

# Phenotypic characteristics and antimicrobial susceptibilities of motile aeromonads isolated from freshwater fish in Korea and Japan

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## 한국과 일본의 담수어에서 분리되는 운동성 aeromonads의 표현형적 특성과 약제감수성

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We investigated the phenotypic characteristics by using API20E, APIZYM and determined minimum inhibitory concentrations (MICs) of 7 antibiotics in motile aeromonads isolated from freshwater fishes in Korea and Japan, and 4 American Type Culture Collection (ATCC) strains. All isolates (n=7) were identified as motile *Aeromonas* species according to API20E test. Lysine decarboxylase activity and acid production from 4 different carbohydrates including mannitol, rhamnose, amygdalin and arabinose were observed in various strains. In enzymatic activities by APIZYM, all isolates showed negative reactions in valine and cystine arylamidases,  $\alpha$ -chymotrypsin,  $\alpha$ -galactosidase,  $\beta$ -glucuronidase,  $\alpha$ -glucosidase,  $\alpha$ -mannosidase and  $\alpha$ -fucosidase. Although the intensities of each enzymatic activity were diverse in alkaline phosphatase, esterase-lipase, leucine arylamidase,  $\beta$ -galactosidase and N-acetyl- $\beta$ -glucosaminidase, all isolates showed positive reactions. All isolates were resistant to ampicillin sodium (MIC>100 $\mu$ g/ml), but sensitive to chloramphenicol (MIC $\leq$ 1.6 $\mu$ g/ml). However, recently isolated strains (AC9804, AC0202 and GMA0361) were commonly resistant to tetracycline (MIC=50 $\mu$ g/ml). Furthermore, AC9804 was resistant to oxolinic acid (MIC=12.5 $\mu$ g/ml). GMA0361 was resistant to kanamycin sulfate (MIC>100 $\mu$ g/ml) and streptomycin sulfate (MIC>100  $\mu$ g/ml).

*Key words:* Motile aeromonads, Freshwater fish, APIZYM, MIC

### Introduction

The genus *Aeromonas*, oxidase-positive, facultatively anaerobic and Gram-negative rods, includes opportunistic and primary pathogens in fish (Aoki, 1999). Motile aeromonads infection is a scourge of warm freshwater fish farming worldwide and is

considered as a major economic problem (Austin and Adams, 1996.). Pathogenic motile aeromonads have been recovered from a wide range of freshwater fishes, e.g. brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), ayu (*Plecoglossus altivelis*), carp (*Cyprinus carpio*), channel catfish (*Ictalurus puncta*), clariid catfish (*Clarias batra-*

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chus), goldfish (*Carassius auratus*), snakehead fish (*Ophicephalus striatus*) and tilapia (*Tilapia nilotica*) (Aoki, 1999). Pathologic conditions attributed to members of the motile aeromonads may include dermal ulceration, tail or fin rot, ocular ulcerations, erythrodermatitis, hemorrhagic septicemia, red sore, red rot, and scale protrusion (Aoki, 1999; Austin and Adams, 1996).

The phenotypic and genetic diversities of motile aeromonads have posed a great difficulty in diagnosis and classification as species (Janda and Abbott, 1998). The phenotypic and genetic associations amongst strains were demonstrated according to their geographic origin, source or associated diseases (Moyer et al., 1992; Paniagua et al., 1990). However, there is not enough phenotypic characterization of motile aeromonads in cultured freshwater fish of Korea and Japan.

Antimicrobials are valuable tools to treat motile aeromonad infections in fish, but the extensive use of antibiotics has led to an increase in the resistant strains in recent years (Aoki, 1999). An increase in resistance levels of the genus *Aeromonas*, particu-

larly to  $\beta$ -lactam antibiotics (Saavedra et al., 2004) and tetracyclines (Nawaz et al., 2006) has been observed. The studies of recent years show the existence of multiple antibiotic resistances in these bacteria, and therefore there is difficulty in selecting suitable antibiotic for treatment.

The aim of this study is to obtain fundamental data for diagnosis and treatment of motile aeromonad infections in freshwater fish. It was also intended to compare the extended spectrum of antibiotic resistance and existence of drug resistance in strains isolated since 1964. We have identified phenotypic characteristics of motile aeromonads by using API20E, APEZYM and determined the antibiotic susceptibility patterns of motile aeromonads isolated from freshwater fishes in Korea and Japan.

## Materials and Methods

### Bacteria

Eleven strains of motile aeromonads were used in this study (Table 1). Seven strains were isolated from freshwater fishes (carp, eel, ayu and goldfish)

**Table 1.** Source and phenotypic profiles as determined by the API 20E system of the motile aeromonads isolated from freshwater fish

Strain	Source	Geographical location	Isolated Year	API20E resulting no.	$\beta$ - hemolysis on 5% rabbit blood agar	Identification
A10	Eel	Japan	1964	7047137	+	<i>Aeromonas sp.</i>
67P-24	Ayu	Japan	1967	7047127	+	<i>Aeromonas sp.</i>
Y62	Eel	Japan	1978	7047135	+	<i>Aeromonas sp.</i>
AC9804	Carp	Korea	1998	7047127	+	<i>Aeromonas sp.</i>
AC0202	Carp	Korea	2002	7047127	+	<i>Aeromonas sp.</i>
GF1	Goldfish	Korea	1995	3047127	+	<i>Aeromonas sp.</i>
GMA0361	Ayu	Japan	2003	3047127	+	<i>Aeromonas sp.</i>
ATCC19570	Pike			7047027	+	<i>A. hydrophila</i>
ATCC14715	Sliver salmon			7047127	+	<i>A. hydrophila</i>
ATCC15467	Used oil-emulsions			7047125	+	<i>A. caviae</i>
ATCC7966	Tin of milk with a fishy odor			7047124	+	<i>A. hydrophila</i>

**Table 2.** Enzymatic profiles of the motile aeromonads from freshwater fish as determined by the API ZYM system

Strain	Enzymatic activities										
	AKP	E	EL	L	LA	T	ACP	NP	GA	GL	AG
A10	++	++	+++	++	+++++	++	+++++	-	+++	++	+++
67P-24	+	+	+++	++	+++	+	++++	-	++++	+++++	+++
Y62	+	+	+	-	++	++	++++	-	++	-	++
AC9804	+++	-	+++	-	++++	-	+++++	-	++++	-	+++
AC0202	++	++	+++	-	+++	+	+++++	-	++++	-	+++
GF1	++	++	++++	-	++++	-	++++	-	++++	+++	+++
GMA0361	+	+	+++	+	+++	-	-	+	++++	-	++++
ATCC19570	+	+	+++	+	++++	+	++	-	++	++	+++
ATCC14715	+++	++	++++	++	+++++	+	+++++	-	+++	+++++	+++
ATCC15467	+++	+	+++	+	+++	-	+++++	-	+++	-	++
ATCC7966	++	+++	++++	+	++++	+	++++	-	++		+++

AKP; Alkaline phosphatase, E; Esterase (C4), EL; Esterase-lipase (C8), L; Lipase (C14), LA; Leucine arylamidase, T; Trypsin, ACP; Acid phosphatase, NP; Naphthol-AS-BI-phosphohydrolase, GA;  $\beta$ -Galactosidase, GL;  $\beta$ -Glucosidase, AG; N-Acetyl- $\beta$ -glucosaminidase, -; Negative reaction, +; Positive reaction, + to +++++ indicates grade of positive reaction.

**Table 3.** MICs of the motile aeromonads from freshwater fish

Antibiotics (BP)	MIC ( $\mu\text{g/ml}$ )						
	Penicillins	Quinolones	Aminoglycosides		Tetracyclines	Macrolides	Phenicol
	AP ( $\geq 32$ )	OA ( $\geq 2$ )	KM ( $\geq 64$ )	SM ( $\geq 64$ )	TC ( $\geq 16$ )	EM ( $\geq 16$ )	CP ( $\geq 32$ )
A-10	> 100	< 0.1	25	25	0.2	6.25	1.6
67 P-24	> 100	< 0.1	25	50	0.8	6.25	0.8
Y-62	> 100	< 0.1	12.5	6.25	0.2	12.5	0.4
AC9804	> 100	12.5	25	50	50	6.25	0.8
AC0202	> 100	< 0.1	50	25	50	6.25	0.8
GMA0361	> 100	0.8	>100	>100	50	6.25	0.8
ATCC19570	> 100	< 0.1	12.5	12.5	0.2	6.25	1.6
ATCC14715	> 100	< 0.1	50	100	0.2	25	1.6
ATCC15467	> 100	< 0.1	25	50	0.4	6.25	1.6
ATCC7966	> 100	< 0.1	12.5	25	0.4	25	1.6

AP; Ampicillin sodium, OA; Oxolinic acid, KM; Kanamycin sulfate, SM; Streptomycin sulfate, TC; Tetracycline hydrochloride, EM; Erythromycin, CR; Chloramphenicol.

<sup>1</sup> Equivalent MIC breakpoint  $\mu\text{g/ml}$ ; Values, with the exception of those for oxolinic acid (Giraud et al., 2004), streptomycin (Yang et al., 2004) and erythromycin (Miller et al., 2005), are based on NCCLS standards (NCCLS 2006).

and four strains were obtained from American Type Culture Collection (ATCC).

### Phenotypic characterization

All strains were tested for 20 biochemical characteristics by using API20E system (bioMerieux, France). The enzymatic activities were detected by using APIZYM commercial kits (bioMerieux, France), according to the manufacturer's protocol. Hemolytic activity was determined by 5% rabbit RBCs containing TSA.

### Antimicrobial susceptibilities

The minimum inhibitory concentrations (MICs) of 7 antibiotics (refer to Table 3) were determined by the agar dilution method following NCCLS guidelines (NCCLS 2006), with Mueller-Hinton agar (Difco, USA) and a multipoint inoculator with an inoculum of  $10^4$  colony forming units (CFU) per spot. After 24h of incubation at 28°C organisms were classified as sensitive or resistant according to NCCLS 2006 guidelines. All antibiotics were purchased from Wako Chemical, Japan.

## Results

### Phenotypic characterization

All seven strains tested were identified as motile *Aeromonas* species according to API20E test (Table 1). Lysine decarboxylase and acid production in four different carbohydrates including mannitol, rhamnose, amygdalin and arabinose were detected in various strains (Table 1). In enzymatic activities by APIZYM test, all isolates showed negative reactions in valine and cystine arylamidases,  $\alpha$ -chymotrypsin,  $\alpha$ -galactosidase,  $\beta$ -glucuronidase,  $\alpha$ -glucosidase,  $\alpha$ -mannosidase and  $\alpha$ -fucosidase. Although the intensities of each enzymatic activity were diverse in alkaline phosphatase, esterase-

lipase, leucine arylamidase,  $\beta$ -galactosidase and N-acetyl- $\beta$ -glucosaminidase, all isolates showed positive reactions (Table 2). The enzymatic activities of esterase, lipase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and  $\beta$ -glucosidase were different in different isolates (Table 2).

### Antimicrobial susceptibilities

All isolates were resistant to ampicillin sodium, and sensitive to chloramphenicol. Three strains, AC9804, AC0202 and GMA0361, were commonly resistant to tetracycline. AC9804 was resistant to oxolinic acid, which had an MIC of 12.5  $\mu$ g/ml. GMA0361 was resistant to two different aminoglycosides; kanamycin sulfate, streptomycin sulfate. Two type strains, ATCC14745 and ATCC7966, were resistant to erythromycin.

## Discussion

Based on Overman and Overley's API20E result (1986), seven isolates except 4 ATCC strains in our study were identified as *A. hydrophila*. However, these isolates were classified in *A. hydrophila*, *A. caviae* or *A. sobria*, and could not be identified at species level according to the other reports (Toranzo et al., 1986; Kozinsaka et al., 2002). At present, 18 species have been reported in this genus *Aeromonas*, which includes the mesophilic species, *A. hydrophila*, *A. caviae* and *A. sobria* well-known as fish pathogens (Aoki, 1999). Identification of the genus has been controversial due to their phenotypic heterogeneity and the taxonomy of *Aeromonas* still confused (Abbott et al., 2003). Although the strains could not be identified at species level in this study, the general phenotypic characteristics by API20E of motile aeromonad isolates were reconfirmed.

APIZYM test is very useful both for determina-

tion of bacteria's enzymatic capabilities and contribution for bacterial identification (Groom et al., 1985; Waltman et al., 1982). In our study, all strains were able to exhibit five different enzymatic activities (alkaline phosphatase, esterase-lipase, leucine arylamidase,  $\beta$ -galactosidase and N-acetyl- $\beta$ -glucosaminidase) and did not produce eight enzymes (valine and cystine arylamidases,  $\alpha$ -chymotrypsin,  $\alpha$ -galactosidase,  $\beta$ -glucuronidase,  $\alpha$ -glucosidase,  $\alpha$ -mannosidase and  $\alpha$ -fucosidase). These characteristic features differ from those of other bacterial species in *Vibrionaceae* (Al-Dagal and Bazaraa, 2001) and they should be a useful indicator for motile aeromonads identification. However, some of enzymatic activities in motile aeromonads showed different reactions in the previous study (Waltman et al., 1982). The  $\alpha$ -glucosidase enzymatic reactions were negative in our study, but motile aeromonads isolated from environment, fish, human and bovine, showed  $\alpha$ -glucosidase positive reaction 21% (Waltman et al., 1982). This implies that all motile aeromonads do not exhibit enzyme activity of  $\alpha$ -glucosidase.

Interestingly, multiple-antibiotic resistance was found in recently isolated three motile aeromonad strains such as AC0202 (resistance to ampicillin and tetracycline), AC9804 (resistance to ampicillin, tetracycline and oxolinic acid) and GMA0361 (resistance to ampicillin, tetracycline, kanamycin and streptomycin). But all the strains isolated before 1978 were found to be sensitive to these antibiotics except for ampicillin. Extensive use of antibiotics in aquaculture might have lead to an increase in resistant strains of motile aeromonads and it may be needed to pay more attention to concern of antibiotics usage to prevent acquisition of multiple-antibiotic resistance.

The incidence of antibiotics resistance in motile aeromonads isolated from cultured fish has been

increased in recent years in other countries except Korea and Japan, and was found to be higher compared to those of naive (Huddleston et al., 2006; Nawaz et al., 2006). In the previous studies with *A. hydrophila* isolates, 62% from rainbow trout were resistant to ampicillin (Saavedra et al., 2004), 48% from tilapia to tetracycline, 57% to streptomycin, and 43% to erythromycin (Rhodes et al., 2000). On the other hand, no strain was found to be resistant to kanamycin, nalidixic acid, ofloxacin and tetracycline in aeromonads (n=282) isolated from lake and river in USA (Huddleston et al., 2006).

The results of the present study will be useful for identification of motile aeromonad isolated from freshwater fish, and also have important clinical implications in the selection of antibiotics for treatment of *Aeromonas* infections in fish.

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## 요 약

본 연구에서는 우리나라와 일본의 담수어에서 분리된 운동성 aeromonads 7균주와 American Type Culture Collection (ATCC)에서 분양받은 *Aeromonas hydrophila* 4균주의 표현형적 특성을 API20E와 APIZYM 방법으로 평가하고, 7종류의 항생제에 대한 최소 성장 억제 농도 (minimum inhibitory concentrations; MICs) 를 측정하였다. API20E 시험 결과 시험한 모든 균주는 (n=7) 운동성 aeromonads로 동정되었다. API20E 시험에서 lysine decarboxylase와 mannitol, rhamnose,

amygdalin, arabinose를 포함한 4종류의 carbohydrates의 산 생성은 균주에 따라 다른 반응을 나타내었다. APIZYM 시험을 이용한 효소 활성능을 평가한 결과, 모든 시험된 균주가 valine arylamidase, cystine arylamidase,  $\alpha$ -chymotrypsin,  $\alpha$ -galactosidase,  $\beta$ -glucuronidase,  $\alpha$ -glucosidase,  $\alpha$ -mannosidase,  $\alpha$ -fucosidase 반응에서 음성 반응을 나타내었으나, 비록 그 효소 활성의 강도에서 차이는 있었으나 alkaline phosphatase, esterase-lipase, leucine arylamidase,  $\beta$ -galactosidase, N-acetyl- $\beta$ -glucosaminidase 모든 균주에서 양성 반응이 나타났다. 최소 성장 억제농도를 시험한 결과, 시험된 모든 균주는 ampicillin sodium (MIC>100 $\mu$ g/ml) 에 내성을 가지며 chloramphenicol (MIC $\leq$ 1.6 $\mu$ g/ml) 감수성을 나타내었다. 그러나 1998년 이후에 분리된 3균주 (AC9804, AC0202, GMA0361)는 tetracycline (MIC=50 $\mu$ g/ml) 모두 저항성이 있었으며, AC9804는 oxolinic acid (MIC=12.5 $\mu$ g/ml), GMA0361는 kanamycin sulfate (MIC>100 $\mu$ g/ml)와 streptomycin sulfate (MIC>100 $\mu$ g/ml)에도 저항성을 나타내었다.

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