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Isolation and characterization of bacteria from the lesion of juvenile sea cucumber *Holothuria scabra* (Jaeger, 1938) with symptom of skin ulceration disease

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Abstract

Echinoderm diseases have attracted little interest in contrast to the other commercial marine organisms. This study reports the pathogenesis pattern of juvenile *Holothuria scabra*. 11 pure bacteria were isolated from lesions of juvenile *H. scabra* with the symptoms of skin ulceration disease. Phylogenetic identification based on 16S rRNA gene sequences analysis revealed that they belonged to the genera *Arcobacter bivalviorum, Pseudoalteromonas citrea, Pseudoalteromonas* sp., *Vibrio azureus, V. fortis, V. owensii, V. parahaemolyticus, V. rotiferianus, V. tubiashi* and *Vibrio* sp..This study is the first report which find *V. owensii, V. azureus* and *V. fortis* as potential pathogens of holothuroids. All isolated bacteria showed *in vitro* susceptibility to the common antibiotics imipenem, chloramphenicol and amoxicillin/clavulanic acid. These antibiotics might be effective in reducing the incidence of the skin ulceration disease in case of emergency.

Keywords: Pathogen, Skin ulceration, *Vibrio*, Sea cucumber, Beche-de-mer

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Introduction

In response to growth in human population and food demands, aquaculture has been developed greatly in the last decades. Resulted products have extensively exploited as food supply (Cao *et al*., 2015) or as raw materials for ornamental, industrial, and pharmaceutical applications (Maki *et al*., 2014; Venkata Mohan *et al*., 2015).

One of the fastest growing aquaculture industries is sea cucumber farming (Conand *et al*., 2014). Sea cucumber, also named Bêche-de-mer or Gamat, are marine invertebrates from the class Holothuroidea, which have long been used in traditional Asian medicines and cuisine. Interest in these kind of animals is growing because of their capability to produce a range of high-value bioactive molecules (Bordbar *et al*., 2011; Pereira *et al*., 2014). Sea cucumber farming has been developed only in recent decades but rapidly expanded in countries such as Australia, China, the Galápagos Islands, Indonesia, Japan, Malaysia and the Philippines. Diseases can lead to big economic losses in this industry but they are less well-studied than diseases in the other aquaculture industries (Yasoda *et al*., 2006).

Skin ulceration syndrome is a highly infectious and a lethal disease in Holothuroids. Symptoms appear as white spots on the skin which rapidly spread over the skin and expand to the lower tissues led to death in infected individuals. In *Holothuria scabra* the invasive organisms have been reported as bacteria, fungi, and protozoa. (Deng *et al*., 2009; Lavitra *et al*., 2009).

During a study in our centre on 50 juvenile *H. scabra* imported from Indonesia, we faced to two dead animals with white spots and some animals with similar but fewer skin lesions. The imported specimens were already kept for ecophysiological experiments in the experimental aquaria facilities (MAREE), of the Leibniz Centre for Tropical Marine Ecology (ZMT) Bremen, Germany, before we lose them due to the disease.

In this study the potential pathogenic bacteria isolated from the lesions of infected juveniles *H. scabra* were identified by using 16S rRNA gene sequence analysis. Then the *in vitro* susceptibility of isolated bacteria was tested against a range of antibiotics.

Materials and methods

Sample collection

Domestic Juvenile *H. scabra* were supplied from Lombok, Indonesia and were shipped with a regular delivery of animals for ornamental purposes, involving commercial companies. Three infected juveniles with skin lesions were used for this study. Before taking samples for microbial experiments, the skin and lesions were washed by 98% ethanol to eliminate the risk of contamination by external contaminants such as waterborne microorganisms and normal aquatic microflora. Skin samples were then taken from the center of each lesion and the soft slimy tissues around each lesion using sterile cotton swabs and a sterile scalpel.

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Bacterial isolation

Skin samples were inoculated into liquid marine broth (Carl Roth, Germany), as well as direct plating on marine agar (1.5% w/v agar). Cultures were incubated at 30º C for 24 hours. 11 different colonies were purified by further streaking onto marine agar plates followed by use of Gram's stain and micro- and also macroscopic observation of colonies. Purified strains subcultured onto agar slant in tubes and stored at 4°C. These were used as working stock cultures. For long term storage, densely grown bacteria were collected from agarised cultures and mixed with 2 mL of an aqueous solution of 16% glycerol and marine broth. 0.5 mL aliquots of these cell suspensions were placed in 1.5 mL cryotubes and preserved at -80 ºC.

Antibiotic sensitivity test

Antibiotic sensitivity of isolated bacteria was tested using antibiotic-impregnated discs (BD BBL™ Sensi-Disc™) on Muller-Hinton Agar (Carl Roth, Germany) with the salinity adjusted to 3.2 % by adding sea salt to the base medium. The following antibiotics were tested using the quantities stated in each disc: tetracycline (30 µg), chloramphenicol (30 µg), ampicillin (10 µg), erythromycin (15 µg), amoxicillin /clavulanic acid (30 µg), ciprofloxacin (5 µg), imipenem (10 µg), penicillin (10 U) and vancomycin (30 µg). The diameters of inhibitory zones were measured with digital callipers after 21 hours incubation at 30ºC.

16S rRNA gene sequence analysis

Bacterial cells were harvested from an overnight culture in marine broth. DNA was extracted from these using Bacterial and Yeast Genomic DNA Purification Kit (EUR_X Gene MATRIX) followed by PCR amplification of the 16S rRNA with universal primer pairs 27F and 1492R (Biomers.net GmbH).

The PCR reactions were performed using a Mastercycler® nexus gradient thermal cycler (Eppendorf, Germany) in 50 µl reaction volumes each containing 0.25 µl of Tag DNA polymerase (Sigma), 1 µl of each primer at a final concentration of 20 pmol, 1 µl of dNTPs at a concentration of 10 mM, 5 µl of PCR buffer, 5 µl $MgCl_2$, DNA template (based on DNA concentration) and DDW (to reach to the reaction volume). The PCR reactions were performed using the following thermal cycling conditions: initial denaturation at 95°C for 45 seconds followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 2 min. The reaction was then cooled to 4°C for 5 mins. 5 µl of the PCR reactions were resolved on a 1% agarose gel stained with Stain G (SERVA, Germany) to verify the size of the amplicons. The remaining PCR reaction was treated with ExoSAP-IT (Affymetrix, USA), and sequenced at StarSEQ (Mainz, Germany).

The closest phylogenetic relative of each isolate was identified by comparison of each 16S rRNA gene sequence to the GenBank database in the National Centre for Biotechnology

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Information (NCBI). The Microbial Nucleotide BLAST program (http://blast.ncbi.nlm.nih.gov) and the Mega6 program (http://www.megasoftware.net) were used to choose closely related strains and identify the 16S rRNA gene sequence similarities of phylogenetic neighbors.

Results

Bacterial isolates

Eleven Gram-negative bacterial strains were isolated from the lesions of infected juveniles *Holothuria scabra*. These were designated as: Strains 1_S1-1, 2_S2-1, 3_S2-2, 4_S2-3, 5_S6-1, 6_3-1, 7_3-2, 8_3-3, 9_4, 10_5-1, and 11_5-2.

Bacterial identification based on 16S rRNA gene sequence analysis

The majority of isolates were species of *Vibrio* and *Pseudoalteromonas* (Gammaproteobacteria), and one isolate was a species of *Arcobacter* (Epsilonproteobacteria).

Isolates shared ≥ 99% pairwise identity to *Vibrio parahaemolyticus, Pseudoalteromonas citrea, Arcobacter bivalviorum, Vibrio* sp., *Vibrio rotiferianus, Pseudoalteromonas* sp., *Vibrio owensii*, *Vibrio azureus*, *Vibrio fortis and Vibrio tubiashii* (Table 1).

Table 1: Identification of bacteria isolated from skin lesions on the surface of juveniles *Holothuria scabra.*

Strains	Accession number	Closest match in Gene Bank	Identity (%)	Phylum
1_S1-1	JN 188420	*Vibrio parahaemolyticus*	99	Gammaproteobacteria
2_S2-1	GU 726872	*Pseudoalteromonas citrea*	99	Gammaproteobacteria
3_S2-2	HE 565358	*Arcobacter bivalviorum*	99	Epsilonproteobacteria
4_S2-3	HF 937146	*Vibrio* sp.	100	Gammaproteobacteria
5_S6-1	JF 907572	*Vibrio rotiferianus*	99	Gammaproteobacteria
6_3-1	AB 029824	*Pseudoalteromonas* sp.	100	Gammaproteobacteria
7_3-2	HQ 908697	*Vibrio owensii*	99	Gammaproteobacteria
8_ 3-3	AB 029824	*Pseudoalteromonas* sp.	99	Gammaproteobacteria
9_ 4	HQ 908716	*Vibrio azureus*	100	Gammaproteobacteria
10_5-1	HQ 449972	*Vibrio fortis*	99	Gammaproteobacteria
11_5-2	NR 118093	*Vibrio tubiashii*	99	Gammaproteobacteria

Antibiotic susceptibility results

Compared to the standard susceptibility zone diameters provided by the suppliers of the antibiotics, imipenem, chloramphenicol and amoxicillin/clavulanic acid demonstrated *in vitro* inhibition of all isolated bacteria (Table 2).

Table 1: Antibiotic susceptibility of isolated bacteria. Shaded cells indicate positive sensitivity.

	Antibiotics and inhibitory zones (mm)								
Strains	**AmC**	**C**	**AMP**	**E**	**VA**	**CIP**	**P**	**IPM**	**TE**
1_S1-1	M	S	R	M	R	M	R	S	M
2_S2-1	S	S	S	S	M	S	M	S	M
3_S2-2	S	S	S	S	R	S	S	S	S
4_S2-3	M	S	R	S	R	S	R	S	M

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Table 2 continued:

5_S6-1	S	S	R	M	R	S	R	S	M
6_3-1	S	S	S	S	M	S	S	S	S
7_3-2	S	S	R	M	M	S	R	S	S
8_3-3	S	S	S	S	S	S	S	S	S
9_4	S	S	R	M	M	S	R	S	S
10_5-1	S	S	S	S	S	S	S	S	S
11_5-2	S	S	S	S	M	S	S	S	S

AmC amoxicillin/clavulanic acid, C chloramphenicol, AMP ampicillin, E erythromycin, VA vancomycin, CIP ciprofloxacin, P penicillin, IPM imipenem and TE tetracycline (R; resistant, S; sensitive, M; medium resistant)

Discussion

Diseases in sea cucumbers can be serious obstacles for commercial aquaculture, causing huge economic loss (Xiyin *et al*., 2004; Yin-Geng, *et al*., 2004; Purcell and Eeckhaut, 2005; Liu *et al*., 2010). Skin ulceration or white spot quickly expands to an invasive lesion that destroys the vicinal and sub-epidermal tissues and led to death within 3-5 days (Becker *et al*., 2004).

Skin ulceration disease with almost the same symptoms has been reported worldwide on different Holothuroids including *Apostichopus japonicus*, *Isostichopus fuscus* and *H. scabra* (Becker *et al*., 2004; Deng *et al*., 2009).

A comparison between isolated bacteria in this study to those isolated from other similar studies showed that species of *Vibrio* always have been the majority of bacteria isolated from infected samples. This was the case for *H. scabra* in Australia and New Caledonia (Morgan *et al*., 2000), *I. fuscus* in Ecuador (Mercier *et al*., 2005) and *A. japonicus* in China (Yin-Geng *et al*., 2004; Ma *et al*., 2006; Deng *et al*., 2009). *Vibrio* belongs to a cluster of closely related species, named the Harveyi clade which are major common pathogens of aquatic organisms such as fish, shellfish, and crustaceans. The Harveyi clade consists of eleven species, which are major pathogens of many aquatic organisms: *V. harveyi, V.rotiferianus, V. alginolyticus, V. campbellii, V. parahaemolyticus, V. mytili, V. natriegens, V. azureus, V. communis, V. jasicida and V. owensii* (Rivera-Posada *et al*., 2011).

Vibriosis is a common fatal disease in fish and other aquaculture animals (Chatterjee and Haldar, 2012). Rotting edge syndrome and off-plate syndrome, skin ulceration disease, peristome oedema disease and infected dermal tissues of the echinoderm *A. japonicus* (Rivera-Posada *et al*., 2011; Sakai, 2011), and white spot in *H. scabra* are infectious diseases caused by *Vibrio sp*. (Austin and Zhang, 2006; Chatterjee and Haldar, 2012).

V. parahaemolyticus has been reported to be associated with dermal tissue disease in the echinoderm *Amphipholis gracillima* (Rivera-Posada *et al*., 2011), whilst Becker *et al.* (2004) isolated the microorganism from skin lesions in juvenile *H. scabra*.

V. tubiashii is a pathogen for larvae of bivalve mollusks (Hada *et al*., 1984) and has recently been isolated from

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lesions of skin ulcerative syndrome of *A. japonicus*. The pathogenicity of the strain was confirmed by artificial infection (Deng *et al*., 2009). Although not a member of the Harveyi clade, it should be considered as a potential pathogen for juveniles *H. scabra*.

V. rotiferianus has been isolated from skin necrotic lesions in echinoderms *Acanthasther planci* and *Linckia guildingi* (Rivera-Posada, 2012), but there is no evidence of its pathogenicity in sea cucumbers.

Pseudoalteromonas sp. and *P. tetraodonis* have been shown to be associated with skin ulceration and peristome tumescence in cultured *A. japonicus* (Liu *et al*., 2010). There is no evidence of pathogenicity in marine organisms by *P. Citrea*, but since this bacterium is a lipase producer (Sivasubramanian *et al*., 2011), the assumption here is that bacterial lipase enhances the primary lesions by decomposition of fatty acids within the skin tissue.

A. bivalviorum was first isolated from shellfish by Levican *et al*. (2012). Some species of the genus have been found to be human and farm animal enteropathogens (Collado and Figueras, 2011), but its pathogenicity in holothuroids has not been reported.

This study is the first to find *V. owensii*, *V. azureus* and *V. fortis* as potential pathogens of holothuroids.

The present work suggests that the white spot in juveniles *H. scabra* is a bacterial disease. To confirm that, an *in vivo* study is required in which each isolate is tested for pathogenicity in healthy animals. Infection could be attempted using inoculation of healthy animals by each of the cultured isolates. If lesions develop and typical symptoms of infection observe, then re-isolation of a particular strain from those lesions would be further confirmation of its pathogenicity.

Exploring the origin of an infection is crucial to the development of effective methods for disease control. Antibiotic therapy could be effective in an emergency to decrease the loss of capital after observation of disease symptoms in commercial aquaculture facilities. Although, use of such inhibitory agents in aquaculture is not encouraged. Estimation of the necessity of an antibiotic, duration of application and the MIC are essential knowledge to reduce the risk of development of pathogenic bacteria to antibiotic resistance.

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