Short Communication

Expression of AheABC Efflux System and Plasmid Profile of *Aeromonas hydrophila* Isolates from Farmed Nile Tilapia (*Oreochromis niloticus*)

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Abstract

Fifty *Aeromonas hydrophila* isolates from farmed Nile Tilapia were characterized for expression of AheABC multidrug efflux system and plasmid profile. Thirty nine isolates (78%) expressed AheABC with production of 1.7 to 221.3 folds more than AheB of *A. hydrophila* ATCC35654, the reference. This suggests that the AheABC efflux system is not always constitutively expressed in the *A. hydrophila* clinical isolates. The isolates with less AheB expression were more resistant to antibiotics than those with high AheB expression. Seventeen isolates were found to carry plasmids that were arranged into 11 plasmid profiles. This is the first study of the AheABC efflux system in *A. hydrophila* isolates from fresh water fish, Nile Tilapia.

Keywords: *Aeromonas hydrophila*, the AheABC efflux system, Nile Tilapia

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บทคัดย่อ

การแสดงออกของระบบ AheABC efflux และรูปแบบพลาสมิดใน *Aeromonas hydrophila* ที่แยกได้จากปลานิลเพาะเลี้ยง (*Oreochromis niloticus*)

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ศึกษาการแสดงออกของระบบ AheABC และรูปแบบพลาสมิดใน *Aeromonas hydrophila* จำนวน 50 isolates ที่แยกได้จากปลานิลเพาะเลี้ยงพบว่าเชื้อจำนวน 39 isolates (ร้อยละ 78) มีการแสดงออกของระบบและคิดเป็น 1.7–221.3 เท่าของ AheB ใน *A. hydrophila* ATCC35654 ซึ่งเป็นเชื้ออ้างอิง แสดงว่าระบบ AheABC ไม่มีการแสดงออกตลอดเวลา โดยเชื้อที่มีการแสดงออกของ AheB ในระดับสูงไม่ดื้อต่อยาปฏิชีวนะในระดับที่สูงกว่าเชื้อที่มีการแสดงออกของ AheB ในระดับต่ำ พบพลาสมิดในเชื้อจำนวน 17 isolates และจัดรูปแบบพลาสมิดได้ 11 รูปแบบ การวิจัยครั้งนี้เป็นการศึกษาระบบ AheABC ครั้งแรกใน *A. hydrophila* ที่แยกได้จากปลานิล

คำสำคัญ: แอร์โรโมนาส ไฮโดรฟิลลา ระบบ AheABC ปลานิล

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Introduction

Nile Tilapia (*Oreochromis niloticus*) is one of the most profitable fish in Thai aquaculture. Tilapia cultivation has rapidly grown from a family-farming system to an intensive-farming industry to supply both domestic consumption and export. Such mass production has provoked stress and increased susceptibility of Tilapia to pathogenic infections. As a result, Tilapia farms have confronted several disease outbreaks. Therefore, various antibiotics have been extensively used during farmed-Tilapia production for prevention of bacterial infections. However, some of these drugs are in the same classes as those used for medical treatment in humans. This raises public concern regarding development and widespread of antimicrobial resistance among aquatic bacterial pathogens that could adversely affect both fish and human health and eventually cause economic losses.

Aeromonas hydrophila has emerged as an opportunistic pathogen causing infections in warm water fish including Tilapia (Kaskhedikar and Chhabra, 2010). This pathogen plays a role as a zoonotic agent that is usually acquired through an open wound exposed to contaminated water *A. hydrophila* is also considered a foodborne pathogen that is transmitted to humans via ingestion of contaminated food and water (Daskalov, 2006).

A. hydrophila exhibits resistance to multiple drugs (Son et al., 1997; Kaskhedikar and Chhabra, 2010). It has been well documented that multi-resistance in most Gram-negative bacteria is mainly contributed to expression of multidrug efflux systems (Nikaido, 1996; Poole, 2005). These systems simultaneously extrude the structurally-unrelated substrates including drugs in different classes (Poole, 2004). To date, only the AheABC efflux system of the resistance nodulation and cell division family has been demonstrated in *A. hydrophila* (Hernould et al., 2008). The *aheA*, *aheB* and *aheC* genes encode for membrane fusion protein, inner membrane transporter and outer membrane protein, respectively. These three genes are chromosomally located and organized in an operon with the same direction (Hernould et al., 2008). In addition, antimicrobial resistance can be mediated by resistance determinants extrachromosomally located on plasmids. Plasmids carrying resistance-encoding genes have been shown to play a major role in distribution of antimicrobial resistance among bacteria (Gebreyes and Thakur, 2005; Rodriguez et al., 2006).

Up to date, contribution of the AheABC efflux system was determined only in an *A. hydrophila* laboratory strain (Hernould et al., 2008) and there is limited data on plasmids in clinical isolates (Son et al., 1997). Expression of the AheABC efflux system and plasmid profile of *A. hydrophila* isolated from freshwater fish, particularly farmed Nile Tilapia has never been reported. The aims of this study were to measure expression of the AheABC efflux system and its involvement in multidrug-resistance phenotypes and determine plasmid profile in *A. hydrophila* isolated from farmed Nile Tilapia.

Materials and Methods

Bacterial isolates: A total of 50 *A. hydrophila* isolates were included in this study. They were originated from clinical cases of farmed Nile Tilapia in Thailand during 2007 and 2009. All strains were stored in strain collection of Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University. The *A. hydrophila* species was confirmed genetically in our previous study (Lukkana et al., 2011).

All the *A. hydrophila* isolates were tested for their antimicrobial susceptibilities in the former study (Lukkana et al., 2011). All exhibited resistance to at least one antibiotic and 94% were multidrug-resistant. Resistance rates to amoxicillin, carbenicillin, ciprofloxacin, enrofloxacin, erythromycin, kanamycin, minocycline, oxytetracycline, streptomycin, sulphamethoxazol, tetracycline, trimethoprim were 86%, 82%, 34%, 32%, 12%, 22%, 2%, 34%, 92%, 62%, 36%, 88%, respectively. None of the isolates were resistant to gentamicin, chloramphenicol and cefoperazone.

PCR and sequencing analysis: PCR-template DNAs were obtained using whole cell boiled lysate protocol (Levesque et al., 1995) or Isoquick Nucleic acid extraction kit (IsoQuick®, WA, USA). All PCR reactions were carried out using Fermentas® PCR master mix (Fermentas, Burlington, Canada) according to the manufacturer's instructions. The PCR amplicons were gel purified using Nucleospin Gel Extraction kit (Nucleospin®, Gutenburg, France) and submitted for DNA sequencing at Molecular Informatic Lab, NT, Hong Kong. The nucleotide sequences were analyzed by using the Blast algorithm (http://www.ncbi.nlm.nih.gov).

Reverse transcription-PCR (RT-PCR): All the *A. hydrophila* strains were screened for transcription of the *aheB* gene using conventional RT-PCR. Total RNA was isolated using QIAGEN RNeasy mini kit (Qiagen® Hilden, Germany) and treated with DNaseI (Fermentas). One µg of total RNA was converted to cDNA using SuperScriptTM III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer and used as DNA template for PCR. Specific primer pairs used were AhyBF (5′ GAGGAGTTCAACGACATCCTG-3′) and AhyBR (5′-GTACGGGTAGACAATCTCCATGTT-3′). *A. hydrophila* ATCC35654 was used as a positive control.

Quantitative real-time PCR (qRT-PCR): The *A. hydrophila* isolates with the expression of *aheB* were randomly selected (n=10) for measurement of the transcription level of *aheB* by qRT-PCR using BiotoolsQuantiMix EASY SYG Kit (Biotools B&M Labs SA, Madrid, Spain) as previously described (Chuanchuen et al., 2008). First cDNA was synthesized from 1 µg of total RNA. The cDNA concentrations were measured and diluted to generate the threshold cycle (Ct) within the limits of standard curve($r^2 > 0.990$) that was a graph relating the average Ct values to the log of given concentrations of *A. hydrophila* ATCC35654 DNA template. Specific primer sets for *aheB* and the housekeeping gene *rpsL* were AhyBUp (5′-TATCGGTCACCCTCACCTTC-3′)/AhyBDown (5′-CTGCTGGAGGTCTTCTGGAC-3′)/ and AhyrpslUp (5′-CCACGGATCAGAACAACAGA-3′)/Ahyrpsl Down (5′-TATACACCACCACCCCGAAG-3′), respectively and the PCR amplification was carried out in triplicate. The average *aheB* cDNA copy number for each clinical sample was calculated from the Ct values from two separate experiments (n=6, SD<0.1) and normalized using the average *rpsL* copy number of the same sample. The relative level of *aheB* expression in each strain was calculated by comparison to that of *A. hydrophila* ATCC35654.

Isolation of plasmid DNA: Plasmid DNA isolation was carried out in all the *A. hydrophila* strains using the alkaline lysis technique (Liou et al., 1999).The plasmid pattern from each strain was visualized in 0.5% agarose gels and photographed. The experiment was repeated in three separate occasions.

Results and Discussion

Of all the *A. hydrophila* strains, expression of the *aheB* gene was detected in 39 isolates (78%). Since transcription of the *aheB* gene was not detected in all isolates, it suggests that the *aheABC* efflux is not always constitutively expressed in the *A. hydrophila* clinical isolates. However, it should be also noted that some isolates may have expression levels lower than the detection limit. All 10 *A. hydrophila* isolates tested produced 1.7 to 221.3 folds more than AheB of A. hydrophila ATCC35654 (Table 1). As the expression level of aheB varied, the isolates with high *aheB* expression did not show more resistance to antibiotics than those with less *aheB* expression. AH194 with the highest *aheB* expression (i.e. 221.3 folds) was resistant to a single antibiotic, while AH173 with the lowest *aheB* expression (i.e. 1.7 folds) was resistant up to 9 antimicrobials. This suggested that the *aheABC* expression level did not correlate with multidrug resistance phenotype in these clinical isolates. Based on a previous report, the preferential antibiotic substrates of the AheABC systems included cefuroxime, cefoperazone, pristinamycin and minocycline (Hernould, et al., 2008). Since the antibiotics tested were not the favored substrates for the systems, this could be a possible explanation for the non-associated findings. When consider individual antibiotics, the AheABC expression did not correlate with the susceptibility level. For instance, MIC value to amoxicillin, carbenicillin, streptomycin, tetracycline and trimethoprim of AH194 was lower than those of AH173. Based on the standard breakpoints, all the isolates tested were susceptible to cefoperazone, chloramphenicol, gentamicin and minocycline. Still, it cannot be concluded that the system did not efflux these antimicrobials by any means. Contribution of the AheABC efflux pump in antibiotic extrusion may not be considerable (Hernould et al., 2008). Therefore, further studies are suggested to elucidate the actual involvement of the system in clinical isolates in particular by using the genetic inactivation of the aheABC operon. Existence of other efflux systems is another topic of interest.

Table 1 Transcription level of *aheB* and antimicrobial susceptibilities of *A. hydrophila* (n=10)

Strain	**Transcription level of *aheB***	**MIC (μg/ml)[a], [b]**														
		AMO	**CAR**	**CEF**	**CHP**	**CIP**	**ENR**	**ERY**	**GEN**	**KAN**	**MIN**	**OTC**	**STR**	**SMZ**	**TET**	**TMP**
AH194	221.3	2	2	0.06	0.5	0.06	<0.06	2	2	4	0.06	0.06	8	**256**	0.5	1
AH200	213.6	**128**	4	0.06	0.5	**4**	0.25	**128**	2	**256**	0.25	0.25	**256**	**512**	1	**16**
AH198	112.8	**≥128**	**256**	0.13	2	0.25	0.25	8	2	8	0.5	**16**	**256**	**512**	**16**	**16**
AH179	71.5	**≥128**	**64**	0.13	0.5	0.25	0.125	0.06	0.5	4	0.5	0.25	**16**	**512**	1	**16**
AH195	51.8	**64**	4	0.5	0.5	2	**4**	8	2	**16**	0.5	0.25	**128**	**512**	1	**16**
AH106	38.3	**≥128**	**128**	1	0.5	0.125	0.125	**128**	0.5	2	1	0.25	8	8	0.25	**16**
AH172	21	**≥128**	**≥128**	0.5	0.5	**4**	**8**	0.06	0.5	8	2	**16**	**64**	**64**	**16**	**4**
AH193	4.9	2	2	0.06	0.5	0.06	<0.06	2	2	4	0.06	0.06	8	**256**	0.5	0.5
AH160	4.8	**128**	**≥128**	0.03	0.5	1	<0.06	4	1	4	0.03	0.25	**16**	8	0.5	**16**
AH173	1.7	**≥128**	**≥128**	0.5	0.5	**8**	**8**	8	0.5	8	2	**16**	**128**	**1024**	**32**	**≥128**

Abbreviations: AMO: amoxicillin, CAR: carbenicillin, CEF: cefoperazone, CHP: chloramphenicol, CIP: ciprofloxacin, ENR: enrofloxacin, ERY: erythromycin, GEN: gentamicin, KAN: kanamycin, MIN: minocycline, OTC: oxytetracycline, STR: streptomycin, SMZ: sulphamethoxazol, TET: tetracycline, TMP: trimethoprim

[a] Values in boldface indicate MICs identified as being resistant (Lukkana et al., 2011).

[b] The MIC_{90} for each antimicrobial agent was AMO(128), CAR(128), CEF(0.5), CHP(0.5), CIP(4), ENR(8), ERY(128), GEN(4), KAN(16), MIN(2), OTC(16), STR(128), SMZ(1,024), TET(16), TMP(128)

Table 2 Plasmid profiles and antimicrobial susceptibility of the *A. hydrophila* strains (n=17)

PP	Strain	Plasmid size (Kb)	Antimicrobial resistance pattern[a]
I	AH200	2, 2.5, 3, 4, 5, 8	AMO-CIP-ERY-KAN-SMZ-STR-TMP
II	AH173	3, 3.8, 4.5, 5, 6.5,7.5	AMO-CAR-CIP-ENR-OTC-SMZ-STR-TET-TMP
	AH175		AMO-CAR-CIP-ENR-OTC-SMZ-STR-TET-TMP
III	AH171	3, 3.8, 4.5, 5, 6.5	AMO-CAR-CIP-ENR-OTC-STR-TET
IV	AH196	2.9, 4.3, 5, 7	AMO-ENR-SMZ-STR-TMP
V	AH108	2.3, 3.3, 3.5, 5.4	AMO-CAR-ERY-SMZ-STR-TET-TMP
VI	AH172	3.3, 5.4	AMO-CAR-CIP-ENR-OTC-STR-TET
	AH146		AMO-CAR-CIP-ENR-SMZ-STR-TMP
	AH195		AMO-KAN-ENR-SMZ-STR-TMP
	AH197		AMO-CAR-CIP-ENR-OTC-SMZ-STR-TET-TMP
VII	AH147	3.3, 4.5	CAR-CIP-SMZ-STR-TMP
	AH199		AMO-STR-OTC-SMZ-TET-TMP
	AH198		AMO-CAR-OTC-SMZ-STR-TET-TMP
VIII	AH162	<1, 2, 2.7, 3, 3.5, 4.5, 6, 12, 16	AMO-CAR-OTC-STR-TET-TMP
IX	AH158	<1, 2.2, 3.5, 4,5, 8, 10	AMO-CAR-STR-TMP
X	AH179	1.5, 2.2, 3.5, 4.8	AMO-CAR-SMZ-STR-TET-TMP
XI	AH181	3, 5, 7.3	AMO-CAR-CIP-ENR-OTC-SMZ-STR-TET-TMP

[a] The data were obtained from a previous study (Lukkana et al., 2011).

Seventeen *A. hydrophila* isolates harbored plasmids with size ranging from 2-17 kb that could be categorized into 11 plasmid profiles (PP-I to PP-XI) according to the size and number of plasmids (Table 2). The most common plasmid pattern was in PP-VI (4/17). Multiple plasmids of varied sizes were detected in approximately one-third of the isolates. This is in agreement with a previous study (Son et al., 1997) with the exception that the plasmids identified in this study were smaller. While each plasmid-containing isolate had the variable number of plasmids with different molecular weights, it should be noted that very large or low-copy number plasmids may have been missed due to the DNA extraction procedure used. All the plasmid-bearing isolates were multidrug resistant, but the relationship of the resistance phenotypes and the existence of plasmid were not investigated in this study. Horizontal transfer of resistance gene-carrying plasmid has been a major concern for distribution of antimicrobial resistance among pathogenic bacterial strains. However, this was not pursued in the current study as well.

In summary, the results obtained in this study demonstrated the possible contribution of the AheABC pump in multidrug-resistance among *A. hydrophila* isolated from farmed Nile Tilapia. In aquaculture practice, the antimicrobials are generally mixed with feed and spread into the farming area resulting in residual deposits in the environment. These antimicrobial agents could provide selection pressure for expression of multidrug efflux system leading to cross resistance among antimicrobials of different classes. Therefore, appropriate and prudent use of antimicrobials is mandatory. Studies of mechanisms underlying antimicrobial resistance need to be encouraged for better understanding of the resistance dynamics and conducting the strategic plan to reduce and prevent development and spread of antimicrobial resistance among aquatic bacteria.

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