CLONING AND IMMUNOLOGICAL CHARACTERIZATION OF RECOMBINANT *VIBRIO CHOLERAE* O-ANTIGEN TRANSPORT PROTEIN EXPRESSED IN *LACTOCOCCUS LACTIS*

HANA FARIZAH BINTI ZAMRI

FPSK(m) 2011 10
CLONING AND IMMUNOLOGICAL CHARACTERIZATION OF RECOMBINANT VIBRIO CHOLERAE O-ANTIGEN TRANSPORT PROTEIN EXPRESSED IN LACTOCOCCUS LACTIS

By

HANA FARIZAH BINTI ZAMRI

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

July 2011
CLONING AND IMMUNOLOGICAL CHARACTERIZATION OF
RECOMBINANT Vibrio cholerae O-ANTIGEN TRANSPORT PROTEIN
EXpressed IN Lactococcus lactis

By

HANA FARIZAH BINTI ZAMRI

July 2011

Chair: Mariana Nor Shamsudin, PhD
Faculty: Faculty of Medicine and Health Sciences

The food grade Lactococcus lactis is a potential vehicle for protein delivery via the oral route. This study used lactococcal strains as models for producing the wzIm gene that codes for the porin protein involved in transport of Vibrio cholerae lipopolysaccharide O-antigen. The 750 bp gene fragment was PCR-amplified from Vibrio cholerae O1 clinical isolates and cloned into the L. lactis nisin-controlled gene expression vector pNZ8048. The constructs were electrotransformed into L. lactis NZ9000 host strains where transcription of the gene on the RNA level was confirmed by reverse transcriptase PCR. Sequence comparison and multiple alignment of the translated cDNA nucleotides with that of known proteins reveal the presence of conserved structural domains of the ABC-2 membrane superfamily integral membrane protein component. Due to its hydrophobic
nature, whole cell \textit{L. lactis} protein extract was subjected to solubilisation with the detergents sodium dodecyl sulfate (SDS) and Triton X-100 before being separated on SDS-PAGE and analysed on Western blot. In the case of the current study, solubilisation using SDS was found to be more efficient when compared to Triton X-100 in retrieving the expressed \( \approx 34 \text{ kDa} \) \textit{wzm} porin as observed upon western blot analysis.

ELISA readings showed that oral administration of recombinant \textit{L. lactis} into New Zealand White rabbits elicited a statistically significant increase of both IgG and IgA levels \((P < 0.05)\) when compared to the control group given only the preparation buffer. Challenge study with virulent \textit{V. cholerae} O1 strains via the oral route evoked watery diarrhoea in rabbits given only the buffer throughout the immunization period, but fecal passing of both the recombinant and non-recombinant \textit{L. lactis} groups were normal. This indicates a positive effect of the Lactococcal cells itself, probably towards the intestinal microbiota, in protecting against the adverse effects of \textit{V. cholerae} and in evading diarrhoea. The diarrhoea lasted approximately two days in the control group, while the others were observed to be diarrhoea-free until the end of the study.

Bioinformatics and molecular methods have enabled prediction and detection of the \textit{wzm} protein product which, as shown in this study, possesses the potential to elicit antibody production and enhance immunity. Administration of the \textit{L. lactis} bacterium through the oral route was shown to increase mucosal immunity and assist in conferring protection against the diarrhoeal-causing disease cholera. These results provide more insight into the relatively unknown product of \textit{V. cholerae} \textit{wzm}, while providing a potential alternative
for health improvement against cholera that could be further developed for a safer, convenient, and effective method in protection and prevention of this disease.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGKOLONAN DAN PENCIRIAN KEIMUNAN PROTEIN PENGANGKUT O-ANTIGEN Vibrio cholerae REKOMBINAN YANG DIEKSPRES DALAM Lactococcus lactis

Oleh

HANA FARIZAH BINTI ZAMRI

Julai 2011

Pengerusi: Mariana Nor Shamsudin, PhD

Fakulti: Fakulti Perubatan dan Sains Kesihatan

Lactococcus lactis bergred makanan berpotensi untuk penghantaran protin secara oral. Kajian ini menggunakan stren-stren Lactococcus sebagai model untuk menghasilkan gen wzm yang mengkod untuk protin porin yang terlibat dalam pengangkutan O-antigen lipopolisakarida Vibrio cholerae. Cebisan gen 750 bp ini telah digandakan melalui PCR daripada isolat klinikal Vibrio cholerae O1 dan diklon ke dalam vektor pengekspres gen yang dikawal nisin, pNZ8048. Konstruk terhasil telah dielektrotransformasikan ke dalam stren hos L. lactis NZ9000 di mana transkripsi gen pada tahap RNA disahkan melalui PCR transkrip terbalik. Perbandingan jujukan serta penjajaran berbilang nukleotida cDNA yang diterjemah dengan protin-protin yang diketahui jujukannya menunjukkan kehadiran domain struktur terpelihara komponen protin membren integral dari keluarga membren ABC-2. Disebabkan sifat gerun airnya, ekstrak protin L. lactis telah dilarutkan dengan detergen sodium dodecyl sulfate (SDS) dan Triton X-100 sebelum diasingkan
melalui SDS-PAGE dan dianalisa dengan Western blot. Dalam kes kajian ini, larutan menggunakan SDS didapati lebih berkesan berbanding Triton X-100 dalam mendapatkan porin wzm \( \approx 34 \text{ kDa} \) yang diekspres seperti yang diperhatikan dari analisa Western blot.

Bacaan ELISA menunjukkan bahawa \( L. \text{lactis} \) rekombinan yang diberi secara oral kepada arnab-arnab New Zealand White menyebabkan kenaikan tahap IgG dan IgA yang ketara dari segi statistik \( (P < 0.05) \) bila dibandingkan dengan kumpulan kawalan yang hanya diberi buffer penyediaan tanpa bakteria. Ujikaji cabaran dilakukan dengan memberikan stren \( V. \text{cholerae} \) O1 secara oral menyebabkan cirit-birit berair kepada arnab-arnab yang hanya diberi buffer penyediaan sepanjang tempoh imunisasi, tetapi bahan buangan kedua-dua kumpulan yang diberi \( L. \text{lactis} \) biasa dan rekombinan adalah normal. Ini menandakan kesan positif sel-sel \( Lactococcus \), kemungkinan terhadap mikrobiota usus, dalam melindungi daripada kesan-kesan negatif \( V. \text{cholerae} \) serta mengelak daripada cirit-birit. Cirit-birit ini berlangsung selama dua hari dalam kumpulan kawalan yang diberi buffer, sementara yang lain didapati bebas dari jangkitan sehingga tamat kajian.

Bioinformatik serta pengkaedahan molekul telah membolehkan ramalan dan pengesanan produk protin wzm yang seperti ditunjukkan dalam kajian ini mempunyai potensi untuk mencetus penghasilan antibody dan meningkatkan tahap imun. Pemberian \( L. \text{lactis} \) secara oral telah menunjukkan peningkatan imun mukosa dan membantu dalam memberikan perlindungan terhadap penyakit kolera yang menyebabkan cirit-birit. Keputusan-keputusan kajian ini memberikan maklumat yang lebih mendalam berkaitan produk wzm \( V. \text{cholera} \) yang agak kurang dikenali, sekaligus menyediakan alternatif berpotensi bagi
peningkatan kesihatan terhadap kolera yang boleh diperkembangkan lagi untuk menghasilkan cara yang lebih selamat, mudah dan berkesan dalam melindungi dan mengelak dari penyakit ini.
ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to my supervisor, Associate Professor Dr. Mariana Nor Shamsudin, for her endless supply of guidance, time, and effort throughout my study. Words cannot describe how grateful I am, thanks so much! I am also indebted to Professor Dr. Raha Abdul Rahim for patiently co-supervising me, and for her always useful advice and ideas.

Many thanks also to my lab members, especially the following people, whose assistance and contributions helped me tremendously throughout the entire run of this research: Encik Zainan, Puan Farrah, Yaya, Ms. Hanim, Dr. Vasu, Kak Yan, Sabrina, Anu, Ms. Lai, Weng, and Dr. Neela. I will be forever grateful. A special mention also to Dr. Ehsanollah and Mr. Hamed for their generous time and technical assistance.

Last, but not least, my heartfelt appreciation to my husband and family for their love and support, endless encouragements, and for always believing in me. Alhamdulillah.
I certify that a Thesis Examination Committee has met on 6 July 2011 to conduct the final examination of Hana Farizah binti Zamri on her thesis entitled “Cloning and Immunological Characterization of Recombinant Vibrio cholerae O-antigen Transport Protein Expressed in Lactococcus lactis” in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the degree of Master of Science.

Members of the Thesis Examination Committee were as follows:

**Zulhairi Hj Amom, PhD**
Associate Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Chairman)

**Chong Pei Pei, PhD**
Associate Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Internal Examiner)

**Syahril Abdullah, PhD**
Lecturer  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Internal Examiner)

**Roohaida Othman, PhD**
Associate Professor  
Institute of Systems Biology  
Universiti Kebangsaan Malaysia  
(External Examiner)

____________________________

NORITAH OMAR, PhD  
Professor and Deputy Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date: 23 August 2011
The thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

**Mariana Nor Shamsudin, PhD**  
Associate Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Chairman)

**Raha Abdul Rahim, PhD**  
Professor  
Faculty of Biotechnology and Biomolecular Sciences  
Universiti Putra Malaysia  
(Member)

_______________________________  
HASANAH MOHD GHAZALI, PhD  
Professor and Dean  
School of Graduate Studies  
Universiti Putra Malaysia  

Date:
DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

_____________________
HANA FARIZAH ZAMRI

Date: 6 July 2011
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>INTRODUCTION</td>
</tr>
<tr>
<td>2</td>
<td>LITERATURE REVIEW</td>
</tr>
<tr>
<td>2.1</td>
<td>Vibrio cholerae</td>
</tr>
<tr>
<td>2.2</td>
<td>Cholera and Immunity</td>
</tr>
<tr>
<td>2.3</td>
<td>Outer Membrane Proteins</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Porin Proteins</td>
</tr>
<tr>
<td>2.3.2</td>
<td>Vibrio cholerae wzm- an ABC Transport Porin</td>
</tr>
<tr>
<td>2.3.3</td>
<td>Isolation of Membrane Proteins</td>
</tr>
<tr>
<td>2.3.4</td>
<td>Solubilization</td>
</tr>
<tr>
<td>2.3.5</td>
<td>Overexpression of Membrane Proteins</td>
</tr>
<tr>
<td>2.3.6</td>
<td>Role of Membrane Proteins in Immunity</td>
</tr>
<tr>
<td>2.4</td>
<td>Lactic Acid Bacteria (LAB)</td>
</tr>
<tr>
<td>2.4.1</td>
<td>Lactococcus lactis</td>
</tr>
<tr>
<td>2.4.2</td>
<td>The Lantibiotic Nisin</td>
</tr>
<tr>
<td>2.4.3</td>
<td>The Nisin-Controlled Expression (NICE) System</td>
</tr>
<tr>
<td>2.4.4</td>
<td>Recombinant L. lactis: Transformation</td>
</tr>
<tr>
<td>2.5</td>
<td>Vaccine and Immunity</td>
</tr>
</tbody>
</table>
2.5.1 The Role of Mucosal Immunity 34
2.5.2 Oral Immunization 36
2.5.3 Lactococcus lactis as an Oral Delivery Vehicle 38

3 MATERIALS AND METHODS 40
3.1 Bacterial strains and growth conditions 40
3.2 DNA Extraction 42
  3.2.1 Total Genomic DNA Extraction from Vibrio cholerae 42
  3.2.2 Plasmid Isolation from Lactococcus lactis 43
3.3 Polymerase Chain Reaction (PCR) 45
  3.3.1 Design and Synthesis of Primers 45
  3.3.2 Hot-Start PCR 47
  3.3.3 Reverse-Transcriptase PCR (RT-PCR) 48
  3.3.4 Agarose Gel Electrophoresis 51
  3.3.5 Gel Purification 52
  3.3.6 Sequencing 53
3.4 Cloning 53
  3.4.1 Preparation of Competent Cells for Cloning 53
  3.4.2 Enzymatic Digestion 54
  3.4.3 Ligation 54
  3.4.4 Transformation 55
  3.4.5 Analysis of Positive Clones 56
3.5 Protein Work 57
  3.5.1 Protein Expression and Purification 57
  3.5.2 Protein detection by SDS-PAGE 58
  3.5.3 Gel Staining and Destaining 60
  3.5.4 Western Blotting 61
3.6 Immunization of rabbits 63
  3.6.1 Animal Models 63
  3.6.2 Preparation of Inoculant 63
  3.6.3 Oral Administration 64
3.6.4 Challenge with *Vibrio cholerae* 64
3.6.5 Serum Collection and Storage 65
3.7 Indirect Enzyme-Linked Immunosorbent Assay (ELISA) 66
3.8 Statistical analysis 67

4 RESULTS 69
4.1 Detection of *Vibrio cholerae wzm* gene 69
  4.1.1 Genomic DNA Extraction 69
  4.1.2 *wzm* Primer sequences and PCR 70
4.2 Cloning of *wzm* 72
  4.2.1 Isolation of *Lactococcus lactis* pNZ8048 Plasmid 72
  4.2.2 Ligation and Electroporation 74
  4.2.3 Transcription of the *wzm* gene in *Lactococcus lactis* 77
4.3 Sequencing 79
4.4 Protein Expression 80
  4.4.1 Protein Extraction 80
  4.4.2 Detection by Western Blot 81
4.5 Immunological evaluation of NHwzm inoculated rabbits 82
  4.5.1 Culture of rabbit fecal samples 82
  4.5.2 Rabbit antibody titer- IgG and IgA 84

5 DISCUSSION 90

6 SUMMARY, CONCLUSION, AND RECOMMENDATIONS 107 FOR FUTURE RESEARCH

7 REFERENCES 110
8 APPENDICES 123
  4.1 BLAST analysis of the *wzm* gene sequences of *Vibrio cholerae* Ogawa and Inaba 124
4.2 BLAST Analysis of Recombinant *L. lactis* NHwzm & *V. cholerae* Ogawa wzm gene

4.3 BLAST analysis of *V. cholerae* Wzm protein sequence with other known ABC Transport proteins

4.4 IgG Analysis of Variance (ANOVA) statistical output

4.5 IgA Analysis of Variance (ANOVA) statistical output

9 BIODATA OF STUDENT

10 LIST OF PUBLICATIONS