Marine Pollution Bulletin 77 (2013) 325–332

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Impact of milk fish farming in the tropics on potentially pathogenic vibrios

W.T. Reichardt *, J.M. Reyes, M.J. Pueblos, A.O. Lluisma

Marine Science Institute, University of the Philippines, Diliman, 1101 Quezon City, Philippines

ARTICLE INFO

Keywords:
Sucrose-negative vibrios
Enterovibrionaceae
Health risk
Milkfish mariculture
Philippines

ABSTRACT

Ratios of sucrose-negative to sucrose-positive vibrios on TCBS agar (suc−/suc+) indicate the abundance of potential human pathogenic non-cholera vibrios in coastal mariculture environments of the Lingayen Gulf (Philippines. In guts of adult maricultured milkfish (*Chanos chanos*) of suc− vibrios reached extreme peak values ranging between 2 and 545 million per g wet weight. Suc− vibrios outnumbered suc+ vibrios in anoxic sediments, too, and were rarely predominant in coastal waters or in oxidized sediments. Suc−/suc+ ratios in sediments increased toward the mariculture areas with distance from the open sea at decreasing redox potentials. There is circumstantial evidence that suc− vibrios can be dispersed from mariculture areas to adjacent environments including coral reefs. An immediate human health risk by pathogenic *Vibrio* species is discounted, since milkfish guts contained mainly members of the *Enterovibrio* group. A representative isolate of these contained proteolytic and other virulence factors, but no genes encoding toxins characteristic of clinical *Vibrio* species.

1. Introduction

Marine coastal pollution caused by intensified milk fish farming in the Lingayen Gulf (South China Sea, Philippines) shows chemical and biochemical effects in water and sediment (Holmer et al. 2003; Reichardt et al. 2011; Nacorda et al. 2012). Repeated fish kills, diseases of maricultured invertebrates, a cholera epidemic in 2005, and enhanced mortality of reef-building corals raise concerns about sustainability of intensive milk fish mariculture in relation to environmental health (Villanueva et al. 2006; Reichardt et al. 2007). In terms of bacterial health risks, vibrios with at least a dozen pathogenic *Vibrio* species play a dominant role in coastal marine environments used for mariculture (Tantillo et al. 2004; Das et al. 2009, Senderovitch et al., 2010). As copiotrophic heterotrophs, marine vibrios are frequently associated with maricultured organisms including fish intestines (De Paola et al., 1994, Beneduce et al. 2010; Ganesh et al. 2010; Matsunaga et al. 2011). Diversified use of coastal waters at Cape Bolinao (Lingayen Gulf, Philippines) for mariculture, restoration of marine wildlife, and tourism offers a promising basis for assessing the impact of tropical mariculture on potential health risks. Whereas numerous marine vibrios have been classified as pathogens affecting marine animals as well as humans (Farmer and Hickman-Brenner 2006; Austin 2010), only a small fraction of these pathogenic species may be virulent in their marine environment (Oberbeckmann et al. 2011; Bier et al., 2013). Yet, this assumption can be biased, as currently available information stems from non-tropical marine environments. Virulent pathogenic *Vibrio* species may be expected more frequently in tropical marine environments, since the expression of virulence genes seems to increase at elevated environmental temperatures (Mahony et al., 2010). Hence tropical coastal waters with year-round temperatures near 30 °C would ensure most favorable growth conditions for these pathogens.

Initial steps to examine this bacteriological health risk relied on selective viable counts of (presumptive) vibrios on TCBS agar (Bolinches et al., 1988). This highly selective medium allows distinction between sucrose positive and sucrose negative phenotypic subgroups of vibrios that comprize valid *Vibrio* species as well as reclassified former *Vibrio* species (Farmer and Hickman-Brenner 2006). Since sucrose negative phenotypes harbor the bulk of potential human pathogens – ratios of sucrose negative to sucrose positive vibrios (suc^-/suc^+) are considered as useful health risk indicator in sea food microbiology (Lopez-Joven et al. 2011).

Human pathogenic vibrios such as *Vibrio vulnificus* and *Vibrio parahaemolyticus* can occur in the intestine of certain finfish (de Paola et al. 1994; Das et al. 2009). But our information about selective forces governing both the enrichment in fish guts and dispersal of milkfish-borne vibrios is scarce (Matsunaga et al. 2011). Routine bacteriological surveys of fish farming sites leave only limited scope for diagnostic analyses at the species level. Therefore this investigation examines suc^-/suc^+ ratios as possible candidate for a practicable indicator of the likely presence of non-cholera human pathogenic vibrios that would be suited for low cost and

* Corresponding author. Tel.: +63 2 922 3959; fax: +63 2 924 7678.
E-mail address: wtreichardt@yahoo.com (W.T. Reichardt).

0025-326X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.marpolbul.2013.09.018

routine environmental monitoring. At the same time it addresses as yet unresolved environmental health risks associated with certain bacterial loads that accompany the dispersal of fish farming waste in adjacent marine environments.

Bacteriological monitoring data in 2007 had suggested a temporary spillover of sucrose-negative water-borne vibrios from a fish cage site into the waters of a nearby coral reef. Subsequent analyses of intestinal contents of milk fish, water and sediment samples in 2011/12 were to provide suc^-/suc^+ ratios for vibrios on a larger scale during NE monsoonal dry season. This choice minimized the possibility of interfering selective salinity effects on *Vibrio* populations during the SW monsoonal wet season and covered both peak harvest and near fallow periods for milk fish. Intestinal contents were obtained exclusively from adult milk fish, because only these had indicated a predominance of sucrose-negative vibrios in previous analyses (Reichardt et al. (2007).

2. Materials and methods

Mariculture zones at Cape Bolinao are located in close proximity to coral reefs and areas devoted to tourism. Milkfish farming using 18 × 18 m cages in the Anda–Bolinao sections of the Lingayen Gulf has been restricted to a southern (Anda) and a northern fish farming zone (Bolinao) along the Caquiputan Strait that diverges at Siapar Island (Fig. 1). Seasonal distribution patterns of water borne viable bacterial counts were obtained at monthly intervals in 2006/2007 from a fish cage site at Siapar (N16°20.9′, E119°57.5′ (11 m water depth) and from a neighboring shallow coral reef site at Cangaluyan Island (N21°58.9′, E119° 58.3′ (1–2 m w.d.).

A survey of surface water and sediment extended over a distance of more than 10 km from the northern fish farming zone of the Caquiputan Strait toward the mouth of the main tidal channel. This survey was conducted on eight sampling dates at intervals of roughly three weeks during NE monsoonal seasons in 2011/2012. It included the peak harvest season in December followed by a subsequent nearly fallow period in January. Water samples were taken (A) at the main tidal channel exit near Lucero, N16°24.606′, E 119°53.916′ at 18 m water depth (w.d.), (B) at a coral reef site at Malilnep (Fig. 1,# 1), N16°26.320′, E119°56.458′ (5 m w.d.), (C) near inhabited shoreline at Bolinao (Fig. 1,# 9), N16°22.840′, E119° 54.626′ (21 m w.d.), (D) in the fish cage area of Bolinao (Fig. 1, #10), N16°23.160′, E119°55.480′ (15 m w.d.), (E) at Poro Panaien islet (Fig. 1, # 11), N16°22.247′, E119°, 55.775′ (6 m w.d.), and (F) near Siapar island (Fig. 1, # 12), N16°21.071′, E119°57.631′ (7 m w.d.). – On each sampling date single specimens of adult milk fish (*Chanos chanos*) were obtained from the fish cage site of Bolinao (Fig. 1, # 10).

Complimentary sediment samples were obtained from the same locations as the water samples, except for replacing the coral reef site at Malilnep with fine sandy sediment from Gawa (Fig. 1, #4), (N 16°23.540′, E 119°54.539′,13 m water depth). In March 2012 additional sediment samples were obtained from a total of 17 locations extending from the southern fish cage areas of Anda to Lucero near the open sea. These sediment sampling sites are indicated by numbers in Fig. 1.

Triplicate water samples from 1 m depth were collected using a Niskin sampler and were immediately filled into sterile 50 ml Corning tubes. Triplicate sediment subsamples were obtained using plexiglass tubes (inner diameter: 5 cm) either by scuba divers or with a gravity corer. The top 2 cm layers of sediment were subsampled using sawed-off 5 cm^3 sterile syringes from which 0.5 ml aliquots were distributed into serial dilution tubes containing sterile sea water. A special sediment survey in 2012 followed a redox potential gradient stretching from anoxic and sulfidic sediments in the fish farming zones of Anda and Bolinao toward oxidized sediments northwest of Santiago Island. Redox potential readings were taken with a Pt redox electrode (WTW) in combination with an Ag/AgCl electrode serving as reference at 194 mV.

Freshly caught milkfish were aseptically dissected within 1–2 h. Weighed contents of the distal end of the intestine were distributed into 50 ml Corning tubes containing 40 ml of sterile seawater for serial dilutions and dispersed three times for 5 s using a high speed ultraturrax blender, before serial dilutions were completed. For comparison with a filter feeding organism as alternative, the same procedure was applied to the aseptically dissected soft tissue of freshly caught bivalve "tahong" (*Perna viridis*). Samples of this bivalve were collected at the same time from immersed structures of the same fish cages that provided the milkfish specimens.

From serial dilutions of water, sediment, and animal samples targeting roughly estimated titer ranges, 0.1 ml aliquots were distributed and spread plated onto petridishes. Means of viable counts of presumptive vibrios (mesophilic Vibrionaceae and other closely related vibrios) were based on triplicate plate counts after 1–2 d of incubation at 37–38 °C on selective thiosulfate-citrate-bile salts sucrose (TCBS) agar (Difco; Bolinches et al. 1988). Sucrose fermenting and sucrose non fermenting colonies (yellow and green in the presence of bromothymolblue) were counted separately. To mark the predominance of sucrose negative (sucrose non fermenting) colony forming units (CFUs), ratios of sucrose-negative to sucrose positive CFUs (suc^-/suc^+) were recorded according to Lopez-Joven et al. (2011). For comparison, plate counts of total copiotrophic bacteria were obtained on ZoBell's marine agar 2216 (HiMedia) after incubation for two weeks at 30 °C using 3–4 replicates.

Sucrose-negative isolates from TCBS agar were grown on marine agar plates and maintained in alkaline peptone "CDC 1494" liquid medium (Farmer and Hickman-Brenner 2006). Phenotypic characterization focused mainly on tests recommended by Noguerola and Blanch (2008) using an API 20E kit (Biomerieux, France).

A representative isolate from milkfish intestine (V6) was chosen for whole genome sequencing using the Roche 454 GS Junior System. The protocols and kits recommended by the manufacturer were used for the preparation of the sequencing libraries. The sequences were then assembled using the software Newbler (ver. 2.7). The resulting contigs were searched for sequences encoding putative virulence factors, including the known toxin genes of clinically pathogenic vibrios (Chen et al., 2005). In addition, phylogenetic analysis of selected markers (*16S rRNA*, *rpoA*, *pyrH*; Thompson et al. 2005a) obtained from the genomic data was also carried out using MEGA5 (Tamura et al. 2011) to provide the basis for the taxonomic classification of the isolate.

Statistics were based on GraphPad InStat 3, version 3.06 for Windows (Graph Pad Software, Inc.).

3. Results

3.1. Bacterial load of vibrios in maricultured organisms

Intestinal contents of freshly collected adult milkfish from the Bolinao fish farming zone showed strong anoxia with redox values ranging between -150 and −290 mV. Viable counts of vibrios yielded almost exclusively sucrose negative CFUs. Ratios of sucrose non-fermenting to sucrose fermenting CFUs (suc^-/suc^+) exceeded by far the equilibrium ratio of one. Milk fish specimens analyzed on seven sampling dates between January 2011 and January 2012 contained from 2.0 × 10^6 to 545 × 10^6 CFUs per g of wet weight (median: 52.3 × 10^6) on TCBS agar (Table 1). Suc^-/suc^+ ratios ranging between 2.1 and 59, with a mean of 22.4, indicated an extreme