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PRODUCTION OF HEPATITIS B VIRAL ANTIGENS AND ANTIBODIES USING PHAGE DISPLAY TECHNOLOGY FOR THE DEVELOPMENT OF A DIAGNOSTIC TEST

TAN GEOK HUN.

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TAN GEOK HUN

DOCTOR OF PHILOSOPHY
UNIVERSITI PUTRA MALAYSIA

2006

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By

TAN GEOK HUN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

April 2006

DEDICATION

I dedicate this work to my beloved mother Goh Beng Eng, darling husband Chau Sawang, brothers (Tan Kee Song, Tan Ke Chuan) and sisters (Tan Geok Huwai, Tan Hui Geok) for their love, support and encouragement during my study.

Thank you so much.......

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

PRODUCTION OF HEPATITIS B VIRAL ANTIGENS AND ANTIBODIES USING PHAGE DISPLAY TECHNOLOGY FOR THE DEVELOPMENT OF A DIAGNOSTIC TEST

By

TAN GEOK HUN

April 2006

Chairman : Associate Professor Tan Wen Siang, PhD

Faculty : Biotechnology and Biomolecular Sciences

Hepatitis B is one of the most common infectious diseases in the world. It is caused by the hepatitis B virus (HBV) which is estimated to infect more than one third of the world's population and there are about 400 million carriers of HBV worldwide. The infection can now be prevented through immunization with a vaccine based on the surface antigen (HBsAg) produced in yeast by genetic engineering. In order to provide additional means to control the disease, rapid, easier and cheaper diagnostic assay have been developed using phage display technology in these studies.

From the present studies, bacteriophage T7 was employed to display the immunodominant region of S-HBsAg, amino acid residues 111-156, on the exterior of the phage particles. The expressed immunodominant region of S-HBsAg remained antigenic and it was displayed on the coat protein of the recombinant phage particle, T7-HBsAg$_{111-156}$, which has the potential to be used as an

immunological reagent for the detection of anti-HBsAg antibody in human serum samples at as low as 0.25 mIU/ml. In addition, the fusion phage also applied on dot-blot for detection of anti-HBsAg antibody. However, the sensitivity of this assay is low as compared to ELISA method.

A phage heptapeptide random library was used to identify peptide ligands that interact with HBsAg. From the third round of panning, 75% of phages screened carried the peptide sequence C-ETGAKPH-C which is the most frequently identified phage clones in this round. The phage clone was characterized and a cyclic synthetic peptide bearing the identical peptide sequence was synthesized. The phage was able to compete with anti-HBsAg monoclonal antibody as well as the synthetic peptide for the binding site on HBsAg. The optimum pH and temperature for phage binding was around 4 to 8 and 4°C, respectively. An equilibrium binding assay in solution showed that the phage binds tightly to HBsAg with a relative dissociation constant (K_d^{rel}) of 2.9 ± 0.9 nM, illustrating that the phage bearing ETGAKPH has the potential to be used as a diagnostic reagent for detecting HBsAg in human sera.

As a preliminary effort to study the detection of HBcAg in the serum samples, single chain variable fragment (scfv) of anti-HBcAg antibody library was constructed by fusion to gpIII protein of bacteriophage M13, which allows for the display of the fusion protein (scfv) at the tip of the filament. Truncated HBcAg was inoculated into female Balb/C mice before the antibody and spleen cells were harvested for the construction of library. Multiple reactions of PCR were carried out,

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and the size of the antibody library was 2.18 x 10^7 cfu/ml. This library was further panned against HBcAg to get the specific phage clone that interacts with HBcAg. The phage clone with higher absorbance value was further rescued by helper phage M13K07, and the phagemid was digested to determine the insert of scfv. In this study, a phage-ELISA assay was established and the minimum amount of HBcAg that can be detected was about 10 ng with 1.0 x 10^{12} pfu/ml of purified fusion phage. This fusion phage scfv showed a promising result for the detection of HBcAg in the human treated serum samples.

In conclusion, development of phage-ELISA for the detection of anti-HBsAg antibody, HBsAg and HBcAg based on phage display can be an alternative choice to reduce the cost of detection kits. This study also provides a model in the development of diagnostic test for the detection of other biological samples based upon phage display technology.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGHASILAN ANTIGEN DAN ANTIBODI VIRUS HEPATITIS B DENGAN MENGGUNAKAN TEKNOLOGI PAMERAN FAJ UNTUK PERKEMBANGAN UJIAN DIAGNOSIS

Oleh

TAN GEOK HUN

April 2006

Pengerusi : Profesor Madya Tan Wen Siang, PhD

Fakulti : Bioteknologi dan Sains Biomolekular

Hepatitis B merupakan salah satu penyakit berjangkit merbahaya di dunia. Penyakit tersebut yang disebabkan oleh virus hepatitis B (HBV) dianggarkan telah menjangkiti lebih daripada satu per tiga daripada penduduk dunia dan lebih 400 juta penduduk dunia dikategorikan sebagai pembawa. Pada masa kini, jangkitan daripada virus HBV boleh dielakkan dengan menggunakan vaksin berdasarkan antigen permukaan virus hepatitis B (HBsAg) yang dibentuk daripada kejuruteraan genetik yis. Untuk mengawal penularan penyakit ini, satu teknik diagnosis yang cepat, senang dan murah telah dibangunkan berdasarkan kepada teknologi pameran faj.

Dalam kajian ini, bacteria faj telah dieksplotasikan bagi mempamerkan bahagian immunodominan antigen permukaan virus pada bahagian luar faj. Bahagian immunodominan antigen permukaan virus ini kekal menjadi antigenik dan

dipersembahkan pada bahagian permukaan protein faj rekombinan T7-$HBsAg_{111-156}$ yang berfungsi untuk mengesan antibody anti-HBsAg yang terdapat di dalam sampel serum manusia sehingga 0.25 mIU/ml. Selain itu, faj ini juga digunakan dalam dot-blok asai. Namun demikian, tahap kepekaan asai ini adalah kurang kalau dibandingkan dengan cara ELISA.

Suatu perpustakaan faj digunakan untuk mengenalpasti ligan peptida yang boleh bertindak balas dengan HBsAg. Tujuh puluh lima peratus daripada jumlah faj yang disaring membawa jujukan asid amino C-ETGAKPH-C yang menunjukkan kebarangkalian paling tinggi dalam pusingan ketiga pemilihan berafiniti. Pencirian faj tersebut telah dilaksanakan dan peptida berkonformasi sintetik yang beridentikal dengan faj tersebut telah disintesiskan. Faj ini didapati mampu bersaing dengan antibodi monoklon anti-HBsAg dan peptida sintetik untuk tapak pengikatan pada HBsAg. pH dan suhu yang optima untuk pengikatan faj masing-masing ialah sekitar 4-8 dan 4°C. Esai keseimbangan pengikatan dalam cecair menunjukkan faj tersebut mengikat dengan kuat kepada HBsAg dengan pemalar penceraian relative (K_d^{rel}) iaitu 2.9 ± 0.9 nM. Ini menggambarkan bahawa faj pembawa ETGAKPH berpotensi sebagai reagen diagnostik bagi pengesanan HBsAg dalam serum manusia.

Sebagai permulaan untuk kajian mengesan HBcAg dalam sampel darah, penghasilan perpustakaan antibodi anti-HBcAg yang mengandungi scfv terikat protein gpIII pada faj M13 telah dijalankan. Mencit betina Balb/C diinokulasikan dengan HBcAg sebelum limpanya dikeluarkan dan digunakan untuk penyediaan

perpustakaan tersebut. Pelbagai tindak balas PCR telah dijalankan dan saiz perpustakaan antibodi tersebut dianggarkan sebanyak 2.18 x 10^7 cfu/ml. Perpustakaan antibodi tersebut seterusnya dipilih melalui afinitinya terhadap HBcAg untuk mendapatkan faj yang bertindak balas secara spesifik dengan HBcAg. Faj klon yang menunjukkan penyerapan OD yang tinggi diselamatkan dengan faj pembantu M13KO7 dan fajmid diuraikan untuk menentukan kandungan scfv. Dalam kajian ini, faj ELISA sudah diperkenalkan dan minimum kuantiti HBcAg yang dapat dikesan oleh faj pada 1.0 x 10^{12} pfu/ml ialah sekitar 10 ng. Faj ini menunjukkan keputusan yang memberangsangkan dalam pengesanan HBcAg dalam sampel darah manusia.

Pengembangan esai faj untuk ELISA pengesanan antibodi anti-HBsAg, HBsAg dan HBcAg berdasarkan pameran faj berupaya berfungsi sebagai alternatif bagi mengurangkan kos pengesanan. Selain daripada itu, kajian ini juga menyediakan satu model bagi pengembangan diagnosis untuk pengesanan sample biologi lain berdasarkan teknologi pameran faj.

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I certify that Examination Committee has met on 3rd April 2006 to conduct the final examination of Tan Geok Hun on her Doctor of Philosophy thesis entitiled "Production of Hepatitis B Viral Antigens and Antibodies Using Phage Display Technology for the Development of A Diagnostic Test" in accordance with the 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:

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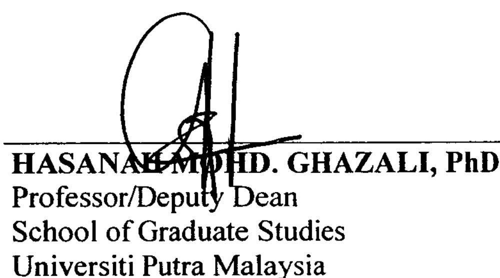

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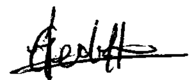
Date: 11 MAY 2006

DECLARATION

I hereby declare that the 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.



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Date: 13 April 2006.

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LIST OF ABBREVIATIONS

α	alpha
β	beta
κ	kappa
λ	lambda
° C	degree centrigrade
μg	microgram (10^{-6} g)
μl	microlitre (10^{-6} l)
μM	micromolar (10^{-6} M)
pmole	picomole
ALT	alanine aminotransferase
Amp	ampicillin
ATP	adenosine triphosphate
anti-HBsAg	HBsAg antibody
anti-HBcAg	HBcAg antibody
anti-HBeAg	HBeAg antibody
bp	basepair
BSA	bovine serum albumin
ccc	covalently closed circular
C-terminus	carboxy terminus
Carb	Carbenicillin
DHBV	duck hepatitis B virus
DNA	deoxy-ribonucleic acid

dNTP	deoxynucleoside triphosphate
DR	direct repeat
dsDNA	double-stranded DNA
DTT	1,4-dithiothreitol
DMF	dimethylformamide
ELISA	enzyme-linked immunoabsorbent assay
ER	endoplasmic reticulum
g	gram
GSHV	ground squirrel hepatitis virus
h	hour
HHBV	herons hepatitis B virus
HBcAg	hepatitis B core protein
HBsAg	hepatitis B surface antigen
HBeAg	hepatitis B e protein
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HIV	human immunodeficiency virus
HSV	herpes simplex virus
IPTA	isopropyl-β-d-thiogalactopyranoside
IgG	Immunoglobulin G
Kan	Kanamycin
kb	kilobase
K_d	dissociation constant
kDa	kilodalton

K_d^{rel}	relative dissociation constant
l	litre
IFN	interferon
LB	Luria broth
L-HBsAg	large surface antigen
M	molar
MHR	major hydrophilic region
mAb	monoclonal antibody
mg	milligram (10^{-3} g)
mIU/ml	miliinternational unit
M-HBsAg	medium surface antigen
MSC	multiple cloning site
min	minute
ml	millilitre (10^{-3} l)
mm	millimeter (10^{-3} m)
mRNA	messenger ribonucleic acid
NDV	Newcastle disease virus
nM	nanomolar (10^{-9} M)
N-terminus	amino terminus
OD	optical density
ORF	open reading frame
PAGE	polyaccrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol