MEDWELL PUBLISHING
Research Journal of Biological Sciences 4 (4): 482-486, 2009
ISSN: 1815-8846

Novel Antibacterial Activity of Peptide Gene Extracted from Malaysian Sea Cucumber

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Abstract: The emergence and spread of antimicrobial resistance in bacteria trigger the discovery of new antibiotics to be ushered in the new age of medicine. Diseases caused by gram positive bacteria become more deadly as the bacteria acquire new virulence factors, develop resistance to modern drug therapy, thus requiring new sources of antimicrobial agents. The sea represents the most promising source of medicinal and natural products of the future. Echinoderms are benthic organism, constantly exposed to high concentration of bacteria, viruses and fungi. Their survival depends on antimicrobial mechanism to protect themselves against microbial infection. In the search for antimicrobial peptide genes, utilization of molecular technique and antimicrobial activity bioassay of a species of sea cucumbers not commercially exploited from Beting Darat, Johore, were carried out. The gene of an antimicrobial peptide that acts by permeabilizing the membrane of bacterial pathogens were amplified at position 222 bp from one species of sea cucumber. The identity of the gene was confirmed by commercial sequencing and similarity, to published gene was determined through BLAST analysis. Four species of sea cucumbers and one species of tunicate were screened for antimicrobial activity using moist disc diffusion technique. Out of 5 samples, the methanol extract of sea cucumber give inhibition zones to 2 strains of *Staphylococcus aureus* and a strain of *Streptococcus pyogenes*. The utility of the sea cucumber not commercially exploited as biomedicinal resources offers an alternative horizon in drug discovery programmed from marine resources. Further, investigations on the importance of the antimicrobial peptide presence in the sea cucumber will be elucidated.

Key words: Methicillin resistant *Staphylococcus aureus*, nosocomial infections, sea cucumber, polymerase chain reaction, disc diffusion, minimum inhibitory concentrations, Malaysia

INTRODUCTION

Sea cucumber or Holothurian belongs to phylum of echinodermata. There are 5 classes of living echinoderms: Crinoidea (sea lilies), Asteroidea (startfish), Ophiurodea (brittle star or snake stars), Echinoidea (sea urchins and san dollars) and Holothuroidea (sea cucumber). The cured products termed beche-de-mer (meaning seaworm), trepang (meaning sea slug) and in Chinese it is called hoi-sum. Sea cucumbers are believed to have some aphrodisiac qualities as well as curing effects on variety of elements. In traditional, Malay medicine, air gamat is used in post-natal treatment and minyak gamat is used for healing open wounds. According to Ridzwan *et al*. (1995), the current research focus on sea cucumber in Malaysia are based on studies, which include identification of species, their distributions, nutrition evolution, kinetic properties of crude lactate dehydrogenase and medicinal value. These studies indicate that there were great potential of sea cucumbers and this finding were needed in the next major commitment to develop selected lines in drugs discovery for treatment of multiple resistant *Staphylococcus aureus*. Diseases caused by *S. aureus* become more deadly as the bacteria acquire new virulence factors; develop resistance to modern drug therapy, thus requiring new sources of antimicrobial agents. Clinicians are continually being challenged by infection of *S. aureus*, a major caused of community acquired and health care associated infections around the world. *S. aureus* infection is a major cause of skin, soft tissues, respiratory, bone, joint and endovascular disorders. These variety of infections due to *S. aureus* cause bacterimia, endocarditis, sepsis, metastatic infection, furunculasis and toxic shock syndrome showed high virulencity of this pathogen.

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S. aureus is one of the most common pathogens in nosocomial and prosthetic valve endocarditis, the mortality rate of this disease is higher then 56%. *S. aureus* endocarditis is characterized by high fever, frequent involvement of normal cardiac valve and the absence of physical stigmata (Franklin, 1998). Approximately 20% of community acquired and nosocomial bacteremias in the United States are cause by *S. aureus* (Tenover *et al*., 2001). The mortality rate of staphylococcal bacterimia range from 11-43%, while the frequency of complication of staphylococcal bacterimia also high until 53% to get endocarditis, metastatic infection or others. The emergence of *Staphylococcus aureus* resistance to antibiotics has become a serious problem in hospitals worldwide (Gemmel *et al*., 2006). Methicillin resistance first appeared among nosocomial isolates of *S. aureus* in 1961, since that Methicillin Resistant *S. aureus* (MRSA) has become wide spread in hospitals and intensive care units around the world (Huang *et al*., 2006). Centers for Diseases Control and Prevention's (CDC) National Nosocomial Infections Surveillance (NNIS) System reported among the patients in intensive care units, the proportion of *S. aureus* nosocomial infection that were MRSA surpass 50% in 1999 (Fridkin *et al*., 2003). MRSA is now one of the most common causes of bacterial nosocomial infections, accounting for 40-70% of *S. aureus* infections in intensive care units (Zetola *et al*., 2005). The emergence of high levels of penicillin resistance followed by the development and spread of strain resistant to the semi synthetic penicillin (methicillin, nafcillin and oxacilin), macrolides, tetracyclines and aminoglycoside has made therapy of staphylococcal diseases a global challenge. Another potential fatal infection caused by a gram positive pathogen is the acute *Streptococcus pyogenes* infections, mildly presents as pharyngitis, scarlet fever, impetigo or cellulites but recently results in increase in variety and severity and describe, leading to facial necrosis resembling flesh eaten infection.

MATERIALS AND METHODS

Sample collection: Live specimens of sea cucumber (5 species) and tunicate (1 species) were collected from Beting Darat, Gelang Patah, Johore. The animals were kept in an aerated sea water tank until arrived to the laboratory. The animals were cut into pieces and wash thoroughly with sterile distilled water and kept at -20°C until organic extraction. For genomic extraction, samples were cut in to small pieces and preserved in TNEAS buffer.

Genomic extraction and organic extraction: Genomic extraction was carried out using Master Pure™ Complete DNA and RNA Purification Kit, following Tissue Samples and Precipitation of Total DNA protocols. The purity and concentration of genomic DNA were measured using a spectrophotometer. Preparations of organic extracts using methanol and Phosphate Buffer Saline (PBS) solvent were carried out using 25 g wet weight of sea cucumber in 50 mL of solvent. The samples were blended in the solvent, stirred for 48 h and kept at 4°C until further used.

Molecular screening: Molecular screenings were carried out by Polymerase Chain Reaction (PCR) amplification using primers specifically designed for the specific antimicrobial gene of interest. Primers were design using Primer Premium 5 software based on region of interest. The reaction mixture volume of 25 μL contained 1X BST buffer (Biosyntech Inc.), 1.8 mM $MgCl_2$, 200 μm dNTPs, 20 pmole of reverse primer and forward primer, 1 U taq polymerase. The PCR programs were, initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 5 sec. annealing temperature depended on the particular primer for 1 min, extension at 72°C for 2 min and final extension at 72°C for 3 min.

Antibacterial activity testing: Antibacterial activity determination for each extract was performed using moist disc diffusion technique, whereby discs of 6 mm diameter were made from Whatman No.1 filter paper using paper borer and discs were then placed in a labeled McCartney bottle. The bottle was then sterilized by autoclaving at 121°C for 15 min. Extract at predetermined dilution for the test was then filled in sterile beaker with the autoclave discs and left for a few minutes. The immersed discs in the beaker was then picked up using forceps and suspended until no sign of the extract dripping. The moist discs were then placed on the surface of bacterial lawn previously spread on the agar in the petri dishes. The plates were then incubated and the zone of inhibition was observed after 24-48 h. Discs immersed in the solvent of the extract were also placed on the bacterial lawn as negative control (Table 1). Commercially available, antibiotic discs of known positive inhibitory activity were used as positive control. The Minimum Inhibitory Concentrations (MIC) of the extracts against bacterial growth were determined using the serial dilution method. Total 900 μL of freshly prepared Mueller Hinton broth was placed in 2 mL of eppendorf tube (Table 2). An aliquot of 100 μL of bacterial suspension at a concentration of 0.5 Mc Farland were pipette into the first tube, followed by 1.0 mL of the respective extracts and mixtures were re suspended accordingly. Then 1 mL of mixture were taken out and re suspend in another tube contain the same mixture and repeat 10 times.

Table 1: Disc diffusion and standard antimicrobial susceptibility test results for gram-positive and gram-negative microorganism

	Methanol extracts			PBS extracts			Positive control
Microorganism	Clear zone	Partial inhibition	No inhibition zone	Clear zone	Partial inhibition	No inhibition zone	(Have clear inhibition zone)
S. aureus (STR 5)	√					√	Vancomycin 30 μg
S. aureus (STR 9)	√					√	Vancomycin 30 μg
S. aureus (N8)	√					√	Vancomycin 30 μg
S. aureus (20)	√					√	Vancomycin 30 μg
Streptococcus pyogenes (sp 168)	√					√	Penicillin 10 μg
Streptococcus pyogenes (sp 246)	√					√	Penicillin 10 μg
Escherichia coli (6)		√				√	Cefuroxime 10 μg
Escherichia coli (9)		√				√	Cefuroxime 10 μg
Pseudomonas aeruginosa			√			√	Imepenem 10 μg

Table 2: Minimum Inhibitory Concentrations (MIC) of the extracts against gram-positive bacterial growth

Microorganism	MIC 70% MeOH SC3	MIC 70% MeOH SC5
Staphylococcus aureus (STR9)	125 mg mL^{-1}	125 mg mL^{-1}
Staphylococcus aureus (N8)	250 mg mL^{-1}	125 mg mL^{-1}
Streptococcus pyogens (sp 168)	125 mg mL^{-1}	125 mg mL^{-1}

RESULTS AND DISCUSSION

Nosocomial infections are an important source of morbidity and mortality in hospital settings, afflicting an estimated 2 million patients in United States each year. This number represents approximately 5% of hospitalized patients and results in an estimated 88,000 deaths and 4.5 billion dollars in excess health care costs (Chang and Chui, 1998; McGowan *et al*., 2001). Although, viruses, fungi and parasites are recognized as sources of nosocomial infections, bacterial agents remain the most commonly recognized cause of hospital-acquired infections (Haley *et al*., 1985). Increasingly, hospital-acquired infections with multidrug-resistant pathogens represent a major problem in patients. Several risk factors for acquiring an infection have been commonly cited, including the presence of underlying conditions (such as diabetes, renal failure, or malignancies), long hospitalizations, surgical procedures, receipt of prior antimicrobial therapy and the presence of indwelling catheters. Major antimicrobial resistance problems are typically associated with gram-positive nosocomial pathogens, which include glycopeptide (vancomycin)-resistant enterococci (Vergis *et al*., 2001), Methicillin-Resistant *Staphylococcus aureus* (MRSA) and more recently, glycopeptide-intermediate and resistant *S. aureus* (Rodley *et al*., 1995). Among the gram-negative bacilli, extended-spectrum-beta-lactamase-producing strains of *Escherichia coli* and *Klebsiella pneumoniae* and fluoroquinolone-resistant strains of *Pseudomonas aeruginosa* and *E. coli* have been the primary concerns (McNeil *et al*., 2001). Understanding pathogen distribution and relatedness is essential for determining the epidemiology of nosocomial infections and aiding in the design of rational pathogen control methods. The role of pathogen typing is to determine if epidemiologically related isolates are also genetically related. Historically, this analysis of nosocomial pathogens has relied on a comparison of phenotypic characteristics such as biotypes, serotypes, bacteriophage or bacteriocin types and antimicrobial susceptibility profiles.

This approach has begun to change over the past 2 decades, with the development and implementation of new technologies based on DNA, or molecular, analysis. These DNA-based molecular methodologies, which will be examined extensively in this study, include Pulsed Field Gel Electrophoresis (PFGE) and other restriction-based methods, plasmid analysis and PCR-based typing methods. The incorporation of molecular methods for typing of nosocomial pathogens has assisted in efforts to obtain a more fundamental assessment of strain interrelationship (Cockerill and Smith, 2004; Emori and Gaynes, 1993). Establishing clonality of pathogens can aid in the identification of the source (environmental or personnel) of organisms, distinguish infectious from noninfectious strains and distinguish relapse from reinfection. Many of the species that are key hospital-acquired causes of infection are also, common commensal organisms and therefore, it is important to be able to determine whether the isolate recovered from the patient is a pathogenic strain that caused the infection or a commensal contaminant that likely is not the source of the infection. Likewise, it is important to know whether a second infection in a patient is due to reinfection by a strain distinct from that causing the initial infection or whether the infection is likely a relapse of the original infection. If the infection is due to relapse, this may be an indication that the initial treatment regimen was not effective and alternative therapy may be required (Singh *et al*., 2006).

In this study, of screening for antimicrobial activity using disc diffusion test on gram positive and gram negative bacteria, bacteria that are commonly found, pathogenic and can develop resistant to antibiotic, were used studied. The discs used were moist disc absorption technique that allows the absorption of active substances

Fig. 1: Alignment between sequencing result of permeabilization gene size 222 bp (seq1) and clavaspirin gene

and prevent formation of complexes during absorption through the agar. Two methods of extraction were utilized, methanol extraction and Phosphate Buffer Saline (PBS). The aim was to find the most suitable and effective extraction method to yield active antibacterial substances. The differences in methods used were in obtaining water soluble or hydrophobic fraction. Antibacterial activity was only found using methanol solvent, whereby inhibition in the growth of gram positive pathogens, *Staphylococcus aureus* and *Streptococcus pyogenes*, were observed. The identity of the active compound in the extract cannot be confirmed in current report. However, potential antimicrobial agent in tissue of sea cucumber was investigated by molecular screening. The study successfully designed a primer pair for amplifying an antimicrobial peptide gene encoding for membrane permeablization as evidenced from the amplification of the gene at the expected size of 222 bp (Fig. 1). Commercialized sequencing further confirmed the identity of the gene after Blast analysis with the published gene in the public domain. The memebrane permeablization gene primers were design from tunicates but could amplify the gene from tissue of sea cucumber 5. Alignment between the sequenced product and the permeabilize gene from tunicates showed minor changes in sequenced product due to different species.

REFERENCES

Chang, N. and L. Chui, 1998. A standardized protocol for the rapid preparation of bacterial DNA for pulsed-field gel electrophoresis. Diagn. Microbiol. Infect. Dis., 31: 275-279. DOI: 10.1016/S0732-8893(98)00007-8. PMID: 9597387.

Cockerill, F.R. and T.F. Smith, 2004. Response of the clinical microbiology laboratory to emerging (new) and reemerging infectious diseases. J. Clin. Microbiol., 42: 2359-2365. PMID: 15184405.

Emori, T.G. and R.P. Gaynes, 1993. An overview of nosocomial infections, including the role of the microbiology laboratory. Clin. Microbiol. Rev., 6: 428-442. PMID: 8269394.

Franklin, L.D., 1998. *Staphylococcus aureus* infections. N. Engl. J. Med., 339: 520-532. PMID: 9709046.

Fridkin, S.K., L.K. McDougal and J. Mohammed *et al.*, 2003. Epidemiological and microbiological characterization of infections caused by *Staphylococcus aureus* with reduced susceptibility to vancomycin United States 1997-2001. Clin. Infect. Dis., 36: 429-439. PMID: 12567300.

Gemmell, C.G., D.I. Edwards and A.P. Fraise *et al.*, 2006. Guidelines for the prophylaxis and treatment of Methicillin-Resistant *Staphylococcus aureus* (MRSA) infections in the UK. J. Antimicrob. Chemother., 57: 589-608. PMID: 16507559.

Haley, R.W., D.H. Culver, J.W. White, W.M. Morgan and T.G. Emori, 1985. The nationwide nosocomial infection rate: A new need for vital statistics. Am. J. Epidemiol., 121: 159-165. PMID: 4014113.

Huang, H., N.M. Flynn, J.H. King, C. Monchaud, M. Morita and S.H. Cohen, 2006. Comparisons of community-associated Methicillin-Resistant *Staphylococcus aureus* (MRSA) and hospital-associated MSRA infections in Sacramento, California. J. Clin. Microbiol., 44: 2423-2427. PMID: 16825359.

McGowan, J.E. *et al.*, 2001. Economic impact of antimicrobial resistance. Emerg. Infect. Dis. 7: 286-292. PMID: 11294725.

McNeil, S.A., L. Nordstrom-Lerner, P.N. Malani, M. Zervos and C.A. Kauffman, 2001. Outbreak of sternal surgical site infections due to *Pseudomonas aeruginosa* traced to a scrub nurse with onychomycosis. Clin. Infect. Dis., 33: 317-323. PMID: 11438896.

Ridzwan, B.H., M.A. Kaswandi, Y. Azman and M. Fuad, 1995. Screening for antibacterial agents in 3 species of sea cucumber from coastal areas of sabah. Gen. Pharmac., 26: 1539-1543. PMID: 8690242.

Rodley, P.D., U. Romling and B. Tummler, 1995. A physical genome map of the *Burkholderia cepacia* type strain. Mol. Microbiol., 17: 57. PMID: 7476209.

Singh, A., R.V. Goering, S. Simjee, S.L. Foley and M.J. Zervos, 2006. Application of molecular techniques to the study of hospital infection. Clin. Microbiol. Rev., 19: 512-530. PMID: 16847083.

Tenover, F.C., J.W. Biddle and M.V. Lancaster, 2001. Increasing resistance to vancomycin and other glycopeptides in *Staphylococcus aureus*. Emerg. Infect. Dis., 7: 327-332. PMID: 11294734.

Vergis, E.N., M.K. Hayden, J.W. Chow, D.R. Snydman, M.J. Zervos, P.K. Linden, M.M. Wagener, B. Schmitt and R.R. Muder, 2001. Determinants of vancomycin resistance and mortality rates in enterococcal bacteremia. Ann. Int. Med., 135: 484-492. PMID: 11578151.

Zetola, N., J.S. Francis, E.L. Nuermberger and W.R. Bishai, 2005. Community-acquired methicillin-resistant *Staphylococcus aureus*: an emerging threat. Lancet Infect. Dis., 5: 275-86. PMID: 15854883.