IN SILICO STUDY, CLONING AND FUNCTIONAL ANALYSIS OF CjS8 PROTEIN FROM *Campylobacter jejuni*

BY

HATIM ABDULLAH YOUSEF NATTO

A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy

Kulliyyah of Allied Health Sciences
International Islamic University Malaysia

JULY 2020

ABSTRACT

In Gram-negative bacteria, protein secretion plays an important part in pathogenesis. Secretory proteins perform a variety of important role for bacterial survival in the environment and its involvement to cause disease to human. We have identified specific surface protein of *Campylobacter jejuni*. Genomic and protein analysis using bioinformatics tools on that protein reveals the presence of signal peptide at N-terminal of its peptide sequence, thus signifies this protein is highly potential acted as secreted protein. The size of the protein was calculated as 24.1 kDa. The availability of complete genome sequences of *C. jejuni* has allowed this study to make predictions about the composition of bacterial secreted protein that has good similarity in their sequence homology. The prediction of others *C. jejuni* secreted proteins were performed based on 24.1 kDa and several enterobacteriaceae pathogenic bacteria proteins sequences using a set of internet-based programs, including BLAST, ORF Finder and SignalP v 5. *In silico* analysis in this study identified the secreted protein of S8 family serine peptidase in *C. jejuni* genome and designated as CjS8. To investigate the involvement of CjS8 in the pathogenesis of *C. jejuni* infection, a C-terminal fragment of CjS8 was successful amplified, cloned and expressed using a TOPO expression vector. Rabbit polyclonal serum was raised against the purified recombinant CjS8 protein. The CjS8 null-mutant was constructed in *C. jejuni* by natural transformation and allelic exchange. PCR analysis and immunoblot of whole cell lysates with Ab_CjS8 (polyclonal antibody against CjS8) showed that CjS8 is naturally expressed in *C. jejuni* but not in the null mutant. In a strain survey on clinical isolates of *C. jejuni*, using the PCR and immunoblot analysis. Data showed that the CjS8 was presence in twenty out of twenty-three clinical isolates. Importantly, this study revealed the functional analysis results, that showed the CjS8 mutant was shown to affect the *C. jejuni* ability to adhere to the host cells (Caco-2 cells). Invasion was also affected by CjS8 mutant strain as well as the biofilm formation and motility. Thus, as a conclusion the CjS8 was the one, among a few secreted proteins described in *C. jejuni* and may represent a novel virulence factor. These results will be important in furthering our understanding of Campylobacter biology and pathogenesis.

خلاصة البحث

في البكتيريا سالبة الجرام ، يلعب إفراز البروتين دورًا مهمًا في التسبب في المرض. تؤدي البروتينات الإفرازية مجموعة متنوعة من الأدوار المهمة لبقاء البكتيريا في البيئة ومشاركتها في إحداث المرض للإنسان. لقد قمنا بتعريف بروتينًا سطحيًا محددًا للكامبيلوباكتر جيجوني. ان تحليل الجينوم والبروتين باستخدام أدوات المعلوماتية الحيوية على هذا البروتين يكشف عن وجود سيجنال ببتيد في الطرف ان من تسلسل الببتيد، وبالتالي يشير إلى أن هذا البروتين محتمل للغاية ان يعمل كبروتين مُفرز. لقد تم حساب حجم البروتين على أنه 24.1 كيلو دالتون.ان توفر التسلسل الجيني الكامل للكمبيالوباكترجيجوني اتاح لهذه الدراسة اجراء تنبؤات حول تكوين البروتينات البكترية المفرزة التي لها تشابه جيد في التجانس الجيني. تم اجراء التنبؤ بالبروتينات الاخرى غير الكامبيلوباكتر جيجوني التي يتم افرازها والتي لها تشابه جيد في التسلسل الجيني. تم اجراء التنبؤ بالبروتينات الأخرى التي يتم افرازها من الكامبيلوباكتر جيجوني استناداً الى 24.1 كيلودالتون والعديد من متواليات البكتريا المعوية المسببة للأمراض وذلك باستخدام مجموعة من البرامج المستندة الى الانترنت, بما في ذلك بلاست و اوبن ريدينج فريم وسيجنال بي. ان التحليل باستخدام السيلكو في هذه الدراسة حدد بروتين مفرز من عائلة اس 8 سيرين بيبتيديز في جينوم الكامبيلوباكتر جيجوني تم تسميته سي جيجوني اس 8. للكشف عن دور سي جيجوني اس 8 في التسبب بالمرض والاصابة بالعدوى بالكمبيلوباكتر جيجوني, تم بنجاح تكوين السي تيرمنل وزراعته وانتاج البروتين باستخدام توبو اكسبريشن فيكتور. تم انتاج مصل ارنب متعدد النسيلة ضد البروتين المنقى من سي جيجوني اس 8. تم انشاء الطفرة الجينية في السي جيجوني اس 8 بواسطة الانتقال الطبيعي والتبادل الأليلي. نتائج تفاعلات سلسلة البوليمريز والغشاء المناعي الناتج بتحليل كامل الخلايا مع الاجسام المضادة الناتجة ضد سي جيجوني اس 8, اظهرت أن سي جيجوني اس 8 ينتج طبيعياً في الكامبيلوباكتر جيجوني ولكن لاينتج في الطفرة الجينية فيها. في الدراسة الاستقصائية للسلالات على عينات طبية للكمبيلوباكتر باستخدام تفاعلات سلسلة البوليمريز وتحليل الغشاء المناعي الناتج بتحليل كامل الخلايا, اظهرت البيانات ان سي جيجوني اس 8 موجود في عشرون عزلة من اصل ثلاثة وعشرين عزلة طبية. ألاهم من ذلك كشفت هذه الدراسة عن نتائج التحليل الوظيفي حيث اوضحت أن هناك تأثير للسي جيجوني اس 8 الطفرة الوراثية على قدرة الكامبيلوباكتر جيجوني على الالتصاق بخلايا العائل المضيف. كذلك الغزو تأثر بالطفرة الوراثية للسي جيجوني اس 8 وكذلك الحال بالنسبة لكل من تشكيل الأغشية الحيوية والحركة. وهكذا فانه كاستنتاج فان السي جيجوني اس 8 يعتبر واحداً من بين عدد من البروتينات المفرزة الموصوفة في الكامبيلوباكتر جيجوني والذي يشكل عامل ضراوة جديد. سوف تكون هذه النتائج مهمة في تعزيز فهمنا لبيولوجيا الكامبيلوباكتر وطرق احداث المرض.

APPROVAL PAGE

The thesis of Hatim Abdullah Yousef Natto has been approved by the following:

_____
Ridhwan Abdul Wahab
Supervisor

_____
Mohd Zaki Salleh
Co-Supervisor

_____
Robaiza Zakaria
Co-Supervisor

_____
Mohammad Syaiful Bahari Abdull Rasad
Internal Examiner

_____
Tang Thean Hock
External Examiner

_____
Mohd Nasir Mohd Desa
External Examiner

_____
Jesni Shamsul Shaari
Chairman

DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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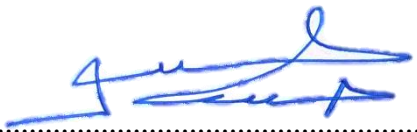
Signature  Date .....

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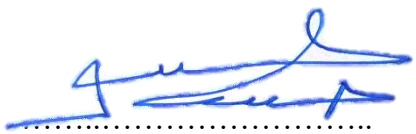

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DEDICATION

It is my utmost pleasure to dedicate this thesis to the beloved parents, may God have mercy on them, who moved to the mercy of God during the final stage of writing on this thesis. Also this thesis is dedicated to my family, who have been my backbone and words cannot express my deepest gratitude and love. Thank you for your support and patience.

ACKNOWLEDGEMENTS

First of all, I would like to thanks my God Almighty who helped and supported me step by step for me to go through on this study. I wish to express my appreciation and thanks to those who provided their time, effort and invaluable support for this study. A special thanks to my supervisor Associate Professor Dr. Ridhwan Abdul Wahab for his continuous and invaluable support, encouragement, friendship and for that, I will be forever grateful. To my Co-Supervisors, Professor Dato' Dr. Mohd Zaki Salleh (Director, Integrative Pharmacogenomics Institute (iPROMISE), UiTM) and Dr. Robaiza Zakaria (Depatment of Medical Microbiology and Parasitology, Medical School, USM) for their valuable encouragement and advice in this study. And also not to forget Professor Dato' Dr. Ridzwan Hashim, Dr Ariffin and many more academic staffs in IIUM that helping me directly or indirectly for the successful of this study. One small smiles from your clean heart, means something to me and motivate me to move on.

I would like to express my thanks to all in Biomedical Science Department in particular, Assistant Professor Dr. Hanani Binti Ahmad Yusof @ Hanafi (Head of Department), KAHS postgraduate office and the Dean, Professor Dr. Suzanah Binti Abdul Rahman for their support during my candidature as a Ph.D student in IIUM.

I would like to extend my warm and sincere thanks to my research team-mates, Sister Alaina, Tara, Faizah, Habibah and Shakirah for their friendship, helps, supports, and encouragement.

I wish to express my sincerest appreciation to Saudi Government in particular, Umm Al-Qura University and the Saudi Embassy and Saudi Cultural Attache staffs for their support and providing me this opportunity to complete the Ph.D degree at IIUM.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Amp	Ampicillin
ATP	Adenosine tri-phosphate
bp	Base pair
C	Celcius
CCDA	Cefoperazone charcoal de-oxy chocolate agar
cDNA	Complimentary DNA
CFU	Colony forming unit
CIAP	Calf Intestinal Alkaline Phosphatase
CjS8	*C. jejuni* S8 family serine peptidase
C. jejuni	*Campylobacter jejuni*
C. coli	*Campylobacter coli*
*C. jejuni*ΔCjS8	*C. jejuni* mutant (S8 family serine peptidase)
CjS8 wsp	*C. jejuni* S8 family serine peptidase without signal peptide
CO_2	Carbon dioxide
dATP	De-Oxy Adenosine Tri Phosphate
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxy ribonucleic acid
EB	Envelope buffer
E. coli	*Escherichia coli*
FCS	Fetal Calf Serum
g	Gram
GBS	Guillain-Barré Syndrome
h	Hour
His	Histidine
HRP	Horse radish peroxidase
Kan	Kanamycin
kb	Kilo base pair
kDa	Kilo Dalton
LB	Luria-Bertani
mÅ	milli Amp
MH	Mueller-Hinton
min	Minute

Ni-NTA	Nickel-nitriotriacetic acid
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	revolutions per minute
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TAE	Tris-acetate EDTA
UV	Ultra-violet
V	Volts
µg	Microgram
µl	Microliter
µM	micro Molar

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

The description of this study must begin from the discovery of the cell surface protein that was found to be specific for *Campylobacter jejuni* (Salleh, 1994) (https://elib.usm.my-Thesis PhD). Genomic and protein analysis using bioinformatics tools on that protein reveals the presence of signal peptide at N-terminal of its peptide sequence, thus signifies this protein is highly potential acted as secreted protein (Abdul Wahab, 2000) (https://elib.usm.my-Thesis MSc). The sequence of this protein was deposited in Uniprot database named as "*Campylobacter jejuni* strain USM1-putative periplasmic protein" and could be retrieved at https://www.uniprot.org/citations/-4646025062340752923. The size of the protein was calculated as 24.1 kDa containing the YCE gene domain (UniProtKB - Q79JB5) identified as putative periplasmic protein at the C-terminal. The gene was also known as "*Campylobacter jejuni* hypothetical protein Cj0419" in the Uniprot database which could be retrieved at https://www.uniprot.org/citations/SIP78F205116FDE3799. Based on the information as described above, principally, the whole main ideas and the initial experimental designed of this study was to construct the 24.1 kDa gene (Cj0419) *C. jejuni* mutant for the characterization of its gene function and how this gene is involved in the pathogenesis of *C. jejuni*. The strategies for identifying secreted protein in established genome sequence of *C. jejuni* (NCTC 11168) was based on the protein sequence information of *C. jejuni* secreted protein 24.1 kDa (Abdul Wahab, 2000). Others secreted proteins which were experimentally validated from selected

enterobacteriaceae pathogenic bacteria using proteomic and genome-based computational prediction (Chen *et al.,* 2019).

The secreted protein of an organism represents the proteins that released by all types of cells of living things (Chua *et al.,* 2012). Secretory protein systems are important for many physiological functions of cells or organisms such as sustaining cell-cell communication, proliferation, metabolism (Zhang *et al.,* 2014), immunomodulation (Toapanta *et al.,* 2018) and invasion into host cells to cause the disease (Jang *et al.,* 2020). Markedly, many secreted proteins have been identified as important biomarkers and therapeutic targets (Walker *et al.,* 2017). Therefore, understanding the biological functional of bacterial secreted proteins have great potential to provide a valuable resource for diagnosis, prognosis, and treatment of bacterial diseases (Brown *et al.,* 2013).

C. jejuni infections are a major cause of diarrheal disease worldwide. The incidence and prevalence of campylobacteriosis have increased in both developed and developing countries over the last 10 years (Kaakoush *et al.,* 2015). They are the leading cause of foodborne illness, with 56,729 cases reported of which almost 96.37 infection over 100,000 populations at England in 2017 (Public Health England, 2017) and among the main confirmed aetiology of bacterial foodborne illness in USA in 2017, infected approximately of 1.5 million people (CDC, 2017). In one of the study in Malaysia it was reported *C. jejuni* was the most predominantly isolated species (69.5%) from broiler chickens and chicken meat (Sinulingga *et al.,* 2020).

1.2 PROBLEM STATEMENT AND SIGNIFICANCE OF THE STUDY

Pathogenic bacteria have the ability to colonized human cells to cause disease. Indeed, bacteria successfully evolved to acclimate at different environments for survival. In addition, bacterial pathogens have to overcome host innate and adaptive immune system, including the microbiome especially in the gut. The secreted proteins of pathogenic bacteria is believed to be the evasion system against the host defence systems. The protein secretion systems of pathogenic bacteria are machineries used to secrete proteins in the extracellular medium, or directly into the targeted cell. Several secretion systems have been described in the scientific literature. Gram-negative bacteria have evolved eight secretion systems and they are made of proteins. The outer membrane proteins (OMPs) or surface protein of the pathogenic bacteria are the main protein that will be translocated through the inner membrane and then inserted in the outer membrane, for full virulence.

C. jejuni contamination is common on chicken in grocery stores (Kramer *et al.,* 2000; Guirin *et al.,* 2019; Walker *et al.,* 2019; Sinulingga *et al.,* 2020), even on the outside of packages (Burgess *et al.,* 2005; Chen *et al.,* 2018), as it is a commensal in birds (Skirrow & Blazer, 1995) allowing it to quickly spread unnoticed through a flock (Hermanns *et al.,* 2011) potentially accelerated by flies (Hald *et al.,* 2008). High throughput carcass processing exaggerates the problem by spreading contamination between birds. Chicken 'liquor', the juices evacuated from thawed chicken carcasses, has been found to be an efficient protectant for *C. jejuni* (Coates *et al.,* 1987). Chicken carcasses have as high as 5 logs of *C. jejuni* per bird with prevelances of 77% to 94% of carcasses contaminated (Kramer et L., 2000; Garin *et al.,* 2012; Nohra *et al.,* 2018; Guirin *et al.,* 2019). All of the reported cases above clearly potrayed the important of

C. jejuni to human health. Human could be easily infected by the *C. jejuni*, thus it is important to understand on the pathogenesis of *C. jejuni* to human host.

In this study we systematically explored the *C. jejuni* genome information for the identification of secreted protein that involved in the pathogenesis of *C. jejuni* infection. For that purposes the 24.1 kDa surface protein of *C. jejuni* (Salleh, 1994; Abdul Wahab, 2000) and others identified secreted protein from pathogenic enterobacteriaceae bacteria were used in the homology searched using BLAST. This study also will further validated and characterized selected *C. jejuni* secreted proteins using several bioinformatics tools. Furthermore, this study will perform the functional analysis of identified secreted protein of *C. jejuni* through constructing the mutant using inverse PCR mutagenesis. It is believed the outcome of this research provides a valuable resource and crucial data for further elaborating the involvement of secreted protein in the pathogenesis of *C. jejuni* infections.

1.3 RESEARCH QUESTIONS

The present study aims to answer the following questions:

- What are the secreted protein in the *C. jejuni* genome that have the closest homology to the 24.1 kDa and selected secreted protein from the pathogenic bacteria?
- What are the best vector system for cloning and protein expression for the *C. jejuni* secreted protein?
- What is the specificity of recombinant secreted protein of *C. jejuni* polyclonal antibody raised in rabbit?