UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT AND EVALUATION OF A DNA VACCINE ENCODING 31-kDa OUTER MEMBRANE PROTEIN OF *BRUCELLA MELITENSIS*

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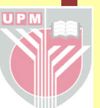

DEVELOPMENT AND EVALUATION OF A DNA VACCINE ENCODING 31-kDa OUTER MEMBRANE PROTEIN OF *BRUCELLA MELITENSIS*

By

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

September 2009

DEDICATED TO……..

My Father and Mother:

Mohammad Ashrafzadeh
Mahnaz Emami

My Brother and Sister:

Pedram Ashrafzadeh
Tanaz Ashrafzadeh

Abstract of thesis presented to the Senate of University Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

DEVELOPMENT AND EVALUATION OF A DNA VACCINE ENCODING 31-kDa OUTER MEMBRANE PROTEIN OF *BRUCELLA MELITENSIS*

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September 2009

Chairman: MD SABRI MOHD YUSOFF, PhD

Faculty: Veterinary medicine

Brucellosis is a widespread disease affecting livestock and humans caused by *Brucella spp.,* which are facultative, Gram negative intracellular pathogens. In Malaysia brucellosis is prevalent especially in animals, which can cause abortion and consequent economic losses. The live attenuated strains like *Brucella melitensis* Rev1 and *B. abortus* S19 and RB51 are being used to control brucellosis in domesticated animals however they have some disadvantages, such as these strains provoke antibodies to their lipopolysaccharide (LPS), making it difficult to distinguish vaccinated animals from those naturally infected. The DNA vaccines offer a new approach because they can stimulate both cellular and humoral immunities. On the other hand, DNA vaccines have many advantages over traditional protein-based vaccines, such as ease of development, inducing a long lived immunity, and minimal preparation costs. On account of the fact that, vaccination is the only way to control and eradicate the disease, the vaccine which can induce antibody in animals and protect them from infection and can overcome the drawbacks of current live attenuated vaccines, is essential.

In this study, the outer membrane proteins (Omps) of *Brucella melitensis* 152, 183 and 293 of local isolates were extracted and characterized using Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE). Accordingly, all the strains showed major bands were approximately 22, 23, 31, 43 and 94 kDa and minor bands were approximately 10, 16, 19, 73 kDa. Immunoblotting was conducted using antisera against the whole cells of *B. melitensis* 183 revealed the antigenicity of Omps of the three isolates. Omp31 was one of the antigenic proteins, chosen as a candidate for this research.

Omp31 gene from all isolates was amplified, cloned in pcDNA3.1 (+) and sequenced. All isolates produced a single DNA fragment approximately at 723 bp. The sequence of all isolates was compared with the published sequences to show the percentage of the similarity to them which displayed 100% similarity of strain 183, 99.8% of strain 152, and 99.8% of strain 293 to strain 16M as the reference strain.

To study the immunization of Omp31, DNA vaccination method applied as a novel approach for vaccination in BALB/c mice. The mice were divided into four groups: vector with insert (pcDNA3.1-Omp31), vector alone (pcDNA3.1), PBS and unvaccinated group. In conclusion, Immunization with pcDNA3.1-Omp31 elicited antibody response which was detectable 30 days after the first immunization. However, No antibody response was detected against other control groups.

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Absrak tesis yang dikemukakan kapada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PEMBANGUNAN DAN PENILAIAN VAKSIN DNA BERKOD 31-kDa PROTIN SELAPUT LUAR *BRUCELLA MELITENSIS*

Oleh

SEYED PARHAM ASHRAFZADEH

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Brucellosis ialah penyakit yang merebak ke atas haiwan ternakan dan manusia yang disebabkan oleh bakteria *Brucella spp.,* di mana ianya fakultatif, patogen di dalam sel dan bersifat Gram negatif. Di Malaysia, brucellosis lazimnya menjangkiti haiwan sehingga menyebabkan keguguran dan kerugian ekonomi yang besar. Bakteria hidup yang dilemahkan seperti *Brucella melitensis* Rev1 dan *B. abortus* S19 dan RB51 digunakan sebagai kawalan brucellosis ke atas haiwan ternakan tempatan. Walaupun terdapat beberapa kelemahan seperti strain merangsang antibodi kepada lipopolisakarida (LPS), ianya menyebabkan sukar dibezakan di antara haiwan yang divaksin dan haiwan yang dijangkiti secara semulajadi. Vaksin DNA dapat menawarkan dengan pendekatan di mana ianya dapat merangsang imuniti ketisuan dan humoral. Dengan kata lain, vaksin DNA mempunyai kelebihan daripada vaksin tradisional berdasarkan protin seperti senang dibangunkan, menginduksikan imuniti berpanjangan, dan kos penyediaan yang rendah. Secara faktanya, pemvaksinan merupakan cara untuk mengawal dan menghapuskan penyakit ini, di mana pemvaksinan menginduksikan antibodi.

Di dalam kajian ini, protin selaput luar (Omp) dari *Brucella melitensis* 152, 183 dan 293 tempatan diekstrak dan diasingkan meggunakan “Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis” (SDS-PAGE). Semua strain menunjukkan perbezaan jalur yang kecil dan besar. “Immunoblotting” yang dijalankan menggunakan antiserum terhadap seluruh sel *B. melitensis* 183 menunjukkan antigenik protin selaput luar (Omp) ke atas tiga isolat ini. Protin selaput luar 31 (Omp31) merupakan salah satu daripada antigenik protin yang dipilih di dalam kajian ini.

Gen protein selaput luar (Omp31) dari semua isolat dilipatgandakan, diklon di dalam vektor pcDNA3.1 (+) dan dijujukkan untuk mempastikan semua susunan berada di dalam bingkainya. Kesemua isolat menghasilkan satu produk, iaitu kira-kira 723 bp. Jujukan kesemua isolat kemudiannya dibandingkan dengan jujukan terbitan untuk menunjukkan peratus persamaan di mana menunjukkan persamaan 100% dengan strain 183, 99.8% dengan strain 152, dan 99.8% dengan strain 293 apabila dibandingkan dengan strain rujukan 16M.

Di dalam kajian terhadap immunisasi protein selaput luar 31 (Omp31), kaedah pemvaksinan DNA digunapakai terhadap pemvaksinan di dalam tikus BALB/c. Tikus dibahagikan kepada 4 kumpulan: vektor dengan kemasukan pcDNA3.1-Omp31, vektor sahaja (pcDNA3.1), PBS dan kumpulan yang tidak divaksin. Immunisasi dengan pcDNA3.1-Omp31 menunjukkan respon antibodi di mana ianya dapat dikesan pada hari ke 30 selepas immunisasi pertama. Walaubagaimanapun, tiada pengesanan antibodi dapat dilihat pada kumpulan-kumpulan yang lain.

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I certify that an Examination Committee has met on 16th September 2009 to conduct the final examination of Seyed Parham Ashrafzadeh on his Master of Science thesis entitled ''Development and evaluation of a DNA vaccine encoding a 31-kilodalton outer membrane protein of *Brucella melitensis*'' in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) regulations 1981. The committee recommends that the student be awarded the master degree. Members of the Examination Committee were as follows:

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Date: 10 December 2009

DECLARATION

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

____

SEYED PARHAM ASHRAFZADEH

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TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	v
ACKNOWLEDGEMENTS	vii
APPROVAL	viii
DECLARATION	x
LIST OF TABLES	xiv
LIST OF FIGURES	xv
LIST OF ABBREVIATIONS	xvi

CHAPTER

1 INTRODUCTION	1.1
2 LITERATURE REVIEW	2.1
2.1 Brucellosis	2.1
2.2 *Brucella* species	2.2
2.3 Ultrastructure of Gram-negative bacteria	2.3
2.3.1 Peptidoglycan	2.4
2.3.2 Lipoprotein	2.4
2.3.3 Lipoplysaccharide	2.4
2.3.4 Fimbriae	2.5
2.3.5 Outer membrane protein	2.5
2.4 Cell envelope of *Brucella* spp.	2.6
2.5 Biochemical tests for identification of *Brucella melitensis*	2.9
2.6 Serological methods	2.9
2.6.1 Complement Fixation Test (CFT)	2.9
2.6.2 Rose Bengal Plate Test (RBPT)	2.11
2.7 Molecular methods	2.12
2.7.1 Polymerase Chain Reaction (PCR)	2.12
2.7.2 Restriction Endonuclease DNA Analysis (REA)	2.13
2.7.3 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)	2.14
2.7.4 Immunoblotting	2.15
2.7.5 Enzyme-linked immunosorbent Assay (ELISA)	2.15
2.7.6 Cloning	2.16
2.8 *Brucella* vaccines	2.17
3 CHARACTERIAZATION AND ANTIGENICITY OF THE OUTER MEMBRANE PROTEINS OF *BRUCELLA MELITENSIS*	
3.1 Introduction	3.1
3.2 Materials and Methods	3.3
3.2.1 Extraction of the Outer membrane proteins (Omps)	3.3

3.2.2 Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS-PAGE) 3.4
3.2.3 Preparation of the antisera against whole cell of *Brucella melitensis* 3.4
3.2.4 Immunoblotting of the Outer membrane proteins 3.5
3.2.5 Electro-transfer of the proteins to PVDF membrane for sequence analysis 3.6
3.2.6 Genebank blast 3.7
3.3 Results 3.8
3.4 Discussion 3.13

4 CLONING AND SEQUENCING OF THE GENE ENCODING 31- KILODALTON OUTER MEMBRANE PROTEIN OF *BRUCELLA MELITENSIS*

4.1 Introduction 4.1
4.2 Materials and methods 4.3
4.2.1 Bacterial culture 4.3
4.2.2 DNA extraction 4.3
4.2.3 Amplification of 31kDa outer membrane protein gene by PCR 4.4
4.2.4 Detection of the PCR products 4.5
4.2.5 Extraction and purification of the PCR products 4.6
4.2.6 Vector 4.7
4.2.7 Double digestion of the plasmid with the insert with restriction enzymes 4.7
4.2.8 Ligation of the insert into the vector 4.8
4.2.9 TOP 10 one shot chemical transformation 4.9
4.2.10 Plasmid extraction 4.9
4.2.11 Analysis of the positive colonies 4.10
4.3 Results 4.12
4.4 Discussion 4.23

5 EFFICACY OF THE OMP31 GENE IN STIMULATING ANTIBODIES IN MICE USING DNA VACCINATION

5.1 Introduction 5.1
5.2 Materials and Methods 5.3
5.2.1 Animals 5.3
5.2.2 Plasmid preparation for DNA vaccination 5.3
5.2.3 Immunization 5.4
5.2.4 RNA extraction 5.5
5.2.5 *In vivo* expression study of Omp31using RT PCR 5.7
5.2.6 Serological tests 5.8
5.2.7 Rose Bengal Plate Test 5.8
5.2.8 Serum Agglutination Test (SAT) 5.8
5.3 Results 5.10
5.4 Discussion 5.15

6 GENERAL DISCUSSION AND CONCLUSION 6.1

REFERENCES R.1
APPENDICES A.1
BIODATA OF STUDENT A.57
LIST OF PUBLICATIONS A.58

LIST OF TABLES

Table		Page
4.1:	The PCR cocktail mixtures for amplification of Omp31	4.5
4.2:	PCR conditions	4.5
4.3:	Double digestion of vector and PCR product with *Bam*H1 and *Xho*1	4.8
4.4:	Ligation of the insert into the vector	4.8
4.5:	Single digestion of the plasmid containing the insert and plasmid alone	4.10
4.6:	Double digestion of plasmid extracts with *Bam*H1 and *Xho*1	4.10
4.7:	Nucleotide sequences similarity of *B. melitensis* isolates	4.21
4.8:	Amino acid sequences similarity of *B. melitensis* isolates	4.21
5.1:	Description of BALB/c mice groups and days of injections	5.5
5.2:	DNase incubation mixture	5.6
5.3	The RT-PCR cocktail mixtures	5.7

LIST OF FIGURES

Figure		page
2.1:	Schematic diagram of the typical of Gram-negative bacteria cell Wall	2.6
2.2:	Diagram shows the polymerase chain reaction steps	2.13
3.1	Gram-negative staining of Brucella showing the coccobacillary shape	3.10
3.2:	Protein profiling of *B. melitensis* 152, 183, and 293 by SDS-PAGE	3.11
3.3	Immunoblot analysis of the outer membrane proteins of three local *B. melitensis* isolates using hyperimmune serum to whole cell of *B. melitensis* 183	3.12
4.1:	Agarose gel electrophoresis of *B. melitensis* genomic DNA amplification products using Omp31F and Omp31R	4.14
4.2:	Comparison of the vector alone and vector with insert	4.15
4.3:	Restriction endonuclease analysis of the extracted plasmid of positive transformant colonies	4.16
4.4:	Complete nucleotide sequence of Omp31 gene of *B. melitensis* 183 with deduced amino acid sequences	4.17
4.5	Omp31 full nucleotide sequence of *B. melitensis* 183 showing T7 promoter (primer binding site), BGH reverse priming site, *Bam*H1, and *Xho*1, start codon and stop codon of Omp31	4.18
4.6:	Alignment of nucleotide sequences of Omp31 of *B. melitensis* 152, 183, and 293	4.19
4.7:	Alignment of the predicted amino acid sequences of Omp31 of *B. melitensis* 152, 183, and 293	4.20
4.8:	pcDNA3.1 (+) with Omp31 Map	4.22
5.1:	Plasmid extraction for immunization	5.12
5.2:	Antibody level in mice following immunization with DNA vaccine in experimental groups	5.13
5.3:	RT-PCR products on 1% gel electrophoresis	5.14

LIST OF ABBREVIATIONS

%	Percentage
ºC	Celsius temperature (centigrade temperature)
µg	Microgram
µl	Microliter
APS	Ammonium persulfate
bp	Basepairs
BSA	Bovine serum albumin
cfu	Colony forming unit
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene-diamine-tetraacetic acid
g	Gram
H_2O	Water
H_2O_2	Hydrogen peroxide
IgG	Immunoglobulin G
Kb	Kilobase pair
kDa	Kilodalton
LB	Luria-bertani
L	Liter
LPS	Lipopolysaccharide
M	Molar
mg	Milligram
$MgCl_2$	Magnesium chloride

min	Minute
mAbs	Monoclonal antibodies
ml	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
OD	Optical density
ORF	Open reading frame
Na_2HPO_4	Di-sodium hydrogen phosphate
NaCl	Sodium chloride
NaH_2PO4	Sodium di-hydrogen phosphate
NaOH	Sodium hydrogen peroxide
nm	Nanometer
Omp	Outer membrane protein
PBS	Phosphate buffer saline
pH	Puissance hydrogen (Hydrogen-ion concentration)
RNA	Ribonucleic acid
rOmp	Recombinant outer membrane protein
rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TAE	Tris-acetate-EDTA buffer
TBE	Tris-bordate-EDTA buffer
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Brucellosis is a zoonotic disease that can happen in both humans and animals. In animals, the most vivid sign of disease is abortion. Transmission happens when animals are exposed to contaminated food or water or lick a newly aborted fetus (Corbel, 1997). Humans can also contract infection by ingesting contaminated milk or other dairy products (Fraser, 1986).

Brucellosis is still endemic in many developing countries causing important economic losses. In Malaysia, the presence of brucellosis in large ruminants was confirmed by the isolation of *Brucella abortus* in 1950 and *Brucella suis* in 1963. The National Program for 'The Area-Wise Eradication of Bovine Brucellosis which started working in 1979 had reduced the prevalence of bovine brucellosis in Malaysia from 3.3% in 1979 to 0.23 in 1988. It was then envisaged that by 1995 bovine brucellosis in Malaysia had been eradicated. However, the prevalence of brucellosis was reported to be relatively high (<2%) again (Anon, 2005).

The attenuated strains like *Brucella melitensis* Rev1 and *B. abortus* S19 and RB51 are applied to control brucellosis in domesticated animals. However, these attenuated vaccines are not efficient enough because of their limited efficacy and

potential to lead to the disease in humans. Moreover, both *B. abortus* S19 and *B. melitensis* Rev1 strains provoke antibodies to their lipopolysaccharide (LPS), making it difficult to distinguish vaccinated animals from those naturally infected (Baldi *et al*., 1996). Recently, *Brucella* spp., have been reported as a dangerous disease by the Centers for Disease Control (Izadjoo *et al*., 2004). So, a vaccine that is protective against *Brucella* is desirable. Based on the extensive economic losses that *Brucella* infection in animals can induce, eradication of the disease worldwide is very crucial. Vaccination is an efficient mean of protecting animals that have not already been exposed to the disease (Fraser, 1986).

The DNA vaccines offer a new approach because they can stimulate both cellular and humoral immunities. DNA vaccines have many advantages over traditional protein-based vaccines, such as ease of development, inducing a long lived immunity, and minimal preparation costs (Srivastava and Liu, 2003). Because of the effectiveness, previous studies have shown that DNA vaccination with sodC (Onate, 2003), lumazine synthase gene (Velikovsky *et al*., 2002), and P39 (Al-Mariri *et al.,* 2001) can induce good antibody levels in animals. Cassataro *et al*. (2005b) showed that Omp31 of *B. melitensis* can confer partial protection against *B. melitensis* and *B. ovis* protection, which maybe because of not sufficient antibody production or improper expression vector. Accordingly, using Omp31 with another vector is necessary to evaluate a DNA vaccine using Omp31 of *B. melitensis*. In contrast to live attenuated vaccines, there are no concerns of contracting disease, and DNA vaccines are stable. Identification of *Brucella* spp. antigens with the ability to elicit a protective immune response is of great interest for the development of vaccines. As shown in mouse model, active immunization

with *Brucella* spp. smooth LPS (Pugh, 1991), or O-polysaccharide chain (Jacques, 1991) and passive protection experiments with anti-polysaccharide monoclonal antibodies (mAbs) (Vizcaino and Fernandez-Lago, 1994) smooth LPS plays an important role in protective immunity. mAbs rose against several *Brucella* outer membrane proteins (Omps) gave slight protection in mice against infection caused by smooth *Brucella* strains (Cloeckaert *et al*., 1991; Jacques *et al*., 1992). However, a mixture of anti-Omp mAbs has been proved to protect against rough *Brucella ovis* infection (Bowden *et al*., 1995) and it has also been suggested that the Omps are important in the development of protective cellular immunity, while the transfer of immune T cells protects against *Brucella* infection (Plommet *et al*., 1985). Cloning of the *Brucella* Omps is an attractive approach for the determination of their potential goodness for vaccination purposes.

With regard to the potential of Omps as a vaccine candidate, the objectives of this study were:

1. to determine the Omps profiles of *Brucella melitensis* 152, 183, and 293 using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
2. to determine the antigenicity of the Omps of *Brucella melitensis* 152, 183, and 293 using Western blotting against homologous and heterologous antisera.
3. to amplify, clone and sequence Omp31 as one of the most antigenic protein gene of *Brucella melitensis* 152, 183, and 293.

4. to evaluate the immunization of the DNA vaccine encoding the antigenic protein in mice.

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CHAPTER 2

LITERATURE REVIEW

2.1 Brucellosis

Brucellosis is a widespread, zoonotic and infectious disease of animals and humans caused by groups of *Brucella* genus that is economically important (Alton, 1990). Since the microorganism is transmitted between animals within herd, it is capable of causing abortions during breeding seasons (Wilesmith, 1978). It can be transmitted from animals like cats, sheep, goats, bison and buffalo through direct contact with blood placenta, fetuses, uterine secretions or by consumption of infected and raw animal products especially milk and milk products. Brucellosis in sheep and goats is mainly caused by *Brucella melitensis* (Hamdy, 1992). In Malaysia, the presence of brucellosis in large ruminants was confirmed by the isolation of *Brucella abortus* in 1950 and *Brucella suis* in 1963. The National Program for 'The Area-Wise Eradication of Bovine Brucellosis which started working in 1979 had reduced the prevalence of bovine brucellosis in Malaysia from 3.3% in 1979 to 0.23 in 1988. It was then envisaged that by 1995 bovine brucellosis in Malaysia had been eradicated. However, the prevalence of brucellosis was reported to be relatively high (<2%) again (Anon, 2005).

The frequency of transmission of *B. melitensis* is low, but the existence of latent infections greatly increases the difficulty of eradicating the disease. This happens as *B. melitensis* persists without having a detectable immune response because of immunotolerance and pathogenicity way.

The intracellular localization of these bacteria shows that the immunity towards *Brucella* requires a cell-mediated immune response, which makes the Th1 arm of the immune response very important for controlling the infection (Splitter *et al*., 1996).The protection against this infection needs a long-lived cellular immune response, depending on the processing of the bacteria by macrophages (Araya *et al*., 1989; Baldwin and Winter, 1994).

2.2 *Brucella* species

Bacteria of the genus *Brucella* are small, facultative intracellular, Gram-negative, non-motile and non-spore forming coccobacilli bacterial pathogens that infect both phagocytic and non-phagocytic cells (Alton 1990; Smith and Fitch, 1990). At the moment, there are six recognized species regarding the host specificity: *B. abortus* in cattle, *B. canis* in dogs, *B. melitensis* in goats, *B. neotamea* in desert wood rats, *B. ovis* in sheep and *B. suis* in pigs, reindeer and hares (Morgan and Corbel, 1976). Recently a large number of *Brucella* strains have been isolated from marine mammals and has proposed to be included in two new species, *B. cataceae* and *B. pinnipediae* (Cloeckaert *et al*., 2001). *B. melitensis* is the most pathogenic species for humans and the lowest species specific, infecting goats, sheep, cows, camels, and dogs (Young, 1995).

Malta fever which is also known as Mediterranean, Gibraltar, or undulant fever and porcine brucellosis caused by *B. melitensis* and *B. suis* infection of humans, respectively, are more apparent clinically than Bang's disease (*B.abortus* infection), whereas among the available species only *B. canis* causes anecdotal mild infections in humans (Corbel, 1997) .

Brucellae are classified in a tight phylogenetic family, the family *Rhizobiaceae* of the alpha-2 subgroup of the class *Proteobacteria,* including *Orchrobactrum*, *Bartonella*, *Rhizobium* and *Agrobacterium* (Velasco *et al*,. 1998). At the DNA level, the different species have more than 90% homology in DNA-DNA hybridization assays. The high similarity of *Brucellae* according to DNA-DNA hybridization suggested that they may represent a monospecies genus (Verger *et al*., 1985).

2.3 Ultrastructure of Gram-negative Bacteria

Gram-negative bacteria have three structural regions, consisting of appendages in the shape of flagella and pili (or fimbriae), cell wall and cytoplasmic region. The cell wall is approximately 10nm in diameter and composing of a single layer of peptidoglycans surrounded by membranous structure called the outer membrane. The outer membrane has unique components like lipopolysaccharides as endotoxin, outer membrane protein, lipoprotein and phospholipid. The cytoplasm surrounded the cell genome which named DNA, contains ribosomes and variable other inclusions. Fimbria is used mostly to describe the conjugative filaments of many Gram-negative bacteria. They are shorter and straighter than flagella and appear anchored through the outer membrane (Jawetz *et al*, 2004) (Figure 2.1).