DOI: 10.19663/j.issn2095-9869.20221211002 http://www.yykxjz.cn/

王会林, 晋怀远, 高晔, 夏苏东, 李杰. 美洲鲥鱼嗜水气单胞菌病原的分离与鉴定. 渔业科学进展, 2024, 45(2): 257–266
WANG H L, JIN H Y, GAO Y, XIA S D, LI J. Isolation and identification of *Aeromonas hydrophila* from *Alosa sapidissima*. Progress in Fishery Sciences, 2024, 45(2): 257–266

美洲鲥鱼嗜水气单胞菌病原的分离与鉴定*

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摘要 2021 年，山东省临沂市一养殖场养殖的美洲鲥鱼(*Alosa sapidissima*)突发疾病并出现严重死亡，日死亡率高峰期达到 2.5%，累积死亡率约为 90%。患病鱼主要症状为体表出血、溃疡，解剖可见腹腔腹水、肝脏暗红，并伴有肠炎。组织病理学检测发现，病鱼肝脏出现弥散性坏死，嗜碱性粒细胞增多和细胞肿胀空泡变性；脾脏出现出血性贫血性坏死灶、核破裂和核固缩；肾脏淋巴细胞坏死脱落，肾小体毛细血管球萎缩，近端小管和远端小管内的细胞出现不同程度的细胞结构消失。发病鱼肉眼和显微镜观察未见明显寄生虫，利用 PCR 方法检测鲤疱疹病毒 2 型(Cyprinid herpesvirus 2)、鲈鱼蛙病毒(largemouth bass ranavirus)等淡水鱼类常见病毒均为阴性。细菌分离培养结果显示，从发病鱼的肝脏、肾脏和脾脏中分离得到形态一致的优势菌，命名为 AS-AH2101。经 16S rRNA 测序比对和生理生化鉴定，确定 AS-AH2101 为嗜水气单胞菌(*Aeromonas hydrophila*)。毒力基因检测结果显示，AS-AH2101 携带气溶素(*aerA*)、溶血素(*hlyA*)、丝氨酸蛋白酶(*ahpA*)、热稳定细胞肠毒素(*ast*)、热敏感细胞肠毒素(*altA*)和密度感应系统(*luxS*)基因。人工感染实验结果显示，AS-AH2101 能引起蓝曼龙(*Trichogaster trichopterus*)发病死亡，半数致死量为 3.23×10^4 CFU/尾。药物敏感性研究表明，AS-AH2101 对头孢拉啶、阿莫西林、氨苄西林和红霉素等 4 种抗生素具有耐药性，对四环素和多西环素等 11 种抗生素敏感。综上所述，本研究报道了我国养殖美洲鲥鱼感染嗜水气单胞菌的典型病例，为美洲鲥鱼在我国养殖过程中的疾病防控以及嗜水气单胞菌病的防治提供了参考和借鉴。

关键词 美洲鲥鱼；嗜水气单胞菌；细菌鉴定；毒力基因

中图分类号 S943 **文献标识码** A **文章编号** 2095-9869(2024)02-0257-10

美洲鲥鱼(*Alosa sapidissima*)又称美洲鲱鱼，隶属于鲱形总目(Clupeomorpha)、鲱形目(Clupeiformes)、鲱科(Clupeidae)、鲥属(*Alosa*)，是世界上个体最长、生长速度最快的鲥鱼之一(高小强等, 2015)。1998 年，美洲鲥鱼率先被上海市水产研究所引入我国进行人工繁殖(施永海等, 2019)。由于美洲鲥鱼肉质鲜美，外形与长江鲥鱼(*Tenualosa reevesii*)相似，受到人们的广泛喜爱，因此，美洲鲥鱼人工养殖迅速在多省市开展(Jia *et al*, 2007; 高小强等, 2017)。随着美洲鲥鱼养殖规模的逐步扩大，病害问题也逐渐严峻，然而，国内目前养殖美洲鲥鱼的病害报道较少，仅有水霉病(董亚伦等, 2015)、应激性出血病(肝胆病)、小瓜虫病(白点病)、粘孢子虫病、车轮虫病(潘庭双等, 2008)等，其中以寄生虫病报道最多，细菌性病害研究较少。

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收稿日期: 2022-12-11, 收修改稿日期: 2022-12-27

嗜水气单胞菌(*Aeromonas hydrophila*)属于气单胞菌科(Aeromonadaceae)、气单胞菌属(*Aeromonas*)。作为一种传统水生动物病原，从 20 世纪 80 年代起嗜水气单胞菌在我国已广泛流行，引起多种养殖淡水鱼类病害，包括草鱼(*Ctenopharyngodon idella*) (邓国成等, 2009)、鲢鱼(*Hypophthalmichthys molitris*) (高汉娇等, 1997)、鲤鱼(*Cyprinus carpio*) (张玉芬等, 2008)、鲫鱼(*Carassius auratus*) (刘亚楠等, 2012)、罗非鱼(*Oreocheomis niloticus*) (董忠典等, 2012)、团头鲂(*Megalobrama amblycephala*) (田甜等, 2010)、白斑狗鱼(*Esox lucius*) (秦莉等, 2014)、黄颡鱼(*Pelteobagrus fulvidraco*) (黄钧等, 2012; 梁正生等, 2012)和翘嘴鳜(*Siniperca chuatsi*) (朱鑫海等, 2022)等，造成严重的经济损失。嗜水气单胞菌在分类地位上与其他气单胞菌相似，检测和鉴定中容易混淆(DebRoy *et al*, 2006; Kawada *et al*, 2008)，在菌种鉴定和基因分型方面常用到多位点序列分型(multilocus sequence typing, MLST)或者平均核苷酸同源性(average nucleotide identity, ANI)分析进行鉴定。

2021 年，山东省临沂市一养殖场养殖的美洲鲥鱼出现严重发病情况，患病鱼主要症状为体表出血、溃疡，并迅速死亡，高峰期日死亡率为 2.5%，超过 1 000 尾，给养殖企业带来严重的经济损失。本研究从发病的美洲鲥鱼内脏中分离得到形态一致的优势菌，并通过 16S rRNA 基因测序、生理生化鉴定和人工感染等实验对病原进行确认和鉴定，以期为美洲鲥鱼的养殖和病害防控提供参考数据。

1 材料与方法

1.1 病鱼来源

2021 年 6 月，山东省临沂市郯城县一养殖场养殖的美洲鲥鱼大规模发病死亡，鱼体重为 200~400 g。初期养殖苗种 4 万尾，养殖模式为室内水泥池养殖(幼苗)和室外水泥池塘养殖(养成)。水源为地下水，换水 1~3 次/d，水温为 18~20 ℃。投喂加州鲈(*Micropterus salmoides*)商品化颗粒饲料，日投喂率为 2%左右。室内池塘先于外塘发病，累积死亡率为 90%左右，患病鱼典型症状为尾部疥疮或溃烂，前期应用恩诺沙星拌料口服，治疗效果不明显。

1.2 细菌分离

取发病鱼的鳃丝、体表粘液、尾鳍和内脏分别在显微镜下观察，检测寄生虫感染情况；取发病鱼的脾脏和肾脏组织冻存于液氮中，按照常规方法检测鲤疱疹病毒 2 型(Cyprinid herpesvirus 2) (罗丹等, 2014)、鲈鱼蛙病毒(largemouth bass ranavirus) (罗晓雯等, 2022)等淡水鱼常见病毒；从患病鱼的肝脏、脾脏和肾脏取样，划线于 TSA 和 LB 平板，在 28 ℃培养 24 h。将获得的优势菌落进行纯化培养，纯化培养后的细菌用甘油保存于–80 ℃冰箱。

1.3 组织病理学观察

选取典型发病症状的美洲鲥鱼，分别取肝脏、脾脏和肾脏切成为 1 cm^3 左右组织块，放置于 10~15 倍体积的 Davidson´s AFA 固定液室温固定 24 h 后，利用 70%的乙醇保存。对组织块进行脱水、包埋、切片和 HE 染色，显微镜观察发病美洲鲥鱼的组织病理变化。

1.4 人工感染实验

蓝曼龙(*Trichogaster trichopterus*)是一种经典的水生动物病原感染模型生物，已被广泛用于嗜水气单胞菌(Leung *et al*, 1997)、杀鱼爱德华氏菌(*Edwardsiella piscicida*)的研究中(Ling *et al*, 2001)。考虑到美洲鲥鱼应激较强，不适合实验室养殖、捕捞和感染等操作，本研究选取蓝曼龙作为实验动物用于毒力评价。蓝曼龙购自青岛市南山花鸟虫鱼市场，平均体长为 7~8 cm，暂养在 60 L 水箱中，养殖水温为 25 ℃。感染实验前，随机取 5 尾鱼麻醉解剖，取肝脏和肾脏，匀浆并涂布于 TSA 和 LB 培养基，确定实验鱼未携带病原。将实验鱼随机分为 4 组，每组 30 尾，养殖于 30 L 整理箱，水温为 25 ℃。用 PBS 缓冲液将培养好的 AS-AH2101 菌液浓度分别调整至 1×10^4、1×10^5、1×10^6 和 1×10^7 CFU/mL。采用背部肌肉注射法，每尾蓝曼龙注射感染 AS-AH2101 菌液 0.1 mL，对照组注射 0.1 mL 的 PBS 缓冲液，饲养于相同条件下，并每天记录各组鱼的发病症状和死亡情况。取感染死亡鱼的肝脏和肾脏，进行病原菌的再次分离和 16S rRNA 基因测序鉴定。感染 14 d 后，根据改进的寇氏法(杨茂成, 1990)计算菌株的半数致死量(lethal dose 50%, LD_{50})。

1.5 病原菌的鉴定和药物敏感性检测

利用 BIOLOG 微生物鉴定系统(美国)，采用 GenⅢ微孔板试剂条根据说明书对细菌进行生理生化特征鉴定。利用引物 27F (5′ AGAGTTTGATCCTGGTCAGAACGAACGCT 3′)和 1492R (5′ TACGGCTACCTTGTTACGACTTCACCCC 3′)对分离菌株的 16S rRNA 基因进行 PCR 扩增和产物测序(Lane, 1991)，将得到的序列在 GenBank 和 EzTaxon 数据库中进行同源性

比对。从 GenBank 中选取气单胞菌属的代表性菌株，使用 MEGA 7.0 软件，采用邻位相连法(Neighbor-Joining)构建系统发育进化树(Bootstrap=1 000)。

采用药敏纸片(杭州微生物试剂有限公司)测定分离株对部分抗生素的耐药性。取培养至 $OD_{600\ nm}$= 0.5 的菌悬液 0.1 mL 均匀涂布于 MH 平板，贴上药敏纸片后将平板在 28 ℃培养 24 h 并测量抑菌圈直径。参照美国临床和实验室标准委员会标准，实验重复 3 次，通过抑菌圈直径大小判定病原菌对药物的耐药性，分别为耐药(R)、中介(I)和敏感(S)。

1.6　病原菌毒力基因检测

针对嗜水气单胞菌的主要毒力因子基因：气溶素(*aerA*)、溶血素(*hlyA*)、丝氨酸蛋白酶(*ahpA*)、热稳定细胞肠毒素(*ast*)、热敏感细胞肠毒素(*altA*)和密度感应系统调控基因(*luxS*)，根据文献报道的引物(表 1)和反应条件(朱大玲等, 2006; 付乔芳等, 2011)对毒力基因进行扩增、测序和比对，确定分离菌株的毒力基因携带情况。

表 1　PCR 引物

Tab.1　PCR primers used in this study

名称 Name	引物序列 Primer sequences	片段大小 Fragment size/bp
hlyA	TGACAGGCAAGTAGAATAACGC	1 750
	TGTCCGCTTTCCACTCCC	
ahpA	GTTAGCGTTGGCAATCTCG	850
	CGCTGGAGTAGGAGGAACG	
altA	TGACCCAGTCCTGGCACGGC	390
	GGTGATCGATCACCACCAGC	
ast	ATCGTCAGCGACAGCTTCTT	480
	CTCATCCCTTGGCTTGTTGT	
aerA	CAAGAACAAGTTCAAGTGGCCA	309
	ACGAAGGTGTGGTTCCAGT	
luxS	GATCCTCTCCGAGGCGTGG	369
	AGGCTTTTCAGCTTCTCTTCC	

2　结果

2.1　病鱼症状

发病美洲鲥鱼的症状包括体表出血，部分鳞片脱落，尾部尤为严重。发病严重时，鱼尾部肿大凸起，内有脓液，呈疥疮病症状，并伴有肠炎和腹水，肝脏暗红坏死，呈败血症症状(图 1)；显微镜和肉眼均未观察到寄生虫感染；未检测到鲤疱疹病毒 2 型、鲈鱼蛙病毒等淡水鱼常见病毒；从 10 尾发病鱼的肝脏、脾脏、肾脏取样培养，12 h 后平板上都得到菌落形态一致的优势菌。16S rRNA 基因测序结果显示，所有分离株的序列一致，命名为 AS-AH2101。

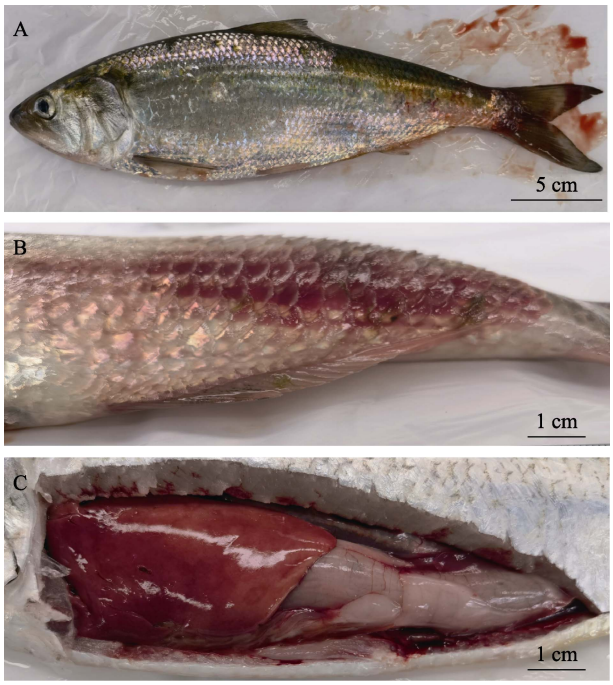

图 1　发病美洲鲥鱼临床症状

Fig.1　The clinical symptoms of diseased *A. sapidissima*

A：尾部渗血；B：尾部肿大凸起，内有脓液，呈疥疮病症状；C：肝脏暗红，呈败血症症状，有肠炎症状。

A: Tail blooding; B: Swollen tail with pus, showing symptoms as furunculosis; C: Dark red liver, showing symptoms of septicemia and enteritis.

2.2　患病鲥鱼组织病理变化

对发病美洲鲥鱼的肝脏、脾脏和肾脏进行切片观察。结果显示，发病鱼肝脏出现弥散性坏死灶、嗜碱性粒细胞增多、细胞肿胀、细胞质空泡变性等症状(图 2A、B)；脾脏中同样发现坏死灶，并呈现出血性贫血状态，细胞核破裂、萎缩和消失(图 2C、D)；肾脏间质疏松、囊腔变大，淋巴细胞坏死脱落。肾小管轻微变形，肾小体毛细血管球萎缩，近端小管和远端小管内的细胞出现不同程度的细胞结构消失、细胞空泡化和核固缩(图 2E、F)。

2.3　人工感染实验

由于美洲鲥鱼应激较强，难以进行实验室操作，本实验选择了蓝曼龙作为实验动物，通过肌肉注射感染检测 AS-AH2101 的致病性。人工感染蓝曼龙在感染后第 3 天出现死亡，感染鱼肌肉注射部位出现红

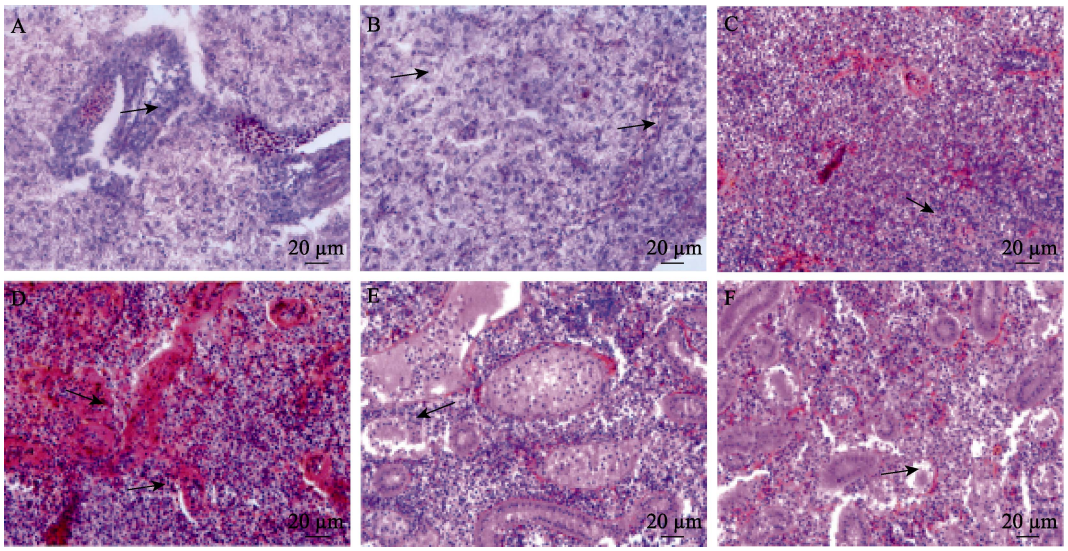

图 2 发病美洲鲥鱼组织病理症状

Fig.2 Histopathological symptoms of diseased *A. sapidissima*

A：嗜碱性粒细胞增多和弥漫性坏死；B：细胞肿胀，空泡变性；C：嗜碱性粒细胞增多；D：脾脏表现出血性贫血坏死、核破裂和萎缩；E：肾间质疏松，肾小管出现变形；F：肾小球萎缩

A: Basophilia, and diffuse necrosis; B: Cell swelling, vacuolar degeneration; C: Basophilia; D: Spleen showed hemorrhagic anemic necrosis, nuclear rupture and atrophy; E: Loose renal interstitial, and tubular variation; F: Atrophic glomerulus

肿、出血和鳞片脱落，鳍条基部充血或出血，解剖可见与自然发病美洲鲥鱼类似的腹腔内腹水、肝脏坏死等症状。人工感染死亡情况如表 2 所示，蓝曼龙注射组感染后死亡率分别为 93.3% (1×10^6 CFU/尾)、60% (1×10^5 CFU/尾)、40% (1×10^4 CFU/尾)和 13.3% (1×10^3 CFU/尾)，对照组实验鱼均未发生死亡。经计算，AS-AH2101 对蓝曼龙的半数致死量为 3.23×10^4 CFU/尾。从感染死亡的蓝曼龙内脏中可重新分离到菌落形态一致的优势菌，经 16S rRNA 扩增、测序和比对，结果与 AS-AH2101 一致，说明 AS-AH2101 是导致美洲鲥鱼发病并死亡的病原。

表 2 蓝曼龙人工感染实验

Tab.2 Experimental infection of *T. trichopterus*

感染剂量/(CFU/尾) Infection dose	死亡数/总数 Dead fish/Total fish	半数致死量 LD_{50}/(CFU/尾)
1×10^6	28/30	3.23×10^4
1×10^5	18/30	
1×10^4	12/30	
1×10^3	4/30	

2.4 病原菌鉴定

生理生化鉴定结果如表 3 所示，AS-AH2101 对龙胆二糖、水苏糖、棉子糖、α-D-乳糖、蜜二糖、3-甲酰葡糖、D-岩藻糖、D-山梨醇、D-阿拉伯醇、肌醇等呈阴性反应；对糊精、D-麦芽糖、D-海藻糖、D-纤维二糖、蔗糖、D-松二糖、β-甲酰-D-葡糖苷等呈阳性反应。经 BIOLOG 鉴定系统分析，AS-AH2101 菌株生理生化特征与嗜水气单胞菌最为接近，可信度为 0.999。

PCR 扩增菌株 AS-AH2101 的 16S rRNA 基因并进行测序(OP787967)，序列比对分析结果显示，菌株 AS-AH2101 16S rRNA 基因在 GenBank 中同源性最高的前 20 位菌株均为嗜水气单胞菌，EzTaxon 比对也与嗜水气单胞菌一致。从 NCBI 数据库中选择气单胞菌属的不同细菌，使用 MEGA 7.0 软件构建了 16S rRNA 基因的系统进化树。分析结果显示，AS-AH2101 与嗜水气单胞菌聚为一支(图 3)，与生理生化鉴定结果吻合。综合以上结果，将 AS-AH2101 病原菌鉴定为嗜水气单胞菌。

药敏实验结果显示，AS-AH2101 菌株对阿莫西林、氨苄西林、红霉素和水产养殖禁用的头孢拉啶等 4 种抗生素具有耐药性，对四环素、多西环素和氟苯尼考等 11 种抗生素敏感(表 4)。

2.5 毒力因子的检测

对嗜水气单胞菌的气溶素(*aerA*)、溶血素(*hlyA*)、丝氨酸蛋白酶(*ahpA*)、热稳定细胞肠毒素(*ast*)、热敏感细胞肠毒素(*altA*)和密度感应系统调控基因(*luxS*)等毒力因子基因进行 PCR 检测。电泳结果显示，6 个

表 3　菌株 AS-AH2101 生理生化特征

Tab.3　Biochemical analysis of AS-AH2101 strain

项目 Items	结果 Results	项目 Items	结果 Results
糊精 Dextrin	+	D-葡糖酸 D-Gluconic acid	+
D-麦芽糖 D-Maltose	+	D-葡糖醛酸 D-Glucuronic acid	+
D-海藻糖 D-Trehalose	+	葡糖醛酰胺 Glucuronamide	–
D-纤维二糖 D-Cellobiose	+	粘酸 Mucic acid	–
龙胆二糖 Gentiobiose	–	奎宁酸 Quinic acid	–
蔗糖 Sucrose	+	糖质酸 D-Saccharic acid	+
D-松二糖 D-Turanose	+	p-羟基-苯乙酸 p-Hydroxy phenylacetic acid	–
水苏糖 Stachyose	–	丙酮酸甲酯 Methyl pyruvate	+
棉子糖 D-Raffinose	–	D-乳酸甲酯 D-Lactic acid methyl ester	+
α-D-乳糖 α-D-Lactose	–	L-乳酸 L-Lactic acid	+
蜜二糖 D-Melibiose	–	柠檬酸 Citric acid	–
β-甲酰-D-葡糖苷 β-Methyl-D-glucoside	+	α-酮-戊二酸 α-Keto-glutaric acid	+
D-水杨苷 D-Salicin	+	D-苹果酸 D-Malic acid	–
N-乙酰-D-葡糖胺 N-Acetyl-D-glucosamine	+	L-苹果酸 L-Malic acid	+
N-乙酰-β-D-甘露糖胺 N-Acetyl-β-D-mannosamine	+	溴-丁二酸 Bromo-succinic acid	+
N-乙酰-D 半乳糖胺 N-Acetyl-D-galactosamine	+	吐温 40 Tween 40	+
N-乙酰神经氨酸 N-Acetyl neuraminic acid	+	γ-氨基-丁酸 γ-Amino-butyric acid	–
α-D-葡糖 α-D-Glucose	+	α-羟基-丁酸 α-Hydroxy-butyric acid	+
D-甘露糖 D-Mannose	+	β-羟基-D, L-丁酸 β-Hydroxy-D, L-butyric acid	+
D-果糖 D-Fructose	+	α-酮-丁酸 α-Keto-butyric acid	+
D-半乳糖 D-Galactose	+	乙酰乙酸 Acetoacetic acid	+
3-甲酰葡糖 3-Methyl glucose	–	丙酸 Propionic acid	W
D-岩藻糖 D-Fucose	–	乙酸 Acetic acid	+
L-岩藻糖 L-Fucose	W	甲酸 Formic acid	+
L-鼠李糖 L-Rhamnose	+	pH 6	+
肌苷 Inosine	+	pH 5	–
D-山梨醇 D-Sorbitol	–	1% NaCl	+
D-甘露醇 D-Mannitol	+	4% NaCl	–
D-阿拉伯醇 D-Arabitol	–	8% NaCl	–
肌醇 Myo-Inositol	–	1%乳酸钠 1% Sodium lactate	+
甘油 Glycerol	+	梭链孢酸 Fusidic acid	–
D-葡糖-6-磷酸 D-Glucose-6-PO_4	+	D-丝氨酸 D-Serine	+
D-果糖-6-磷酸 D-Fructose-6-PO_4	+	L-天冬氨酸 L-Aspartic acid	+
D-天冬氨酸 D-Aspartic acid	+	L-谷氨酸 L-Glutamic acid	+
D-丝氨酸 D-Serine	+	L-组胺 L-Histidine	+
明胶 Gelatin	+	L-焦谷氨酸 L-Pyroglutamic acid	–
甘氨酰基-L-脯氨酸 Glycyl-L-proline	+	L-丝氨酸 L-Serine	+
L-丙氨酸 L-Alanine	+	D-半乳糖醛酸 D-Galacturonic acid	–
L-精氨酸 L-Arginine	+	L-半乳糖醛酸内酯 L-Galactonic acid lactone	–

注：“+”为阳性；“–”表示阴性；“W”表示弱阳性。

Note: “+”: Positive; “–”: Negative; “W”: Weak positive.

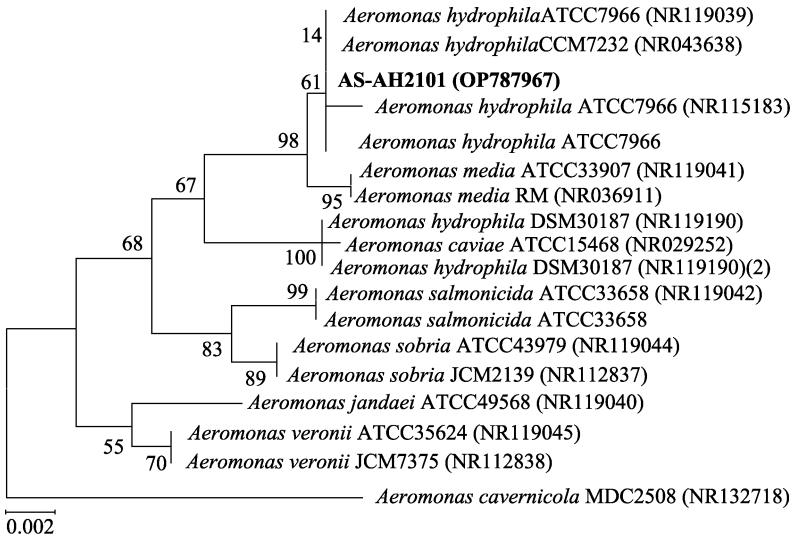

图 3 基于 16S rRNA 基因序列构建的系统发育进化树

Fig.3 Phylogenetic tree constructed by neighbor joining method based on sequences of 16S rRNA

表 4 AS-AH2101 的药敏检测结果

Tab.4 Test results of AS-AH2101 drug sensitivity

抗生素 Antibiotics	敏感性 Sensitivity	抗生素 Antibiotics	敏感性 Sensitivity
四环素 Tetracycline	S	氟苯尼考 Florfenicol	S
多西环素 Doxycycline	S	磺胺异唑 Sulfamethoxazole	S
头孢拉啶 Cephradine	R	链霉素 Streptomycin	S
卡那霉素 Kanamycin	S	氨苄西林 Ampicillin	R
甲氧苄啶 Trimethoprim	S	新霉素 Neomycin	S
恩诺沙星 Enrofloxacin	S	红霉素 Erythromycin	R
阿莫西林 Amoxicillin	R	多粘菌素 B Polymyxin B	S
复方新诺明 Cotrimoxazole	S		

注：S 表示敏感，R 表示不敏感。

Note: “S”: Sensitive; “R”: Resistant.

毒力因子基因在 AS-AH2101 菌株中均扩增出目标条带，测序结果与目的基因的序列一致，证明 AS-AH2101 具有这 6 种毒力基因。

3 讨论

嗜水气单胞菌是鱼类、两栖类和爬行类等淡水养殖经济动物的重要病原，在世界各地均有该病原感染淡水养殖鱼类造成出血性败血症的报道(Austin *et al*, 1996)。嗜水气单胞菌是一种典型的条件致病菌，经常存在于鱼类的体表和肠道中(Allen *et al*, 1983; Boulanger *et al*, 1977)，当鱼体受到环境压力等胁迫时表现出致病性(Leung *et al*, 1995)。在本次调查中，我们了解到在 4 月初水温回升时期，该批次美洲鲥鱼即出现初步症状，并发生少量死亡情况，但养殖企业未引起充分重视。4 月中下旬，日死亡数量迅速增加，发病变得难以控制。推测此次发病类似于“草鱼综合征”，在水温回升后，鱼群摄食量上升，消化道压力增大，排泄增多，引起水质恶化和鱼体应激，进一步导致原本在体表或肠道附生的嗜水气单胞菌表现出致病性，造成养殖群体的大规模发病和大量死亡。在人工感染实验中，也通过蓝曼龙进一步验证了病原菌的致病性。本研究发现，蓝曼龙感染 3 d 迅速出现症状，并发生死亡。感染鱼症状与自然发病的美洲鲥鱼类似。AS-AH2101 对蓝曼龙的半数致死量远低于之前报道的其他嗜水气单胞菌(Leung *et al*, 1997)，因此，根据本研究结果，推断嗜水气单胞菌感染是造成此次美洲鲥鱼大规模死亡的原因。

目前，抗生素是很多国家治疗水产动物细菌性感染的主要药物，且其用量随着养殖周期的延长和规模的扩大而迅速增长。1988 年，挪威三文鱼养殖共使

用抗生素 670 kg，而到 1992 年，抗生素的使用量上升至 27.5 t (Grave *et al*, 1999)。抗生素的大量使用带来了病原菌的耐药性问题。印度一项耐药性调查结果显示，在鱼虾中分离到的 319 株嗜水气单胞菌对甲氧西林和利福平均具有耐药性，99%以上的菌株对新生霉素和短杆菌肽具有耐药性(Vivekanandhan *et al*, 2002)。抗生素耐药性已经成为水产养殖中控制嗜水气单胞菌感染的持续性障碍。在本研究中，我们检测了 AS-AH2101 的耐药性，结果显示，该菌株对头孢拉啶、阿莫西林、氨苄西林和红霉素等 4 种抗生素具有耐药性。考虑到该养殖企业未使用过这 4 种抗生素，且这 4 种抗生素在水产中或者属于禁用药物，或者用量很低，推测 AS-AH2101 具有这些抗性可能已有较长的时间，或者从其他细菌中通过抗性基因水平转移获得了耐药性(Kim *et al*, 1993)。同时，根据抗生素耐药性结果，推荐养殖场改用多西环素进行拌料投喂，施用后取得了显著效果。

嗜水气单胞菌的致病机制复杂，具有多种毒力因子，本研究对美洲鲥鱼病原菌 AS-AH2101 进行了 PCR 检测，结果显示，AS-AH2101 携带 6 种毒力基因。毒力基因 *ahpA* 和 *aerA* 分别编码嗜水气单胞菌丝氨酸蛋白酶基因和气溶素基因，是嗜水气单胞菌的主要毒力因子，与致病性存在相关性，强致病性菌株均具有这 2 个毒力基因(朱大玲等, 2006; 付乔芳等, 2011)。*ast* 编码细胞性肠毒素，*hlyA* 编码溶血素基因，*altA* 编码肠毒素基因，这 3 种基因也是气单胞菌中常见的毒力因子，具有细胞毒性、溶血毒性和肠毒素毒性，能促进宿主细胞凋亡并发挥致病性功能(吴同垒等, 2011; Buckley *et al*, 1999; 单晓枫等, 2011; Rosenshie *et al*, 1993)。*luxS* 编码细菌的密度感应，用以调控毒力基因的表达和分泌，并促进细菌生物膜的形成，增强细菌对宿主免疫杀伤和抗生素药物的抵抗力(Learman *et al*, 2009; Azakami *et al*, 2006)。本研究分离得到的病原菌 AS-AH2101 同时具有 6 种毒力基因，从基因层面上进一步反映了菌株强致病性，与人工感染实验结果吻合，也解释了此次美洲鲥鱼发病过程中的高死亡率原因。

综上所述，本研究从严重发病的养殖美洲鲥鱼中分离得到了一株嗜水气单胞菌 AS-AH2101，通过感染实验和毒力基因分析，证明 AS-AH2101 具有强致病性，是此次美洲鲥鱼大规模发病死亡的病原，并根据药敏结果使用抗生素取得较好的治疗效果。但从长远来看，抗生素防治嗜水气单胞菌感染会面临耐药性风险，因此，疫苗、噬菌体、益生菌和中草药等抗生素替代品的研发将是未来研究的重点。

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(编辑　马璀艳)

Isolation and Identification of *Aeromonas hydrophila* from *Alosa sapidissima*

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Abstract　*Aeromonas hydrophila* (Family: Aeromonadaceae) is a traditional aquatic animal pathogen. It has been widely prevalent in our country since the 1980s and 1990s, causing diseases in a series of freshwater fish, leading to serious economic losses. It has been confirmed to be one of the main pathogens in freshwater aquaculture worldwide. The American shad (*Alosa sapidissima*) is one of the biggest shad in the world and grows much faster than other shad. Because of its delicious taste as reeves shad (*Tenualosa reevesii*), American shad were introduced into China from the USA by the Shanghai Fisheries Research Institute for local farming in 1998 and is widely welcomed in Shanghai, Jiangsu, and Zhejiang Provinces. In recent years, with the rapid development of the American shad culturing, fish diseases have become a major threat to fish farming. However, because most of the American shad are cultured in extensive ponds, few diseases have been reported in China. In this study, we reported a case of *A. hydrophila* infection in American shad. In 2021, the disease outbreak was observed in American shad cultured in Linyi City, Shandong Province, with severe mortality. The daily mortality could be up to 2.5%. The fish were cultured in indoor ponds for breeding and outdoor ponds when fish reached 300 g. The American shad were cultured with underground water and water was changed 1–3 times daily. The water temperature was 18–20 ℃. The fish were fed largemouth bass (*Micropterus salmoides*) commercial feed, and the daily feeding rate was approximately 2%. The disease broke out in the indoor ponds first, and then in outdoor ponds. The cumulative mortality was approximately 90% in 2 months. Enrofloxacin was administered

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orally, but no effects were observed and the disease continued to progress. The typical disease symptoms in the American shad were furunculosis or ulceration, with shedding scales and surface bleeding, especially on the tail, sometimes swollen and pus-filled. In autopsies of the diseased fish, ascites were found in the fish abdomen, and dark red necrosis on the liver, with sepsis and enteritis. The liver, spleen, and kidney of American shad with typical symptoms were collected and cut into about 1 cm^3 tissue blocks, immersed in Davidson's Fixative (Davidson's AFA) for 24 h, and preserved in 70% ethanol. The tissues were mounted onto glass slides with hematoxylin and eosin staining for histological analysis. Histopathological results showed swollen liver cells, vacuolar degeneration, basophilia, and diffuse necrosis; the spleen showed hemorrhagic anemic necrosis, nuclear rupture, and atrophy. Glomerular atrophy of the renal corpuscle, cells in the proximal and distal tubules cytoarchitectural loss, and necrosis and shedding of kidney lymphocytes was also observed. No parasite was found on the fish surface, fins, in the gills, or internal organs with the naked eye and a light microscope. Freshwater viruses, such as Cyprinus herpesvirus type 2, largemouth bass ranavirus, megalocytivirus, and rhabdovirus, were checked by polymerase chain reaction (PCR), and no viruses were detected. The liver, spleen, and kidney of the diseased fish were sampled and cultured in tryptic soy agar medium (TSA) and Luria-Bertani agar medium (LB) plate medium at 28 ℃ for 24 h. Several pure and dominant colonies with the same morphology were observed on all the plates. These colonies were purified and cultured. The 16S rRNA gene sequencing results of all the purified colonies showed that the dominant strains were of the same species. The typical isolate was purified and named AS-AH2101. The results of biochemical identification with Biolog GenⅢ showed that the isolate AS-AH2101 was negative to gentiobiose, stachyose, D-raffinose, α-D-lactose, D-melibiose, 3-methyl glucose, D-fucose, D-sorbitol, D-arabitol, and Myo-inositol, while positive to dextrin, D-maltose, D-trehalose, D-cellobiose, sucrose, D-turanose, and β-methyl-D-glucoside. According to the Biolog GenⅢ identification system database, the biochemical characterization of AS-AH2101 was similar to that of *A. hydrophila*, with a confidence of 0.999. The 16S rRNA gene sequence of AS-AH2101 was submitted to GenBank databases under the accession number OP787967 and blasted in GenBank and EzTaxon. Comparison of the 16S rRNA gene sequences showed 99%–100% identity with those of *A. hydrophila*. The phylogenetic tree was constructed using Mega 7 with the *Aeromonas* typical strains 16S rRNA gene sequences obtained from GenBank, and the phylogenetic analysis also clustered AS-AH2101 with *A. hydrophila*. Thus, the molecular analysis results identified the SC18032201 strains as *A. hydrophila*, and the phenotype also supported this result. Because of the strong stress response of American shad, it is difficult to perform the experimental culturing and infection in the laboratory. As a classic pathogenic infection model organism in aquatic animals, blue gourami (*Trichogaster trichopterus*) is a traditional model for fish pathogen study and has been widely used in the research of *A. hydrophilia* and *E. piscicida*. Therefore, in this study, blue gouramis were used as the model organism for virulence evaluation of AS-AH2101 in the experimental infection. The results of the challenge experiment showed that the death of the blue gourami infected via intramuscular injection was observed on the third day post infection. The infected fish showed redness, bleeding, and scale shedding at the injection site, congestion or bleeding at the base of the fin, abdominal ascites, and liver necrosis, which were similar to the naturally infected American shad. The isolate strain AS-AH2101 showed high virulence to blue gouramis, with the median lethal dose (LD_{50}) of 3.23×10^4 CFU/fish. The virulence genes of *A. hydrophilia* were also detected by PCR, and results indicated that AS-AH2101 possessed six virulence genes, including aerolysin (*aerA*), hemolysin (*hlyA*), extracellular protease (*ahpA*), anti-metalloproteinases (*ast*), enterotoxin (*altA*), and quorum sensing gene (*luxS*). Antibiotic sensitivity studies showed that AS-AH2101 was resistant to cefradine, amoxicillin, ampicillin, and erythromycin. These results provided important information for disease control and *A. hydrophila* prevention and control of American shad culturing in China.

Key words *Alosa sapidissima*; *Aeromonas hydrophila*; Bacteria identification; Virulence genes