Aquaculture 432 (2014) 1–6

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Short communication

Multilocus sequence typing revealed a clonal lineage of *Aeromonas hydrophila* caused motile *Aeromonas* septicemia outbreaks in pond-cultured cyprinid fish in an epidemic area in central China

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ARTICLE INFO

Article history:
Received 9 March 2014
Received in revised form 7 April 2014
Accepted 8 April 2014
Available online 24 April 2014

Keywords:
Aeromonas hydrophila
Motile *Aeromonas* septicemia
MLST
Clonal lineage
Cyprinid fish

ABSTRACT

Motile *Aeromonas* septicemia (MAS) has been increasingly prevalent in cultured fish throughout China since 1989. Over the past two decades, our laboratory conducted two pathogen detection studies in septicemia outbreak fishponds in an epidemic area in central China. One was conducted from May 1990 to October 1991, when MAS was beginning to spread in China, and the other was recently conducted from August 2006 to July 2009. We found that *Aeromonas hydrophila* was responsible for these MAS outbreaks. *A. hydrophila* isolates were previously known to be phenotypically, serologically, and genetically diverse and no dominant clones were found. In this study, multilocus sequence typing analysis was used to observe a clonal lineage of *A. hydrophila*, which was responsible for MAS outbreaks in pond-cultured cyprinid fish in an epidemic area in central China for two decades.

1. Introduction

Aeromonas hydrophila and other motile aeromonads are among the most common bacteria in freshwater habitats, and these bacteria frequently cause diseases among cultured and feral fishes worldwide (Akinbowale et al., 2007; Cipriano, 2001; da Silva et al., 2012; Gopalakannan and Arul, 2006; Nielsen et al., 2001). During the past 30 years, the aquaculture industry in China has evolved dramatically, and the capital-intensive production has become the main culture mode (Hishamunda and Subasinghe, 2003; Nielsen et al., 2001; Zhang and Jostein, 2004). The total area devoted to aquaculture increased from 2.86 million hectares in 1979 to 5.63 million hectares in 2008 in China. Meanwhile, total production rose from 1.23 to 34.13 million tons, garnering a 69.7% share of the total aquatic production, making China the only country in the world where aquaculture production exceeds the wild catch (Li et al., 2011; National Bureau of Statistics of China, 2009). With the rapid development of the aquaculture industry, motile aeromonad infections caused by *A. hydrophila* have become an increasingly prominent problem (Xiao et al., 2011; Nielsen et al., 2001; Xu et al., 1993). Based on different clinical symptoms, *A. hydrophila* can cause several diseases in fish, which include motile *Aeromonas* septicemia (MAS) or hemorrhagic septicemia (da Silva et al., 2012; Nielsen et al., 2001; Xu et al., 1993), hemolytic ascitesosis (Sun et al., 1991), intussusception (Liu et al., 2008), tail or fin rot (Liu et al., 1993; Rahman et al., 2001), stigmatosis (Xu et al., 1980), and epizootic ulcerative syndrome (Austin and Adams, 1996; Roberts, 1997). MAS is the most serious disease and has frequently caused huge economic losses in the cyprinid fish industry throughout China since 1989 (Lu, 1992; Nielsen et al., 2001; Qian et al., 1997).

Many studies have investigated the genetic diversity of human diarrhea and environmental isolates of *A. hydrophila* (Aguilera-Arreola et al., 2007; Sechi et al., 2002; Szczuka and Kaznowski, 2004). All these studies revealed a high intra-specific genetic diversity within *A. hydrophila*, and no dominant clones were found. As an etiological agent in fish and shellfish diseases, *A. hydrophila* is widely identified and characterized at the species level (da Silva et al., 2012; Nielsen et al., 2001; Xu et al., 1993). However, studies concerning the genetic diversity of this widespread fish pathogen are scarce. Thus, systematic investigations and precise delineation on the population structure of *A. hydrophila* from pond outbreaks in epidemic areas are lacking, making prevention difficult. Clarifying the population structure of MAS outbreak isolates of *A. hydrophila* and determining an efficient measure for the prevention of this disease are of utmost importance.

Over the past two decades, our laboratory conducted two pathogen detection studies in septicemia outbreak fishponds in an epidemic area in central China. One was conducted from May 1990 to October 1991 (Xu et al., 1993), when MAS became widespread in China, whereas the other was conducted from August 2006 to July 2009 (Zhang et al., 2013a). We found that *A. hydrophila* was responsible for these MAS

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http://dx.doi.org/10.1016/j.aquaculture.2014.04.017
0044-8486/

outbreaks. *A. hydrophila* isolates were previously studied to be phenotypically, serologically, and genetically diverse (Abbott et al., 2003; Aguilera-Arreola et al., 2005; Szczuka and Kaznowski, 2004; Thomas et al., 1990). Thus, we used a recently developed precise method of multilocus sequence typing (MLST) to evaluate the population structure of MAS outbreak isolates of *A. hydrophila*. The results of this study will help clarify the epidemiological information of outbreak isolates of *A. hydrophila* in pond-cultured fish, and further determine an efficient measure to prevent this pathogen.

2. Materials and methods

2.1. Bacterial strains

Detailed information about all *A. hydrophila* strains evaluated in this study is listed in Table 1. All strains were isolated from diseased cyprinid fish. Among these strains, one strain (ST78-3-3) was isolated from fish with stigmatosis in 1978 (Xu et al., 1980). All the other 22 *A. hydrophila* strains were isolated from pond-cultured cyprinid fish with MAS outbreaks. Among the 22 strains, two strains (NSC90-4-1 and XS91-4-1) were isolated in 1990 and 1991, respectively, when MAS became widespread in China (Xu et al., 1993). The other 20 *A. hydrophila* strains were isolated from August 2006 to July 2009, when MAS was a high risk (Zhang et al., 2013a). The isolation and identification of these strains were described in the previous studies (Xu et al., 1993; Zhang et al., 2013a,b). Briefly, the tissues of diseased fishes, which include blood, spleen, liver, kidney or ascites fluid, were aseptically inoculated on tryptic soy agar (TSA; Becton, Dickinson and Company, USA) plates. All plates were incubated at 28 °C for 24 h. Then, two to three single colonies were randomly selected and re-purified from each TSA plate. *Aeromonas* genus-specific polymerase chain reaction (PCR) detection was carried out as previously described (Lee et al., 2002). All isolates were positively amplified and were further confirmed as members of the genus *Aeromonas* in terms of motility, positive reactions for both oxidase and catalase, fermentation of glucose and resistance to O/129 on a 150 μg disk (Abbott et al., 2003). To further identify the species of these isolates, the *gyrB* gene was amplified and sequenced with previously described primers (Yáñez et al., 2003). The results showed that all the isolates were *A. hydrophila*, indicating a single species outbreak in these fishponds (Xu et al., 1993; Zhang et al., 2013a).

The sampled fishponds in this study are located in five cities in central China (Table 1). The mortality rates in ponds C, D, E, F, G, and H ranged from 20% to 40% (data from the owner). Ponds D and E were 200 m apart; Ponds G and H were adjacent to each other but not connected; and the other ponds were geographically distant from each other. Ponds E and F reared various cyprinid fish species, including crucian carp (*Carassius carassius*), grass carp (*Ctenopharyngodon idellus*), silver loweye carp (*Hypophthalmichthys molitrix*), wuchang bream (*Megalobrama amblycephala*), and bighead carp (*Aristichthys nobilis*). Ponds C and D reared wuchang bream and silver loweye carp, respectively. Ponds G and H reared crucian carp.

2.2. MLST and phylogenetic analysis

MLST was proposed as a universal method to characterize bacteria based on sequence polymorphisms within internal fragments of housekeeping genes. Each gene fragment is translated into a distinct allele, and each isolate is classified as a sequence type (ST) by the combination of alleles of the housekeeping loci (Urwin and Maiden, 2003). MLST can achieve precise strain genotyping, and is a powerful tool for outbreak traceability (Martino et al., 2011). Six housekeeping genes of all strains, namely, *gyrB*, *groL*, *gltA*, *metG*, *ppsA*, and *recA*, were sequenced with the previously described primers and procedures (Martino et al., 2011). The sequences of distinct alleles were deposited in the *Aeromonas* MLST database (http://pubmlst.org/aeromonas) (Jolley and Maiden, 2010) and GenBank under accession nos. KC767928–KC767939. A phylogenetic tree was constructed based on the concatenated sequences of the six housekeeping genes by the neighbor-joining method using MEGA program (version 4.1) (Tamura et al., 2007). Genetic distances were obtained by Kimura's two-parameter model (Fig. 1). Given that the sequences of subspecies of *A. hydrophila* in the *Aeromonas* MLST database are lacking, a phylogenetic tree was constructed based on the *gyrB* gene sequences of these strains and reference *Aeromonas* strains to further analyze the subspecies of these strains (Fig. 2).

3. Results and discussion

Through the MLST and phylogenetic analysis, we confirmed that all the 22 MAS outbreak isolates were *A. hydrophila* (Table 2, Fig. 1). All these strains demonstrated identical ST and alleles. Thus, the 3084-bp concatenated sequences of the six alleles in the MLST loci were identical, whereas the control strain isolated from fish with stigmatosis showed different STs and alleles with MAS outbreak isolates (Table 2).

Table 1
Information of tested *A. hydrophila* strains in this study.

Strain	Host fish species	Source	Disease	Pond	Location	Isolation time
NSC90-4-1	Silver loweye carp	Spleen or liver or kidney	MAS	A	Changsha	1990
XS91-4-1	Silver loweye carp	Spleen or liver or kidney	MAS	B	Xiaogan	1991
IB101	Wuchang bream	Blood	MAS	C	Hanchuan	08/2006
IB336	Wuchang bream	Blood	MAS	C	Hanchuan	08/2006
4LNG101	Silver loweye carp	Liver	MAS	D	Jingmen	06/2008
4LNG201	Silver loweye carp	Liver	MAS	D	Jingmen	06/2008
4LNS301	Silver loweye carp	Kidney	MAS	D	Jingmen	06/2008
JG101	Crucian carp	Liver	MAS	E	Jingmen	06/2008
CG101	Grass carp	Liver	MAS	E	Jingmen	06/2008
LNB101	Silver loweye carp	Blood	MAS	E	Jingmen	06/2008
DWCG101	Wuchang bream	Liver	MAS	F	Wuhan	06/2009
DLNG101	Silver loweye carp	Liver	MAS	F	Wuhan	06/2009
DLNG201	Silver loweye carp	Liver	MAS	F	Wuhan	06/2009
DBHS101	Bighead carp	Kidney	MAS	F	Wuhan	06/2009
JBN1001	Crucian carp	Blood	MAS	G	Wuhan	06/2009
JBN1101	Crucian carp	Blood	MAS	G	Wuhan	06/2009
JBN1201	Crucian carp	Blood	MAS	G	Wuhan	06/2009
JBN1301	Crucian carp	Blood	MAS	G	Wuhan	06/2009
2JBN101	Crucian carp	Blood	MAS	H	Wuhan	07/2009
2JBN301	Crucian carp	Blood	MAS	H	Wuhan	07/2009
2JFN201	Crucian carp	Ascites fluid	MAS	H	Wuhan	07/2009
2WCL101	Wuchang bream	Liver	MAS	H	Wuhan	07/2009
ST78-3-3	Silver loweye carp	Lesions or blood	Stigmatosis	I	–	1978