encoding enzyme enolase. This may be due to this enzyme does not involve only on the pathogenicity, a secreted and surface-expressed glycolytic enzyme binding to human/host plasminogen which leads to production of plasmin (Sha *et al*., 2009), but also in glycolysis pathway associated with the enegy production in bacterial cells. Other virulence genes, *lipase* and *elastase* which involve in host physical barrier degradation as well as genes encoding toxins, *alt* and *aerA*, could detected. These results were comparable to the previous work (U-taynupun *et al*., 2020) but different in the prevalence of virulence factor genes. Moreover, the virulence gene profiles observed were varied among *Aeromonas* isolates which may be associated with the bacterial pathogenesis similarly to those reported by Roges *et al*. (2020), Azzam-Sayuti *et al*. (2021) and Sadique *et al*. (2021).

In conclusion, this work provided the information of prevalence of *Aeromonas* spp., its tetracycline resistance ability together with the elucidated profiles of tetracycline resistance gene and virulence factor gene. The high prevalence of tetracycline-resistant *Aeromonas* spp. with diverse virulence gene profiles is of significant concern to the Nile tilapia culture and warrants continued surveillance and research to develop treatments or management strategies leading effective MAS disease control.

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