UNIVERSITI PUTRA MALAYSIA

CHARACTERISATION OF HYALURONIDASE, BIOFILM FORMATION AND EMM TYPE AMONG INVASIVE AND NON-INVASIVE STREPTOCOCCUS PYOGENES CLINICAL ISOLATES IN TWO HOSPITALS IN MALAYSIA

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By

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

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CHARACTERISATION OF HYALURONIDASE, BIOFILM FORMATION AND *EMM* TYPE AMONG INVASIVE AND NON-INVASIVE *STREPTOCOCCUS PYOGENES* CLINICAL ISOLATES IN TWO HOSPITALS IN MALAYSIA

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Streptococcus pyogenes or group A streptococcus (GAS) is one type of bacterium that causes a wide variety of infectious and immunologically-related diseases. Hyaluronidase enzyme produced by GAS degrades hyaluronic acid, a major component in extracellular matrix of human connective tissues allowing the pathogen to enter. Meanwhile, biofilm formation in GAS is associated with therapeutic failure due to the robustness of biofilms against antibiotics. Till date, little is known on GAS hyaluronidase, its gene (*Hyl*) and biofilms. The aims of this study were to determine the production of bacterial hyaluronidase and its molecular characteristics and biofilm formation as well as *emm* typing among GAS clinical isolates.

A total of 45 *S. pyogenes* clinical isolates were obtained from the previous stock cultures which were collected from Kuala Lumpur and Serdang hospitals. Phenotypic hyaluronidase production was assessed by a solid plate assay. *Hyl* genes (*HylA*, *HylP1*, *HylP2*, *HylP3*) were detected through multiplex PCR using established primers. *emm* typing was done by *emm* gene amplification and sequencing according to established protocols. The enzymatic activity of hyaluronidase from different GAS isolates was determined through hyaluronic acid turbidity reduction assay. Biofilm formation was detected using Congo Red Agar (CRA) and Crystal Violet Assay (CVA).

Forty isolates (88.9%) exhibited hyaluronidase production despite the presence of *Hyl* genes (chromosomal and phage-associated) in all isolates. Hyaluronidase exhibited the maximum activity at 10^{th} minute time point with appropriate conditions that include 3% of bovine serum albumin (BSA), pH 3.5 and 0.1M NaCl. Positive correlation between bacterial growth and enzymatic activity was observed (p value < 0.05). Most of the

strains exhibited moderate biofilm production, (30, 66.66%) in CRA and (23, 51.11%) in CVA. A total of 29 different emm types were detected. The most prevalent *emm* types were *emm*1, 18, 28, 97 and 102 with (6.7%) of the isolates each, while *emm* types 63, 71, 76, 89 and 91 were seen in 4.4% of the isolates respectively. The rest of the *emm* types 12, 15, 44, 56, 65, 81, 98, 101, 105 and 120 were found about 2.2% each. No new *emm* types were detected. In general, there was no strong association between hyaluronidase production, *emm* type, invasiveness and biofilm formation as *p* values were more than 0.05). Hyaluronidase and production of biofilms in GAS may be involved in its pathogenesis as hyaluronidase able to break hyaluronic acid (HA) which is the layer wall on the host cells and the development of biofilm aid bacteria to become antimicrobial resistance towards antibiotics. Diverse *emm* types in the study may signify the heterogeneity of the local GAS strains. More research is warranted in future to establish to link between GAS virulence factors and its biofilms.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
Sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

KARAKTERISASI HYALURONIDASE, PEMBENTUKAN BIOFILM DAN JENIS EMM DENGAN INVASIFIKASI DAN NON-INVASIFIKASI *STREPTOCOCCUS PYOGENES* ISOLAT KLINIKAL DI DUA BUAH HOSPITAL DI MALAYSIA

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Streptococcus pyogenes atau Streptococcus kumpulan A (GAS) adalah sejenis bakteria yang menyebabkan pelbagai jenis penyakit yang berjangkit yang berkaitan dengan imunologi. Enzim hyaluronidase yang dihasilkan oleh GAS memusnahkan asid hialuronik, komponen utama dalam matrik sel luar tisu konektif pada manusia yang membolehkan patogen untuk masuk. Sementara itu, pembentukan biofilm didalam GAS berkaitan dengan kegagalan terapeutik disebabkan oleh ketahanan biofilm terhadap antibiotik. Sehingga kini, hanya sedikit yang diketahui mengenai hyaluronidase dalam GAS, gennya (*Hyl*) dan biofilm. Tujuan kajian ini adalah untuk menentukan penghasilan hyaluronidase oleh bakteria dan ciri-ciri molekulnya dan pembentukan biofilm serta pengetipan *emm* dalam kalangan isolat klinikal GAS.

Sejumlah 45 isolat klinikal *S. pyogenes* diperolehi daripada stok simpanan sebelumnya yang dikumpulkan daripada Hospital Kuala Lumpur dan Hospital Serdang. Penghasilan hyaluronidase secara fenotipik diperolehi melalui ujian plat pepejal. Gen *Hyl* (*HylA, HylP1, HylP2, HylP3*) dikesan melalui multiplex PCR menggunakan primer yang sudah tersedia ada. Pengetipan *emm* dilakukan dengan mengamplikasi gen dan penjujukan mengikut protokol yang ditetapkan. Aktiviti enzim hyaluronidase daripada isolat GAS yang berbeza ditentukan melalui ujian penurunan kekeruhan asid hialuronik. Pembentukan biofilm dikesan menggunakan Congo Agar Merah (CRA) dan Ujian Kristal Violet (CVA).

Hanya empat puluh isolat (88.9%) yang mempamerkan pengeluaran hyaluronidase walaupun terdapat gen *Hyl* (kromosom dan berkaitan phage) di dalam semua isolat. Hyaluronidase mempamerkan aktiviti maksimum pada minit ke-10 dengan kondisi yang sesuai iaitu 3% serum albumin lembu (BSA), pH 3.5 dan 0.1M NaCl. Korelasi positif

antara pertumbuhan bakteria dan aktiviti enzimatik turut diperhatikan (nilai p <0.05). Kebanyakan strain mempamerkan pengeluaran biofilm sederhana, (30, 66.66%) dalam CRA dan (23, 51.11%) dalam CVA. Sejumlah 29 jenis *emm* yang berbeza telah dikesan. Jenis-jenis *emm* yang paling lazim adalah emm1, 18, 28, 97 dan 102 dengan masing-masing (6.7%), sementara jenis *emm* 63, 71, 76, 89 dan 91 sebanyak 4.4% untuk setiap isolat. Selebihnya jenis *emm* 12, 15, 44, 56, 65, 81, 98, 101, 105 dan 120 didapati sekitar 2.2% setiap satu. Tiada jenis *emm* baru yang dikesan. Secara umum, tidak ada hubungan yang kuat antara penghasilan hyaluronidase, jenis *emm*, kadar invasif dan pembentukan biofilm kerana nilai p adalah lebih daripada 0.05. Hyaluronidase dan penghasilan biofilm dalam GAS mungkin terlibat dalam patogenesis memandangkan hyaluronidase dapat menguraikan asid hyaluronik (HA) yang menjadi lapisan dinding pada sel hos dan perkembangan biofilm membantu bakteria untuk menjadi rintangan antimikrobial terhadap antibiotik. Pelbagai jenis *emm* dalam kajian ini mungkin menandakan strain heterogeniti dalam GAS tempatan. Lebih banyak penyelidikan diperlukan pada masa akan datang untuk mewujudkan hubungan antara faktor-faktor virulence GAS dan biofilmnya.

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LIST OF ABBREVIATIONS

APSGN	acute post-streptococcal glomerulonephritis
ARF	Acute Rheumatic Fever
Asn	Aspargine
ATCC	American Type Culture Collection
BSA	bovine serum albumin
BHI	Brain Heart Infusion
CDC	Centers for Disease Control and Prevention
cep A	Cell envelope protease
Cfa	CAMP factor
CLSM	confocal laser scanning microscopy
CO_2	Carbon Dioxide
CovR/S	control of virulence system
CRA	Congo Red Agar
CVA	Crystal Violet Assay
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediamine tetraacetate
Emm	M-protein gene
Enn	M-like protein
G	Gram
GAGs	Glycosaminoglycans
GAS	group A streptococcus
gm/l	Gram per Litre
gpo A	Glutathione peroxidase

HA	hyaluronic acid
HEp-2	Human epithelial type 2
His	Histidine
hyl A	Hyaluronidase
HylP	Phage encoded hyaluronidase
ideS	IgG-degrading enzyme
IU	International unit
Kb	Kilobyte
kDa	Kilo Dalton
Kg	Kilogram
M	Molar
MA	Moderately adherent
mA	Miliamps
Mac	MAC-1, Sib35, MspA
Mg	Milligram
MHBA	Mueller Hinton blood agar
Ml	Mililiter
Mm	Millimetre
Mrp	M-related protein
NA	Non adherent
NaCl	Sodium chloride
Nga	NAD glycohydrolase
ndoS	EndoS (EndoS2)
ndoS2	EndoS2
nm	Nanometre
OD	Optical density

PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
PIA	polysaccharide intercellular adhesion
PS/A	capsular polysaccharide/adhesion
PSAGN	Poststreptococcal Acute Glomerulonephritis
RHD	rheumatic heart disease
RNase	Ribonuclease
SA	Strongly adherent
sag A	Streptolysin S
scp A	C5a peptidase
sda D2	SdaD2
sibA	SibA
Sic	Inhibitor of complement
Ska	Streptokinase
Slo	Streptolysin O
SNPs	Single-nucleotide polymorphism
Sof	Serum opacity factor
sod A	Superoxide dismutase
Spa	Streptococcal protective antigens
spd 1	SpdI
spe A	Streptococcal exotoxinA
spe B	Cysteine proteinase
SPESs	streptococcal pyrogenic exotoxins
Sse	Secreted esterase
STSS	streptococcal toxic-shock syndrome

TBE	Tris-Borate-EDTA
TE	Tris-EDTA
THY	Todd Hewitt Yeast
Tyr	Tyrosine
U	Atomic mass weight
V	Volt
WA	Weakly adherent
w/v	Weight/ volume
β	Beta
µg	Micro gram
µl	Microliter

CHAPTER 1

INTRODUCTION

1.1 Introduction

According to Center for Diseases Control and Prevention (CDC), non-invasive bacteria are the pathogens that does not spread onto organ or tissues of the host body and can be found at wound, throat and pus. Whereas, invasive bacteria are the pathogens that able to invade deep tissues, spinal cord, brain and bloodstream as this type of invasiveness often associated with meningtis (infection to the brain or spinal cord) and sepsis (infection to the bloodstream). Meanwhile, hyaluronidase is an enzyme capable of degrading hyaluronic acid, a major or sole component of the capsular material of certain bacteria as well as being the major component of the extracellular matrix of body tissues (Laurent and Fraser, 1992). In order for bacteria to survive in harsh environments, some bacterial species developed a defence mechanism that was referred as biofilms. It has an exopolysaccharide matrix layer which is composed of water, polysaccharides and many extracellular components (Sutherland, 2001). Whereras, *emm* typing is a modern molecular approach that uses the polymerase chain reaction and DNA sequencing of the *emm* gene encoding the M protein (Steer, et al., 2009).

Streptococcus pyogenes or group A streptococcus (GAS) is one of Gram-positive pathogens that is responsible for causing many diseases ranging from pharyngitis, cellulitis, pneumonia, necrotizing fasciitis and toxin-mediated conditions such as streptococcal toxic-shock syndrome (STSS) and acute post-streptococcal glomerulonephritis (APSGN). According to Markowitz, (1994), since the development of antibiotics, GAS infection has now become less harmful but this devastating illness is still prevalent in many developing countries. For instance, 1.78 million people have been infected with invasive GAS strains in 2005 (Carapetis, *et al*., 2005). In addition, 2.21 million children in Asia, ranging from 5 to 14 years have been diagnosed with rheumatic heart disease (RHD) caused by GAS (Carapetis, 2008).

GAS itself produces many compounds known as virulence factors that are involved in its pathogenesis. Apart from the ability of producing a large repertoire of exotoxins, GAS also produces hyaluronic acid (HA) which adheres to the mucosal surface and thwarts phagocytosis by neutrophils (Kreil, 1995). However, its structure mimics the structure of mammalian hyaluronic acid, which is a known substrate for streptococcal hyaluronidase or hyaluronate lyase. Hyaluronic acid can be found in most human tissues such as skin. Thus, this enzyme is commonly known as a spreading factor as it is able to cleave the hyaluronic acid and enables the pathogen to easily invade human tissues by increasing the tissue permeability to fluids (Sandson, *et al*., 1968). In snake and insect venoms, hyaluronidase plays a major role by degrading host hyaluronic acid thus alllowing the spread of toxin (Kreil, 1995). Meanwhile, hyaluronidase produced by *Clostridium perfringens* contributes to the initiation of its spread in tissues by degrading

hyaluronic acid in connective tissues (Canard, *et al*., 1994). Based on these observations and the tendency for GAS to spread rapidly in soft tissues, it has been widely assumed that its hyaluronidase could serve a similar function (Hynes, 2004). Ironically, only certain serotypes of GAS can produce bacterial hyaluronidase, and whether its production is related to invasiveness has not been well explored (Hynes, *et al*., 2000).

In addition, biofilm is considered as one of the virulent factor produced by *S. pyogenes*. Nonetheless, little is known on the contribution of GAS biofilms to human disease. Moreover, bacteria that produce biofilm would have its own phenotypic characteristics due to an alteration in the growth rate and the level of gene transcription (Donlan and Costerton, 2002). A bacterial biofilm is defined as a sessile microcolony that is enclosed in the slime-like matrix and tends to attach with each other or at any surface layer including tissue membrane or medical devices. Mainly, the biofilm or slime is considered as the protection mechanism for the microorganism in terms of their pathogenicity. Surprisingly, the available data in the literature gives conflicting results. A study has shown that *emm* types and invasiveness could play an important role in biofilm formation (Kalia and Bessen, 2004). In contrast, the development of biofilm was not related to the GAS serotypes as reported in another study (Lembke *et al.*, 2006). Thus, it would be interesting to know whether certain *emm* types are associated with GAS invasiveness, hyaluronidase production and biofilm formation among the local strains.

An effective method is needed for the identification and typing of GAS in terms of understanding the pathogenicity and epidemiology of these bacteria. The serotyping system which was developed by Lancefield (1928) is a system that is based on the antigenic dissimilarity at the membrane surface of the group A streptococcus, specifically on *emm* types. This protein plays an important role in the pathogenesis of GAS. M-typing scheme is an old method used to characterize the GAS by using antisera, but now it has largely been substituted with *emm* typing scheme. This *emm* typing is based on the different sequences of *emm* genes that encode the M protein. It is now considered as an important epidemiological marker in GAS infection (Kreikemeyer, *et al*., 2004). Moreover, this *emm* typing could offer the identification of predominant *emm* types among local GAS strains that might be important in the development of potential vaccines in future.

Investigation of hyaluronidase in GAS is an important as other virulence factors because the ability of the enzyme that potentially aid in invasion into many parts of host tissue cells. Whereas, biofilm protect bacteria from antimicrobial treatment and the emm types that encodes the cell surface M virulence protein. Lack of studies conducted especially in Malaysia on the particular topic thus making this investigation relevant to interpret the local data with other data globally. Potential usage of the hyaluronidase GAS has also not much investigated. As many previous research shows a potential of others hyaluronidase members in commercial applications including therapeutic and cancer diagnostics. As the investigation on GAS hyaluronidase might also promise potential application in term of commercial application fields. To fill the gap of knowledge, this study was design to identify the phenotypic and genotypic characteristics of GAS hyaluronidase and the correlation with invasiveness, emm type and biofilm formation. The findings may benefits for future research on virulence determinants and improve understandings of pathogenesis of GAS infection.

1.2 Objectives

1.2.1 General Objective

The general objective of this study is to determine inter relationship production of bacterial hyaluronidase and its molecular characteristics and biofilm formation as well as *emm* typing among GAS clinical isolates.

1.2.2 Specific Objectives

The specific objectives of this study are as follows:

i. To determine the production of bacterial hyaluronidase among invasive and non-invasive *S. pyogenes* clinical isolates by plate assay method.
ii. To determine the presence of bacterial hyaluronidase genes among invasive and non-invasive *S. pyogenes* clinical isolates by PCR.
iii. To determine the relationship between bacterial hyaluronidase with *emm*-typing patterns among invasive and non-invasive *S. pyogenes* clinical isolates.
iv. To determine the enzymatic activity of bacterial hyaluronidase by enzymatic assay.
v. To determine the formation of biofilms by Congo Red Agar (CRA) and Crystal Violet Assay (CVA) and its relationship with *emm* types among *S. pyogenes* clinical isolates.

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APPENDICES

Appendix A

Ethical Clearance

PEJABAT TIMBALAN NAIB CANSELOR (PENYELIDIKAN DAN INOVASI)

Reff. : UPM/TNCPI/RMC/1.4.18.1 (JKEUPM)/F2
Date : 24th February 2016

Assoc Prof Dr Rukman Awang Hamat
Department of Medical Microbiology
Faculty of Medicine and Health Science
Universiti Putra Malaysia
Serdang, Selangor

Dear Madam/Sir,

RESEARCH PROJECT: HYALURONIDASE DETECTION ASSAYS, HYALURONIDASE GENES AND emm TYPING AMONG INVASIVE AND NON-INVASIVE *STREPTOCOCCUS* PYOGENES CLINICAL ISOLATES

RESEARCHER : WAN MUHAMMAD ZAMIR BIN WAN MANSOR
SUPERVISOR : ASSOC PROF DR RUKMAN AWANG HAMAT

The Ethics Committee for Research involving Human Subjects of University Putra Malaysia (JKEUPM) has studied the proposal for the above project and found that there were no objectionable ethical issues involved in the proposed study.

Please find the list of documents received and reviewed with reference to the study and committee members who reviewed the documents (as attached)

Notwithstanding above, we will not be responsible for any misconduct on the part of researcher in the course of carrying out the research.

Thank you.

"WITH KNOWLEDGE WE SERVE"

Sincerely yours,

PROF. DATO' DR. ABDUL JALIL NORDIN
Chairperson
Ethics Committee for Research involving Human Subjects (JKEUPM)
Universiti Putra Malaysia

Pejabat Timbalan Naib Canselor (Penyelidikan dan Inovasi), Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
Pejabat Timbalan Naib Canselor (PA) (603-8947 1283 603-8945 1641), Pejabat Pengarah TNCPI (603-8947 1408 603-8963 1673),
Pejabat Pengurus, Pusat Pengurusan Penyelidikan (RMC) (603-8947 1401 603-8945 1656), Pejabat Pengurus, Putra Science Park (PSP)
(603-8947 1291 603-8946 4121 http://www.tncpi.upm.edu.my

Appendix B

Preparation of Media, Buffers and Reagents

TE buffer	a.10Mm Tris-HCL, pH 8.0 (1.31g Tris-HCL in 200ml of distilled water at room temperature and adjusted to pH to 8.0) b. 1mM Na-EDTA, pH 8.0 (add 0.05g Na-EDTA in 200ml distilled water and adjusted to the pH to 8.0) c. The buffer was autoclaved at 1210C for 15 min and stored at room temperature
TBE buffer (x10)	108 g/l Tris-base (C14H11NO3) 55 g/L Boric acid (H3BO3), 9.13 g/L Ethylenediamine tetraacetic acid. The above chemicals were dissolved in distilled water and the pH was adjusted to 8.3 adding NaOH until dissolved, then topping up with distilled water to a final volume of 1000ml. The buffer was autoclaved and stored at room temperature.
Lysozyme stock solution	0.5mg of lysozyme powder (Sigma, Germany) was dissolved into 1ml of distilled water (0.5mg/ml), It was sterilized by using 0.5µm filter paper and stored at room temperature.
0.85% (w/v) Nacl	0.125g of Nacl was measured and dissolved in 100ml of distilled water. The prepared saline was autoclaved and stored at room temperature
Mueller-Hinton broth	500ml of distilled water was measured into a clean 1L bottle, measure using a graduated cylinder. 12.5g of Dehydrated LB broth was added to the bottle and was dissolved using magnetic stirrer. It was Autoclaved and stored in -4oC fridge.
70% (v/v)ethanol	70 ml of absolute ethanol was measured into a dispensing container. 30 ml of distilled water was added to make up 100ml of 70% ethanol

Appendix C

GAS Hyaluronidase Gene (*HylA, HylP1, HylP2, HylP3*) Nucleotide Homology Blast Through NCBI Database

1). *Streptococcus pyogenes* extracellular hyaluronate lyase (*hylA*) gene, complete cds
Sequence ID: AF218838.1 Length: 3510 Number of Matches: 1
Range 1: 1786 to 2186

Score	Expect	Identities	Gaps	Strand
708 bits(383)	0.0	396/402(99%)	1/402(0%)	Plus/Minus

```
Query  3     TGTAGTGTCCTAAATCGTTATTGTATAGGTAAAACATTCCATCAGAAGTAAACCAGCCAT  62
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2186  TGTAGTGTCCTAAATCGTTATTGTATAGGTAAAACATTCCATCAGAAGTAAACCAGCCAT  2127
Query  63    GAAGATTTTCATTATTCATAGCTTCATAATTTTGAGTTCGATTCGAAAACATTGATAGGC  122
             ||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  2126  GAAGATTTTCATTATTCATAGCTTCATAATTTTGAGTTCGATTCGAAAACATGGATAGGC  2067
Query  123   CAAAAGCAAAATCGTGTTTATTATTATATAGTGCCAATTTATCCATACTATTGAAACTAG  182
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2066  CAAAAGCAAAATCGTGTTTATTATTATATAGTGCCAATTTATCCATACTATTGAAACTAG  2007
Query  183   CTACGTAACTATCAAGTTTTTGGACTGGAACAAAAGTATCACTTAGTAGTTCTTTCATAA  242
             |||||||||||||||||||||||||||||||| ||||||||||||||| ||||||||||
Sbjct  2006  CTACGTAACTATCAAGTTTTTGGACTGGAACAGAAGTATCACTTAGTAGCTCTTTCATAA  1947
Query  243   GTTTGATATCGTGATAGGTTTTCAAATTATCATAGACATTGTAAAAAGCATTCCCTTGTG  302
             |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  1946  GTTTGATATCGTGATAGGTTTTCAAATTATCATAGACATTGTAAAAAACATTCCCTTGTG  1887
Query  303   TGACGAGTGTTTTTATTCGTGTTTTAAGTGCCAAACGGTGAGGCTCTTCAGACATGTCAG  362
             ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1886  TGACGAGTGTTTTTATACGTGTTTTAAGTGCCAAACGGTGAGGCTCTTCAGACATGTCAG  1827
Query  363   CAATACGTAAAATAGCACGAAGTGCTTCAATGCCCAGCAACA  404
             |||||||||||||||||||||||||||||||||| |||||||
Sbjct  1826  CAATACGTAAAATAGCACGAAGTGCTTCAATGCC-AGCAACA  1786
```

Query=sequence of *S. pyogenes* isolate
Subject= GenBank sequence for AF218838.1

2). Bacteriophage H4489A (from GAS) hyaluronidase (*hylP1*) gene, complete cds
Sequence ID: M19348.1 Length: 3222 Number of Matches: 1

Range 1: 646 to 853

Score	Expect	Identities	Gaps	Strand
351 bits(190)	3e-93	202/208(97%)	0/208(0%)	Plus/Plus

```
Query  2    AGGTGCTGCTATGGTGATGTATACAAATAAAGATACTACTGATGGACCATTGATGATTTT  61
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  646  AGGTGCTGCTATGGTGATGTATACAAATAAAGATACTACTGATGGACCATTGATGATTTT  705
Query  62   ACGCTCTGACAAAGATACGTTTGATCAGTCAGCTCAATTTGTGGATTACAGAGGTAAGAC  121
            ||| ||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  706  ACGTTCTGACAAAGATACGTTTGATCAGTCAGCTCAATTTGTGGATTACAGCGGTAAGAC  765
Query  122  TAATGCTGTAAATATTGTAATGCGTCAGCCAAGCACACCTAATTTTTCCTCAGCACTTAA  181
            |||||||||||||||||||||||| ||||||||| ||||||||||||||| ||||||||
Sbjct  766  TAATGCTGTAAATATTGTAATGCGCCAGCCAAGCGCACCTAATTTTTCCTCGGCACTTAA  825
Query  182  TATAACCAGTGCTAACGAAGGCGGTAGT  209
            |||||||||||| ||||||||||||||||
Sbjct  826  TATAACCAGTGCCAACGAAGGCGGTAGT  853
```

Query=sequence of *S. pyogenes* isolate
Subject= GenBank sequence for M19348.1

3). *Streptococcus pyogenes* hyaluronidase (*hylP2*) gene, complete cds
Sequence ID: U28144.1 Length: 1197 Number of Matches: 1

Range 1: 902 to 1043

Score	Expect	Identities	Gaps	Strand
233 bits(126)	6e-58	137/142(96%)	1/142(0%)	Plus/Plus

```
Query  3     ACTC-ACCTCGGGCACGACAGGGAAGTTGCTTAGGATTAGAAACCTTGGTGATGATAAGT  61
             |||| ||||| ||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  902   ACTCAACCTCAGGCACGACAGGGAAGTTGCTTAGGATTAGAAACCTTAGTGATGATAAGT  961
Query  62    TCTACGTCAAGCCTGACGGTGGTTTTTATGCCAAGGCAACTTCGCAGATTGATGGCAACC  121
             |||||||||| |||||||||||||||||||| |||||||||||||||||||||||||||
Sbjct  962   TCTACGTCAAGTCTGACGGTGGTTTTTATGCCAAGGAAACTTCGCAGATTGATGGCAACC  1021
Query  122   TGAAACTCAAGGACCCCACAGC  143
             |||||||||||||||||||||
Sbjct  1022  TGAAACTCAAGGACCCCACAGC  1043
```

Query=sequence of *S. pyogenes* isolate
Subject= GenBank sequence for U28144.1

4). *Streptococcus pyogenes* NZ131, complete genome (*HylP3*)
Sequence ID: CP000829.1 Length: 1815785 Number of Matches: 2

Range 1: 781550 to 78183

Score	Expect	Identities	Gaps	Strand
516 bits(279)	1e-142	286/289(99%)	1/289(0%)	Plus/Minus

```
Query  1       ATCGCT-CTCCTACGGAAGATGAGGGTTTAATACCACTTTTATTAGGTTTAAACTGTAGT  59
               |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781838  ATCGCTCCTCCTACGGAAGATGAGGGTTTAATACCACTTTTATTAGGTTTAAACTGTAGT  781779
Query  60      TGTCCTGTCACTATGCCGCCTGTCAAACTCAATTTTTCGTCTAGCTTTGCATTTGACTCT  119
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781778  TGTCCTGTCACTATGCCGCCTGTCAAACTCAATTTTTCGTCTAGCTTTGCATTTGACTCT  781719
Query  120     GCTTTTAAGTAAACAGCGTTTTTATCTGCTTTATTTGATTTTAATTCGGTGATTTTACTA  179
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781718  GCTTTTAAGTAAACAGCGTTTTTATCTGCTTTATTTGATTTTAATTCGGTGATTTTACTA  781659
Query  180     TCAGTTTCTTGTTTTCGTGCAAACGCATCTAGATTTGGTTTATTTTGGAGTTGATTATAA  239
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781658  TCAGTTTCTTGTTTTCGTGCAAACGCATCTAGATTTGGTTTATTTTGGAGTTGATTATAA  781599
Query  240     TCTGTCGTTCCAGGCTTGCCAGCAGGGCCCCGAGAACCAGTTCCTCCAG  288
               |||||||||||||||||||||||||||||| |||| ||||||||||||||
Sbjct  781598  TCTGTCGTTCCAGGCTTGCCAGCAGGGCCTCGAGGACCAGTTCCTCCAG  781550
```

Range 2: 1469909 to 1470129

Score	Expect	Identities	Gaps	Strand
239 bits(129)	3e-59	194/225(86%)	6/225(2%)	Plus/Plus

```
Query  1        ATCGCT-CTCCTACGGAAGATGAGGGTTT-AATACCACTTTTATTAGGTTTAAACTGTAG  58
               |||||| |||||   ||||||||   | | ||||||||   |||||||||||| | ||||
Sbjct  1469909  ATCGCTCCTCCTGTAGAAGATGA-CTTCTCAATACCAC---TATTAGGTTTAAGCCGTAG
1469964
Query  59       TTGTCCTGTCACTATGCCGCCTGTCAAACTCAATTTTTCGTCTAGCTTTGCATTTGACTC  118
               |||||||||||||||| |||| |||||||||||||| ||||||||  |    || |||||
Sbjct  1469965  TTGTCCTGTCACTATGCCACCTGCCAAACTCAATTTTTTGTCTAGCTCTATTTTGGACTC
1470024
Query  119      TGCTTTTAAGTAAACAGCGTTTTTATCTGCTTTATTTGATTTTAATTCGGTGATTTTACT  178
               |||||||||||||||||||||||||||||||||  |||||| ||||| ||||||||||||
Sbjct  1470025  TGCTTTTAAGTAAACAGCGTTTTTATCTGCTTTGCTTGATTCTAATTTGGTGATTTTACT
1470084
Query  179      ATCAGTTTCTTGTTTTCGTGCAAACGCATCTAGATTTGGTTTATT  223
               || |||||||| |||| |||||||||| |||||| |||||||||
Sbjct  1470085  ATTAGTTTCTTCTTTTTGTGCAAACGCACCTAGATCTGGTTTATT  1470129
```

Query=sequence of *S. pyogenes* isolate
Subject= GenBank sequence for CP000829.1

BIODATA OF STUDENT

Wan Muhammad Zamir bin Wan Mansor was born on 05th September 1992 in Kuala Terengganu, Terengganu, Malaysia. He graduated with Bachelor of Science (Plant Science) from Universiti Malaysia Sarawak (UNIMAS) in 2014. His Master Degree was sponsored by Ministry of Education Malaysia and Universiti Putra Malaysia under Graduate Research Fellowship (GRF). He is currently working at the Prime Minister's Department (JPM) in Putrajaya as a senior assistant coordinator and holding the post of deputy secretary-general in Majlis Kebajikan dan Sukan Anggota-anggota Kerajaan Malaysia (MAKSAK).

PUBLICATIONS

Poster presentation:

Mansor, W.M.Z.W., Hamat, R. A., (2016, December). Hyaluronidase Detection Assay and Hyaluronidase Genes Among Invasive and Non-Invasive *Streptococcus Pyogenes* Clinical Isolates. Poster presented at the 33rd Symposium of the Malaysian Society for Microbiology (MSM2016), Grand Ballroom, Ramada Plaza Melaka.