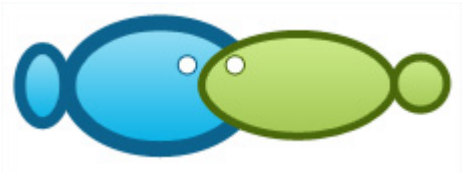

Bioluminescent *Vibrio* spp. with antibacterial activity against the nosocomial pathogens *Staphylococcus aureus* and *Klebsiella pneumoniae*

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Abstract. Though the vast marine ecosystems of the Philippine archipelago are steeped with immense biodiversity, bioluminescent bacteria and their fish hosts remain unidentified and untapped for potential biomedical applications. In this study, 51 out of 60 marine fish samples purchased from selected public seafood markets in the Philippines were found to be associated with bioluminescent bacteria. Purified bioluminescent bacterial colonies isolated from the intestine and skin of the fish samples were screened for antibacterial activity against strains of hospital-acquired pathogens, also known as 'nosocomial' pathogens, *Staphylococcus aureus* and *Klebsiella pneumoniae*. Membrane and cytosolic fractions from 4 bioluminescent bacterial isolates, designated here as strains ADMU-AUF-01 to -04, isolated from *Lagocephalus spadiceus* (half-smooth golden puffer fish), *Leiognathus equulus* (ponyfish), *Scomber japonicus* (Pacific chub mackerel), and *Lutjanus argentimaculatus* (red snapper) respectively, exhibited antibacterial activity. Statistical *t*-test analysis showed that, except from strain ADMU-AUF-01, the fractions from the bioluminescent bacterial isolates showed comparable antibacterial activity with the positive controls Piperacillin and Ceftriaxone. The 16S rRNA gene sequence analysis showed that strain ADMU-AUF-01 has close identity with *Vibrio* sp. (98%), ADMU-AUF-02 with *Vibrio anguillarium* strain X0906 (96%), ADMU-AUF-03 with *Vibrio* sp. (93%), and ADMU-AUF-04 with an uncultured bacterium clone F2G (89%). This study reveals the presence of bioluminescent bacteria in the diverse marine fishes of the Philippines and their potential as important new microbial resources of anti-nosocomial substances especially for deadly pathogens infecting both adult and newborn infants.
Key Words: marine fish, quorum sensing, 16S rRNA gene phylogeny, *Vibrio*.

Introduction. Bioluminescence is the generation of light by living organisms as a result of energy released when the light emitting molecule luciferin undergoes oxidation catalyzed by luciferase in the presence of oxygen (Nealson & Markovich 1970). Bioluminescence functions for attracting mates, capturing prey, and for countershading in nocturnal aquatic organisms to evade predators (Widder 2010; Zarubin et al 2012). It occurs across a broad range of organisms such as fireflies, annelids, fungi, dinoflagellates, mollusks, and fish regulated by biochemical, neural, or endocrine control (Widder 2010; Zarubin et al 2012). Bioluminescence, however in certain fish and cephalopods, is typically achieved by harboring symbiotic bioluminescent bacteria, typically *Photobacterium* spp., which thrive in nutrient-rich skin, intestinal tracts, gills, and light organs which the hosts developed over time (Urbanczyk et al 2011; Naguit et al 2014). Some *Vibrio* spp. thrive in many marine, brackish, and aquacultured fish, shrimps, and crustaceans as commensals or pathogens (Urbanczyk et al 2011).

Bioluminescent bacteria or their *lux* genes are tapped as biosensors for water quality and toxicity testing (Menz et al 2013) and for the detection of antibiotic residues and pathogens in food (Griffiths 1993; Robinsons et al 2011). Bioluminescence is also used in bioluminescence imaging (BLI) making use of luciferase to monitor different biological processes (Badr & Tannous 2011; Gahan 2012). To date, bioluminescent bacteria are not yet explored as potential new bioresources of antibiotics to fight the

global threat of antibiotic resistance. One of the concerns nowadays is the increasing number of antibiotic resistant human pathogens in hospital-acquired infections, also known as 'nosocomial' infection in immunocompromised patients and hospital staff. Nosocomial infection occurs 48 hours or more after admission or after the discharge of patients with prolonged intake of antibiotics, HIV/AIDS, solid organ transplants, and in cancer patients (Bienvenido et al 1983; Ducel et al 2002). The most common and challenging nosocomial cases are pneumonia, surgical site infection, respiratory and urinary tract infections, and bloodstream infections commonly caused by *Staphylococcus aureus* and *Klebsiella pneumonia*e (Munoz-Prize 2009).

Newborn infants or neonates are relatively immunocompromised and prolonged hospitalization increases the risk of their vulnerability (Shane & Stoll 2014). Nosocomial infections are one of the major causes of mortality and morbidity in newborn infants in neonatal intensive care units (Dong & Speer 2015; Shane & Stoll 2014). At a global scale, an estimated 1.4 million neonatal deaths yearly are caused by nosocomial infection (Shane & Stoll 2014). Though there has been an alarming increase of infections by antibiotic-resistant nosocomial bacterial pathogens such as methicillin-resistant *S. aureus* (MRSA) and the incidence of panantibiotic-resistant infections by *Acinetobacter* species, multiple drug-resistant (MDR) *Pseudomonas aeruginosa* and carbapenem-resistant *Klebsiella* spp. (Arias & Murray 2009), there is still an alarming low number of newly approved antibiotics to combat this global human health threat (Donadio et al 2010).

To tap the unexplored rich microbial biodiversity of the numerous aquatic habitats of the Philippine archipelago, this study therefore embarked on the isolation, purification, and cultivation of bioluminescent bacteria from randomly selected marine fish samples, and screening of the bacterial isolates as new sources of antibiotics against two of the most severe nosocomial pathogens encountered in and out of the intensive care units in Philippine hospitals, *K. pneumoniae* and *S. aureu*s (Gill et al 2010).

Material and Method

Collection and morphological identification of marine fish samples. Three specimens each of the 60 marine fish samples were purchased from early morning catches delivered in randomly selected public markets in Olongapo City, Lingayen, Puerto Galera, and Iloilo City, Philippines. The collection was done from January to February 2013. The standard protocol for morphological identification of the fish samples was performed based on taxonomic keys (Carpenter & Niem 2001)

Isolation and purification of bioluminescent bacteria. All fish samples were dissected immediately after collection and cotton swab samples were obtained from the skin and inside the vitreous sac of the eyes. The fish ventral area was cut open exposing the stomach and the intestines which were cut laterally making the mucosa exposed. These areas were swabbed as well. The cotton swabs were streaked on standard bioluminescent agar plates (30 g salt, 10 g peptone, 2 g K_2HPO_4, 0.5 g $MgSO_4$, 2 mL glycerol, and 15 g agar in 1 L distilled water (Nealson & Markovitch 1970) and incubated at 20^oC overnight. Bioluminescent colonies were picked and purified by standard microbiological streakings. Cell morphology was observed through light microscopy.

Preparation of bacterial cytosolic and membrane fractions. Cells were grown for 24 hours in 250 mL bioluminescent broth at 20^oC (Nealson & Markovich 1970) and harvested by centrifugation at 5,000 rpm for 5 min. Cells (1.6×10^{-11} CFU mL^{-1}) were sonicated on ice for 15 min, centrifuged, and the supernatant was obtained as the cytosolic fraction (CF). The pelleted debris were washed 3X with sterile distilled water to remove residual CF and broth, and sonicated for 15 min and centrifuged as described above. The supernatant was obtained as the membrane fraction (MF). The fractions were frozen to -20^oC overnight, freeze-dried, and stored at 4^oC until use (Osborn & Munson 1974).

Screening of CF and MF for anti-nosocomial pathogen activity. Two of the most prevalent hospital-acquired pathogens in the Philippines, *S. aureus* and *K. pneumoniae* were purchased as pure isolates from the Philippine General Hospital (PGH), Manila, Philippines. The Kirby-Bauer method in triplicates was used to test the anti-nosocomial pathogen property of the CF and MF. Ten milligrams (10 mg) each of freeze-dried CF and MF were suspended in 1 mL sterile distilled water and 30 µL were preloaded into sterile Whattman™ 1 blank discs. The discs were laid on nutrient agar plates lawned with $1x10^9$ CFU/mL of either *S. aureus* or *K. pneumoniae*. Piperacillin (0.1 g mL^{-1}) and Ceftriaxone (0.1 g mL^{-1}) were used as positive controls for *S. aureus* and *K. pneumoniae* respectively. Freshly prepared bioluminescent broth was used as the negative control. Zones of inhibition were measured after 24 hours incubation at 37°C. To assess if the mean zones of inhibition are significantly different, the *t*-test in SPSS version 17 was used. The standard error of the mean was also computed.

Molecular identification and phylogenetic analysis of bioluminescent bacterial isolates. Only bioluminescent bacterial isolates with antibiotic activity were processed for identification based on 16S rRNA gene sequence. The InstaGene Matrix™ DNA extraction kit was used in the isolation of the genomic DNA of the bioluminescent bacteria. The 16S rRNA gene was amplified using KAPATaq DNA Polymerase™ PCR Kit (Kapa Biosystems, Inc., MA, USA). The universal primers 27F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5'GGTTACCTTGTTACGACTT3') were used (Abulencia et al 2006). Reactions were carried out using MyGene™ Series Peltier Thermal Cycler Model MG25+, with the following PCR conditions: initial denaturation at 94°C for 5 min, 20 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 1 min, and final extension at 72°C for 5 min. Agarose gel electrophoresis of the amplicons was performed in 1% agarose gel at 100 V for 45 min. The amplicons were sent to the sequencing service laboratory of AITBiotech, Singapore. The sequences were edited using Chromas software (Goodstadt & Ponting 2001) and were assembled using CAP3 software (Huang & Madan 1999). The 16S rRNA gene sequences were analyzed for the presence of chimera using the Database Enabled Code for Ideal Probe Hybridization Employing R (DECIPHER) (Wright et al 2012) and were then compared to deposited sequences in the public databases using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (Altschul et al 1990). ClustalW was used to align the 16S rRNA gene sequences of the bioluminescent bacteria used in this study and the 16S rRNA gene sequences of known species of bioluminescent bacteria which were downloaded from NCBI database (accession numbers: AF493804.1, AY278667.1, DQ860043.1, EU413955.1, FJ227119.1, GU078676.1, HQ908673.1, JQ799072.1, JQ799114.1, JQ799128.1, JQ904733.1, KC210811.1, C210816.1, KC814182.1, KJ841877.1, NR_042736.1, and NR_117890.1). The molecular phylogenetic tree of the bioluminescent bacterial isolates was constructed using Molecular Evolutionary Genetics Analysis version 6 software (MEGA 6) (Tamura et al 2013). Evolutionary history was determined using Neighbor-Joining method (Tamura et al 2004) while the evolutionary distance was calculated using Maximum Composite Likelihood. Bootstrap test was conducted at 1000 replicates (Felsenstein 1985), and only branches with more than 50% bootstrap support were marked with bootstrap values. The tree was rooted with the thermophilic bacterium *Thermococcus celer* strain DSM 2476 (NR_042736.1).

Results

Marine fish samples with associated bioluminescent bacteria. Gram negative curved-rod bioluminescent bacteria were isolated from 51 out of the 60 marine fish samples. The swabbed bacteria emitted blue light after 24 hours incubation at 20°C in bioluminescent agar plates (Figure 1). Four out of the 51 isolates, designated in this study as strains ADMU-AUF-01 to -04, were found to have antibacterial activity against the nosocomial pathogens *S. aureus* and/or *K. pneumoniae*. Their respective marine fish hosts were identified based on morphology as *Lagocephalus spadiceus* (half-smooth golden puffer fish), *Leiognathus equulus* (pony fish), *Lutjanus argentimaculatus* (red

snapper), and *Scomber japonicus* (Pacific chub mackerel) respectively (Figure 2). ADMU-AUF-01 was isolated from the fish skin and ADMU-02 to -04 were isolated from the fish intestines.

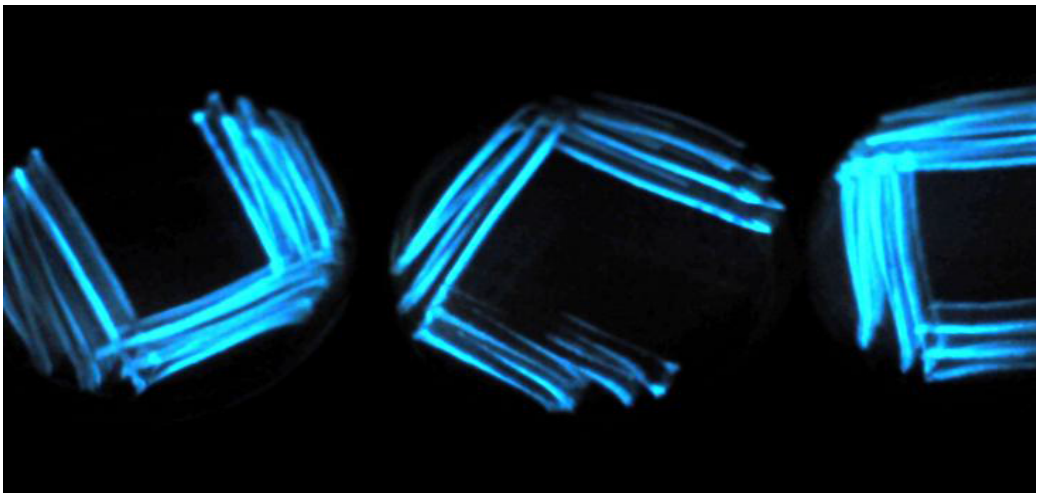

Figure 1. Bioluminescent bacterial swab samples from marine fish.

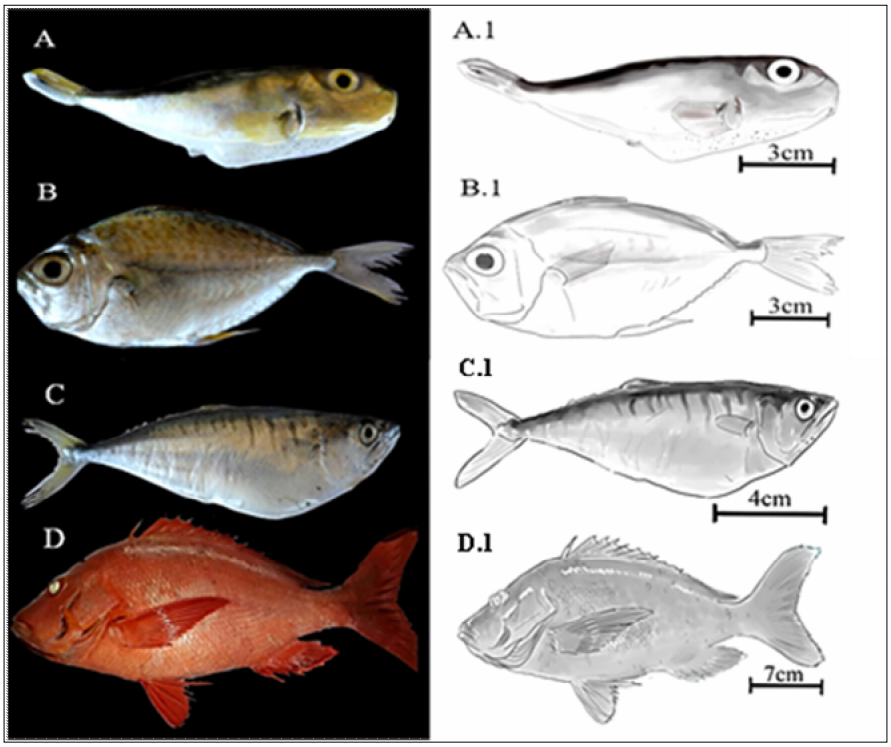

Figure 2. The Philippine marine fishes with associated bioluminescent bacteria. (a) *Lagocephalus spadiceus* (half-smooth golden puffer fish), (b) *Leiognathus equulus* (ponyfish), (c) *Scomber japonicus* (Pacific chub mackerel), and (d) *Lutjanus argentimaculatus* (red snapper). The respective sketches of the marine fishes are also shown (a.1 to d.1).

Screening of CF and MF with anti-nosocomial pathogen activity. Statistical t-test was used to compare the mean zones of inhibition of the extracts from the bioluminescent bacterial isolates from the positive control. Figure 3.A shows the antibacterial activity of bioluminescent bacterial isolates against *S. aureus*. The zones of inhibition of the CFs of ADMU-AUF-01 and -04 are significantly different compared with the control Piperacillin (p-value of 0.013 and 0.034). The CF of ADMU-AUF-02 and ADMU-03 are not significantly different from the control (p-value of 0.168 and 0.055). This

result indicates that the CFs from the bioluminescent isolates strains ADMU-AUF-02 and -03 have antibacterial activity comparable to Piperacillin.

The zones of inhibition of the MFs of ADMU-AUF-01 and ADMU-AUF-02 are significantly different from the positive control Piperacillin (p-value 0.001 and 0.022, respectively) but the zones of inhibition of the MFs of ADMU-AUF-03 and -04 are not significantly different compared with the positive control (p-value 0.530 and 0.237, respectively). This result shows that the MFs from the bioluminescent isolates strains ADMUAUF-03 and -04 have antibacterial activities against the nosocomial pathogen *S. aureus* comparable to the positive control.

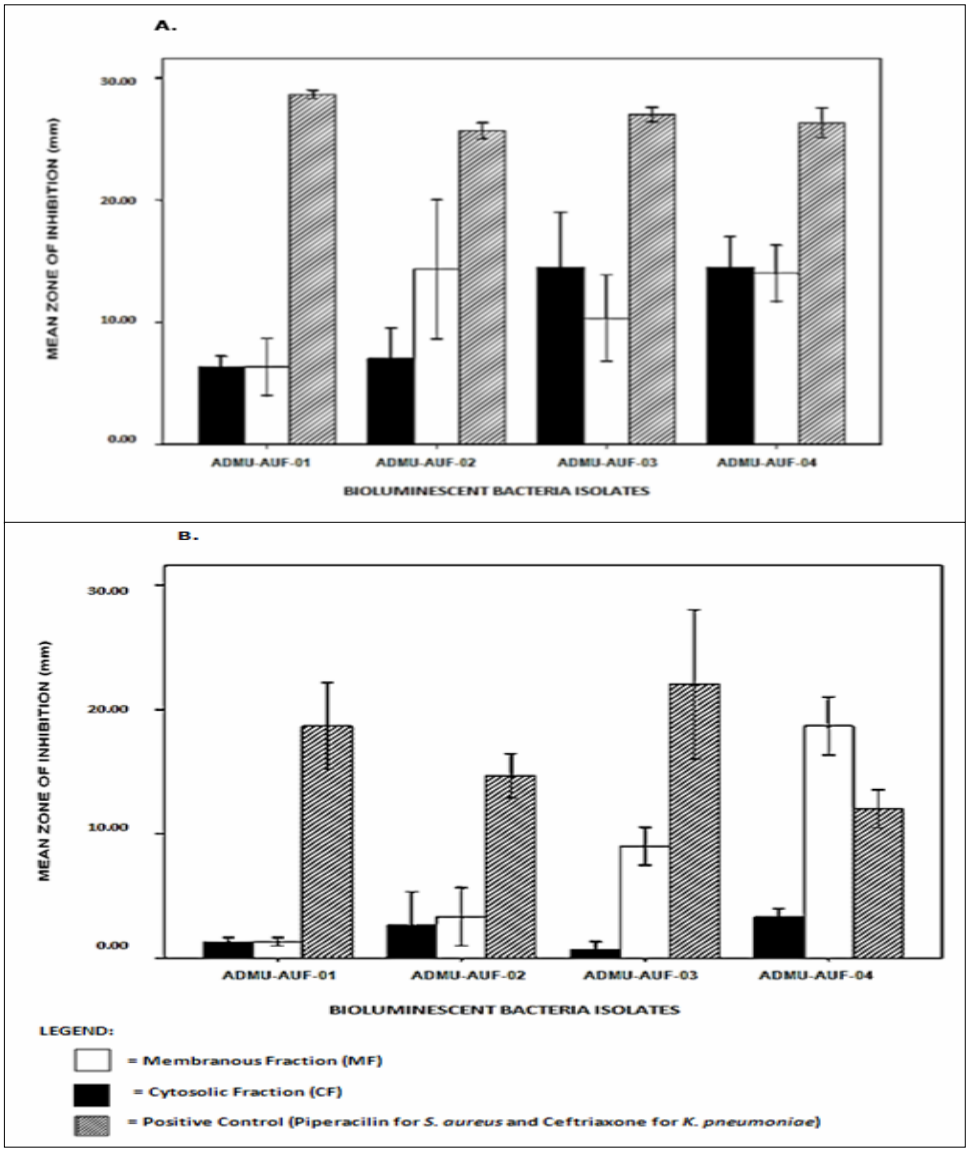

Figure 3. Anti-nosocomial property of bioluminescent bacteria. The mean zone of inhibition from three replicates of membranous fraction (MF) and cytosolic fraction (CF) of bioluminescent bacteria from different fish host against A) *Staphylococcus aureus* and B) *Klebsiella pneumoniae*. Error bars indicate the ± 1 standard error of the mean.

Figure 3.B shows the antibacterial activity of extracts from the bioluminescent bacterial isolates against *K. pneumoniae*. The CF of isolates ADMU-AUF-01 is significantly different with the positive control (p-values 0.04) while isolates ADMU-AUF -02, -03, and -04 are not significantly different from the positive control with p-values 0.07, 0.15, and 0.10 respectively. The highest mean zone of inhibition for the CF was observed for ADMU-AUF-04 at 18.67 mm with significant difference with the positive control (p-value 0.010), but it indicates a very strong antibacterial activity because its zone of inhibition is higher than

the positive control. The CF from isolate ADMU-AUF- 02, -03, and -04 exhibited antibacterial activity against the neonatal nosocomial pathogen *K. pneumoniae*.

The MFs of the isolated strains ADMU-AUF-01 and -04 are significantly different from the positive control Ceftriaxone (p-values 0.03 and 0.01 respectively). The MFs of isolates ADMU-AUF-02 and ADMU-AUF-03 are not significantly different with from the control (p-values 0.10 and 0.08 respectively).

Taken altogether, the bioluminescent bacterial isolates strains ADMU-AUF-02, -03, and -04, except ADMU-AUF-01, possess antibacterial activity against the neonatal nosocomial pathogens *S. aureus* and *K. pneumoniae*.

Molecular identification and phylogenetic analysis of the bioluminescent bacterial isolates. Based on the BLAST analysis of 16S rRNA gene sequences of the bioluminescent bacterial isolates with antibacterial activity, strains ADMU-AUF-01, ADMU-AUF-02, and ADMU-AUF-04 had significant hits with deposited sequences of known genera of bioluminescent bacteria, while strain ADMU-AUF-03 showed close identity to an 'Uncultured Bacterium Clone F2G' (89%). Strains ADMU-AUF-01 and ADMU-AUF-04 showed close identity to *Vibrio* sp. (98% and 93%, respectively). Strain ADMU-AUF-02 had close identity to known fish pathogen *Vibrio anguillarium* strain X0906 (96%). Phylogenetic analysis showed that the bioluminescent bacterial isolates clustered with two major groups: known genera of bioluminescent bacteria and uncultured bacteria (Figure 4). The isolates from the fish *L. spadiceus*, *L. equulus*, and *S. japonicus* clustered with representative species of genus *Vibrio* while the bacterial isolate from *L. argentimaculatus* clustered with uncultured bacteria.

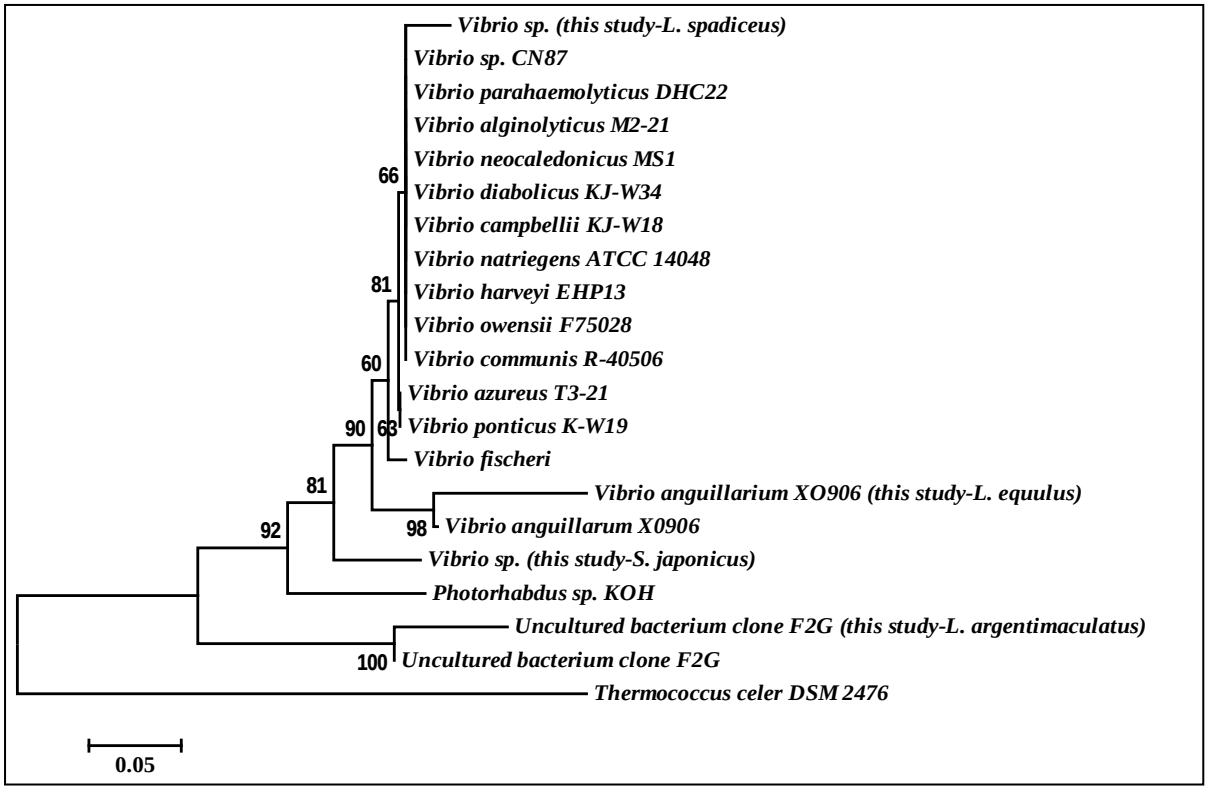

Figure 4. Bioluminescent bacteria phylogenetic tree. Phylogenetic tree (Neighbor-Joining method) of the bioluminescent bacteria isolated from marine fish host samples based on ~ 800 – 900 bp16S rRNA gene sequences. The marine fish hosts of the isolates were indicated in the parenthesis. The branch lengths were based on the units of the number of base substitutions per site. Bootstrap values (1000 replicates) are shown for branches with more than 50% bootstrap support. The tree was rooted using *Thermococcus celer* strain DSM 2476 (NR_042736).

Discussion. Though the vast marine ecosystems of the Philippine archipelago is steeped with immense biodiversity, microbial biodiversity especially bioluminescent bacteria, remain poorly studied and unexplored for biomedical applications. Concomitantly, diverse

Philippine fish hosts possibly associated with this unique group of microorganisms remain unidentified.

Klebsiella and *Staphylococcus* spp. are two of the nosocomial pathogens found to survive 7 to 30 months on surfaces and most frequently isolated from nosocomial infected patients (Neely 2000; Neely & Maley 2000). Studies in Asia including the Philippines show that these pathogens are 2 of the most predominant causes of neonatal infections (Bhutta 1996; Gatchalian et al 1999; Karthikeyan & Premkumar 2001). In a study in 2 neonatal intensive care units in Manila (Gill et al 2009), from the 1827 neonates admitted, 561 (30.7%) arrived from delivery already colonized with drug-resistant bacteria and 578 (45.6%) eventually became colonized with drug-resistant bacteria, 358 (19.6%) became infected in their bloodstream and 615 (33.7%) died. Out of 2903 identified drug-resistant colonizing bacteria, 85% were gram-negative bacilli (primarily *Acinetobacter*, *Pseudomonas*, and *Klebsiella* species) and 14% were MRSA. Given these scenarios, there is therefore truly an urgent need for the discovery of new antibiotics from unexplored microorganisms such as Philippine bioluminescent bacteria. In this study, membrane and cytosolic fractions from 4 bacterial bioluminescent isolates, strains ADMU-AUF-01 to -04 exhibited zones of inhibition in the Kirby-Bauer test against the nosocomial bacterial pathogens *S. aureus* and *K. pneumoniae* from the Philippine General Hospital. The statistical *t*-test showed that cell fractions from 3 out of the 4 isolates showed comparable antibacterial activity with the positive controls Piperacillin and Ceftriaxone. These results provide very promising new microbial resources for treatment of deadly neonatal nosocomial infections.

Based on 16S rRNA gene sequence analysis, the bioluminescent bacterial strains ADMUAUF- 01 to -04 belong to the genus *Vibrio*, one of the two genera under the large family of Vibrionaceae which exhibits bioluminescence (Widder 2010; Zarubin et al 2012). The genus *Vibrio* is composed of greater than 100 species grouped into 14 clades distributed in estuarine but mostly associated with various marine organisms such as fish, corals, abalones, sea urchins, crustaceans, and seahorses (Widder 2010; Zarubin et al 2012). Species of *Vibrio* are also highly abundant in aquaculture farms (Beaz-Hidalgo et al 2010; Johnson et al 2012).

In this study, based on the 16S rRNA gene sequence analysis, the closest match for strain ADMU-AUF-01 isolated from *L. spadiceus (*puffer fish) is *Vibrio* sp. The species of this isolate could still not be established using the approximately 900 bp sequence obtained. Strain ADMU-AUF-02, isolated from *L. equulus* (pony fish), has close identity to *Vibrio anguillarium*, the causative agent of vibriosis, a severe hemorrhagic disease of wild and farmed fish, mollusks, and crustaceans (Denkin & Nelson 1999). A large number of *Vibrio* species are associated with marine organisms as commensals or as pathogens, common examples are *V. anguillarium, V. salmonicida*, and *V. harveyi* (Fidopiastis et al 1999). The closest match for strain ADMU-AUF-03 isolated from *S. japonicus* (Pacific chub mackerel) is also a *Vibrio* sp. Strain ADMU-AUF-04 was isolated from the gut of *L. argentimaculatus* (red snapper) and has close identity to an uncultured bacterium.

The phylogenetic analysis of the fish-associated strains ADMU-AUF-01 to -04 in this study showed distinct separation between known genera and uncultured species of bioluminescent bacteria. The different *Vibrio* species isolated in this study were not observed in the same clade. The bioluminescent bacterial isolates formed four clades (Figure 4). Clade 1 includes strain ADMU-AUF-01 isolated from the puffer fish *L. spadiceus* and clustered with some pathogenic vibrios common in marine waters such as *V. parahaemolyticus* known to cause gastroenteritis though most strains are non-pathogenic to humans (Urbanczyk et al 2011) and *V. alginolyticus* known to reside in puffer fish and produces the potent neurotoxintetrodotoxin known as TTX (Noguchi et al 1987). Among the isolated strains in this study, ADMU-AUF-01 did not exhibit antibacterial activity, though it may produce a toxin like TTX, its action may not be specific for antibacterial activity against the pathogens used in this study. Clade 2 includes ADMU-AUF-02. It clustered with a known vibroid pathogen *V. anguillarium* which is a known causative agent of vibriosis, a fatal hemorrhagic septicemic disease in aquacultured fish causing severe economic losses in the aquaculture industry worldwide (Toranzo & Barja 1993). *V. anguillarium* is also known to infect fish in the wild and strain

ADMU-AUF-02 was isolated from pony fish (*L. equulus*) fished in the wild. Clade 3 includes strain ADMUAUF-03 isolated from chub mackerel (*S. japonicus*) which is also fished in the wild. Clade 4 includes strain ADMU-AUF-04 which has close identity to an uncultured bacterium. It diverged from the lineages of known genera of bioluminescent bacteria. Among the isolates in this study, strain ADMU-AUF-04 showed strong antibacterial activity for both gram positive *S. aureus* and gram negative *K. pneumoniae* nosocomial pathogens.

Conclusions. To date, bioluminescent bacteria remain unexplored as potential new bioresources of antibiotics to fight the global threat of emerging diseases. One of the concerns nowadays is the increasing number of antibiotic resistant human pathogens in hospital-acquired infections, also known as 'nosocomial' infection in immunocompromised patients and hospital staff. Nosocomial infections are one of the major causes of monetary burdens in South East Asia. The discovery of new drugs is therefore imperative. Our results showed that the isolated marine fish-associated bioluminescent bacteria identified as *Vibrio* spp. are potential new microbial resources of antibiotics against known nosocomial pathogens *S. aureus* and *K. pneumoniae*. Given this scenario and the economic condition of the Philippines, the exploration of novel sources of drugs from local and novel microorganisms such as bioluminescent bacteria associated with Philippine marine hosts, provide new potential microbial resources for the treatment of hospital-acquired infections in adult patients as well as in newborn infants or neonates. This study also provides additional perspective to the very limited knowledge of the immense and untapped diversity of microorganisms existing in the vast and numerous Philippine aquatic environments.

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