HAEMATOLOGY AND ANTIBACTERIAL PROPERTIES OF BLOOD FROM HORSESHOE CRABS (*Carcinoscorpius rotundicauda* AND *Tachypleus gigas*)

By

SHAHRAM SHAKIBA ZADEH

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

November 2006

DEDICATION

To my beloved family

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

HAEMATOLOGY AND ANTIBACTERIAL PROPERTIES OF BLOOD FROM HORSESHOE CRABS (*Carcinoscorpius rotundicauda* AND *Tachypleus gigas*)

By

SHAHRAM SHAKIBA ZADEH

November 2006

Chairman : Annie Christianus, PhD

Faculty : Agriculture

Samples of adult *Carcinoscorpius rotundicauda* and *Tachypleus gigas* caught from Pulau Lumut, Selangor, were conditioned in the Hatchery Unit, Universiti Putra Malaysia and used for all the experiments.

Haematological study of the *C. rotundicauda* and *T. gigas* was carried out by blood extraction through cardiac puncture. Light microscopy of blood from both species showed the existence of only one type of blood cell, granulocyte, circulating in their circulatory system. Blood cell count for *C. rotundicauda* and *T. gigas* were not significantly different (27,000 and 33,000 cells/mm^3, respectively), but it was otherwise for the two sexes of C*. rotundicauda.* Blood cell measurements of both species revealed that the size of *T. gigas* blood cells were significantly larger than *C. rotundicauda*. Transmission electron microscopy of

blood cells was carried out for normal and clotted blood of both species. Granulocytes contained of all the necessary organelles: a nucleus, Golgi apparatus, mitochondria, endoplasmic reticulum, ribosome particles, vacuoles, and microtubles. Moreover two types of granules filled the cytoplasmic space, they were large and small granules. Intermediate stages of granules were observed in the cytoplasmic region especially near the Golgi apparatus. Light microscope photography of the degranulation process of granulocytes with one minute interval in both species exhibited similar pattern. Blood cells under un-sterile condition released their granules which are the main source of the immunologic material. Vacuoles with different sizes then filled the cytoplasmic space and at the same time cytoplasmic projections were formed.

Antibacterial effect of horseshoe crabs (*C. rotundicauda* and *T. gigas*) fresh blood against Gram negative (*E. coli* and *V. parahaemolyticus*) and Gram positive bacteria (*S. aureus*, *B. subtilis* and *B. cereus)* were examined. Freshly extracted blood from both species was observed to inhibit the growth of *E. coli*, *V. parahaemolyticus* and *B. cereus*, while partial inhibition for *S. aureus.* On the other hand, *B. subtilis* growth was inhibited by *C. rotunicauda* fresh blood but only partial inhibition by *T. gigas* blood. These results showed that granular blood cells contained antibacterial compounds.

Total RNA was extracted from the blood cells of these two species and complementary DNA was synthesized from it. Amplification of target antibacterial

genes were carried out by using cDNA as template for both species. The purified PCR amplified DNA fragments of tachyplesin and tachycitin of *C. rotundicauda* and *T. gigas* were sequenced. The similarity of the putative genes of both species were analyzed and compared with the Genbank database using the BLAST program of the National Center for Biotechnology Information (NCBI). The amplified PCR fragments from *C. rotundicauda* and *T. gigas* analyzed by the program indicate 92% and 95% similarity to the tachycitin gene of *T. tridentatus*, respectively. While the similarity of the amplified PCR product from the BLAST program of NCBI for *C. rotundicauda* and *T. gigas* exhibit 91% and 93% similarity to the tachyplesin gene of *T. tridentatus*, respectively.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

HAEMATOLOGI DAN KANDUNGAN ANTIBACTERIA DARAH BELANGKAS (*Carcinoscorpius rotundicauda* DAN *Tachypleus gigas*)

Oleh

SHAHRAM SHAKIBA ZADEH

November 2006

Pengerusi : Annie Christianus, PhD

Fakulti : Pertanian

Sampel dewasa *Carcinoscorpius rotundicauda* dan *Tachypleus gigas* dari Pulau Lumut, Selangor, disesuaikan di Unit Penetasan Ikan, Universiti Putra Malaysia dan digunakan untuk semua eksperimen.

Histologi sel dare *C. rotundicauda* dan *T. gigas* dijalankan dengan pengekstrakan darah melalui tebukan kardiak. Mikroskopi cahaya kedua spesies menunjukkan kehadiran satu jenis sel darah sahaja, granulosit, mengalir didalam system peredaran darah. Pengiraan sel darah untuk *C. rotundicauda* dan *T. gigas* tidak menunjukkan perbezaan yang sinifikan (masing-masingnya 27,000 dan 33,000). Tetapi sebaliknya untuk kedua-dua jantina C*. rotundicauda.* Pengukuran sel darah kedua-dua spesies menunjukkan bahawa saiz sel darah *T. gigas* adalah secara sinifikannya adalah lebih besar daripada *C. rotundicauda*. Mikroskopi Transmisi Elektron sel darah dijalankan ke atas darah normal dan

beku kedua-dua spesies. Granulosit terdiri dari semua organel perlu, nukleus, aparatus Golgi, mitokondria, retikulum endoplasmik, partikel ribosom, vakuol dan mikrotubul. Dua jenis granul memenuhi ruang saitoplasmik adalah granul besar dan kecil. Granul peringkat perantara didapati di bahagian saitoplasmik terutamanya berdekatan dengan aparatus Golgi. Fotografi dengan mikroskop cahaya proses degranulasi granulosit dengan interval satu minit pada kedua-dua spesies menunjukkan corak yang serupa. Sel darah pada keadaan tidak steril akan membebaskan granul yang merupakan sumber utama bahan-bahan immunologik. Vakuol dengan saiz berlainan kemudiannya akan memenuhi ruang saitoplasmik dan pada masa yang sama unjuran saitoplasmik terbentuk.

Kesan antibakteria darah segar belangkas (*C. rotundicauda* dan *T. gigas*) ke atas bakteria gram-negatif (*E. coli* dan *V. parahaemolyticus*) dan gram-positif (*S. aureus*, *B. subtilis* dan *B. cereus)* telah dikaji. Darah segar dari kedua-dua spesies didapati menghalang pertumbuhan *E. coli*, *V. parahaemolyticus* dan *B. cereus*, sementara menghalang separa pertumbuhan pada *S. aureus.* Sebaliknya pertumbuhan *B. subtilis* dihalang oleh darah segar dari *C. rotunicauda* tetapi hanya menghalang separa pertumbuhan bagi darah *T. gigas*. Keputusan ini menunjukkan bahawa sel granular darah mengandungi bahan-bahan antibakteria.

Jumlah RNA diekstrak dari sel darah kedua-dua spesies ini dan DNA komplimentari disintesiskan. Amplifikasi gen-gen antibakteria yang dikehendaki dijalankan dengan menggunakan cDNA sebagai templat untuk kedua-dua

spesies. Fragmen DNA amplifikasi PCR yang ditulinkan untuk tachyplesin dan tachycitin *C. rotundicauda* dan *T. gigas* telah dibuat turutannya. Homologi gen putatif kedua-dua spesies dianalisis and dibandingkan dengan pengkalan Genbank menggunakan program BLAST dari Pusat Maklumat Bioteknologi Kebangsaan (NCBI). Fragmen PCR yang diamplifikasikan dari *C. rotundicauda* dan *T. gigas* dianalisis dengan program menunjukkan homologi sebanyak 92% dan 95% masing-masingnya dengan gen tachycitin dari *T. Tridentatus*. Manakala hasil PCR yang diamplifikasikan dari program BLAST program di NCBI untuk *C. rotundicauda* dan *T. gigas* menunjukkan homologi sebanyak 91% dan 93% masing-masingya untuk gen tachyplesin dari *T. tridentatus.*

AKNOWLEDGEMENTS

My full praise to our God for enabling me to complete my study.

I would like to express my sincere appreciation to my supervisor and chair person of the supervisory committee, Dr. Annie Christianus, who was the main scientific guide and her encouragements and trust lead me to finish my study. My grate thanks to her because of friendly environment and support during my study period.

I gratefully acknowledge my supervisory committee members, Associate Professor Dr. Mariana Nor Shamsudin, Associate Professor Dr. Mohd Salleh Kamarudin and Associate Professor Dr. Che Roos Saad, for their valuable contribution and guidance during this study.

I deeply thank to the government of Malaysia for funding this project through the Intensified Research on priority Areas (IRPA) grant no. 01-02-04-0864 EA001.

My special gratitude to Associate Professor Dr. Mariana Nor Shamsudin and Associate Professor Dr. Mohad Salleh Kamarudin who provided me with an opportunity to use their well-equipped laboratories, Professor Dato' Dr. Shariff Mohamad Din and Associate Professor Dr. Hassan Mohd Daud for their histological facilities and guidance (Faculty of Veterinary Medicine), Dr. Fuziah and her staff, Ms. Azilah and Mr. Ho in Electron Microscopy unit (Institute Bio Science), Mr. Jasni of UPM Hatchery unit for his help during my field

experiments, associate Professor Dr. Anuar Abd Rahim, Dr. Osmanu Haruna Ahmad and Dr. Mohamada Boyie Jalloh (Department of Soil Science) for their valuable guidance in statistical analysis of my study.

My thanks also goes to staffs, Mr. Zinan, Ms. Susana, Dr. Neela and students, Naje, Lai, Farah, Hana, and Yaya, who were always helpful during my lab works in Microbiology lab (Faculty of Medicine and Health Science).

My deepest indebtedness to my dear friends specially Dr. Sasan, Dr. Mohamadi and Dr. Haghighi for their friendly help and support.

I also appreciate and acknowledge my dear colleague and friend Ms. Parvaneh for her helpful scientific information, suggestions and responsibility feeling toward my family and me during my study.

I am very much grateful to my uncle, Dr. Mohamad Zaman Khodaey for his continuous support and true love during my life and study.

I deeply appreciate my dear family, my brother, my uncle, my wife and specially my mother for their love and patience.

I certify that an Examination Committee met on 9th Nov. 2006 to conduct the final examination of Shahram Shakiba Zadeh on his Master of Science thesis entitled "Haematology and Antibacterial Properties of Blood from Horseshoe Crabs (*Carcinoscorpius rotundicauda* and *Tachypleus gigas*)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Abd. Razak Alimon, PhD
Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Son Rado, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

Hassan Hj Mohad Daud, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Wan Kiew Lian, PhD
Associate Professor
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
(Independent Examiner)

HASANAH MOHD GHAZALI, PhD
Professor / Deputy Dean
School of Graduate Studies
Universiti Pura Malaysia

Date: **21 DECEMBER 2006**

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

Annie Christianus, PhD
Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Mariana Nor Shamsudin, PhD
Associate Professor
Faculty of Medicine and Health science
Universiti Putra Malaysia
(Member)

Mohd Salleh Kamarudin, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Che Roos Saad, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

AINI IDERIS, PhD
Professor / Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 16 JANUARY 2007

DECLARATION

I hereby declare that this thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

_____

SHAHRAM SHAKIBA ZADEH

Date: 20 DECEMBER 2006

TABLE OF CONTENT