CHARACTERISATION AND SUBTYPE DISTRIBUTION OF *Blastocystis* sp. IN CATTLE: ITS ASSOCIATION WITH POTENTIAL RISK FACTOR AND PROTEASE ACTIVITY

BY

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ABSTRACT

Blastocystis sp. is an eukaryotic protozoan with 17 distinctive subtypes discovered in animals and humans worldwide. Cattle is one of important source of animal protein, which shown an increasing demand for its produce due to increased in growth of population especially in developing countries. However, the production is likely to be affected by infection and disease management of this animal. Currently, the zoonotic pathogen of *Blastocystis* sp. from cattle was identified with multiple concurrent infections with rates of infection as high as 80%. Unlike human, animal particularly cattle infected with *Blastocystis* sp. are commonly healthy carriers and serve as a main reservoir in transmitting the infection to human. Though, *Blastocystis* exhibited protease activity that suggest its pathogenicity, but its effect on host remain unclear. Thus, this study was aimed to determine occurrence of *Blastocystis* sp. isolated from cattle and subtypes variation in the protease activity for better understanding of the host-parasite relationships and effect of location and farm management on *Blastocystis* infection. A total of 120 faecal samples of cattle were collected from three farms in Pahang, Malaysia for *in-vitro* cultivation and microscopy identification. The gender and age of the cattle as well as management system of the farms were also noted during the sampling. Later, DNA extracted from the faecal were subjected to genotyping analysis before protease activity of three selected subtypes were determined using azocasein assay of colorimetric quantification method. The study found 30 out of 120 (25%) cattle infected with *Blastocystis* sp. with vacuolar as the dominant form observed during cultivation. While gender has no association with the occurrence of *Blastocystis* sp. and cattle of age below 3 months as well as Muazam Shah farm with integrated system were significantly ($p<0.05$) associated with the infection. Molecular genotyping revealed that *Blastocystis* ST10 (21.3%) occurred predominantly in the cattle with ST1 (2.5%), ST3 (7.5%), ST4 (2.5%), ST5 (8.8%) and ST14 (1.3%). Phylogenetic analysis found that these subtypes were closely related and had shared common ancestors with high homologous sequences of genetic relation. ST3, ST5 and ST10 exhibited inter-and intra-subtype quantitative protease activity variation, which mainly expressed cysteine protease and partially serine protease, aspartic protease and metallo-protease in the respective subtypes. In conclusion, moderate rates of *Blastocystis* sp. infection with six different subtypes were identified in the cattle. The farm management systems, cleaning and sanitation as well as segregation condition influence the distribution of *Blastocystis* sp. infection which significantly associated with age and condition of the farms. While, protease activity were commonly been reported in ST3, ST4 and ST7, this study has discovered in the ST3, ST5 and ST10 of *Blastocystis*. The variant observed in the protease activity indicate that isolates of different subtypes may exhibit different pathogenic condition upon exhibition of diseases, yet suggests for more studies needed in the future. Nevertheless, this study presented updates on the occurrence of *Blastocystis* sp. in cattle from Pahang, Malaysia. This information is important in understanding host-parasite relationship associated with gastrointestinal diseases and identification of possible virulence factor in the future.

خلاصة البحث

إن المتبرعمة الكيسية هي إحدى الكائنات الأولية حقيقية النواة، وهي مكونة من 17 نوعًا فرعيًا متميزًا تم اكتشافها في الحيوانات والبشر في جميع أنحاء العالم. تعد الماشية أحد المصادر المهمة للبروتين الحيواني، والتي أظهرت زيادة في الطلب على منتجاتها بسبب زيادة النمو السكاني خاصة في البلدان النامية. ومع ذلك فإنه من المرجح أن يتأثر إنتاج الماشية بانتشار العدوى وطريقة إدارة الأمراض. يتم التعرف على العامل الممرض الحيواني المنشأ للمتبرعمات الكيسية في الماشية بواسطة الالتهابات المتزامنة المتعددة وذلك بمعدل إصابة يصل إلى 80%. على عكس الإنسان، فإن الحيوانات وخاصة الماشية المصابة بالمتبرعمات الكيسية عادة ما تكون ناقلات لها وبصحة جيدة وتعمل بمثابة خزان رئيسي في نقل العدوى إلى الإنسان. على الرغم من ذلك فقد أظهرت المتبرعمات الكيسية نشاطا في إنزيم البروتياز والذي يشير إلى احتمال تسببيه للمرض، ولكن تأثيره على المضيف لا يزال غير واضح. ولذلك هدفت هذه الدراسة إلى تحديد وجود المتبرعمات الكيسية المعزولة من الماشية وتحديد أنواعها الفرعية في نشاط إنزيم البروتياز من أجل فهم أفضل للعلاقة بين الطفيل والمضيف ووتحديد تأثير الموقع وإدارة المزرعة على عدوى المتبرعمة الكيسية. تم جمع 120 عينة برازية من الماشية من ثلاث مزارع في ولاية باهانج في ماليزيا للاستزراع داخل المختبر وللتحديد المجهري. تم أيضًا تدوين جنس وعمر الماشية وكذلك نظام إدارة المزرعة أثناء أخذ العينات. في وقت لاحق تم تحليل الحمض النووي المستخرج من البراز للتنميط الجيني قبل أن يتم تحديد نشاط إنزيم البروتياز لثلاثة أنواع فرعية باستخدام اختبار الآزوكاسين للقياس الكمي اللوني. وجدت الدراسة أن 30 من 120 (25%) من الماشية كانت مصابة بالمتبرعمة الكيسية حيث كانت الفجويات الشكل المهيمن لها أثناء الاستزراع. لم يكن للجنس أي علاقة بوجود المتبرعمات الكيسية، وكانت الماشية التي تقل أعمارها عن 3 أشهر ومزرعة ذات النظام المتكامل مرتبطة بشكل كبير ($P<0.05$) بالعدوى. كشف التنميط الجيني الجزيئي أن المتبرعمة الكيسية ST10 (21.3 %) كانت موجودة في أغلب الماشية، والفروع الأخرى كما يلي: ST1 (2.5%)، و ST3 (7.5%)، ST4 (2.5%)، و ST5 (8.8%)، و ST14 (1.3%). وجد التحليل الوراثي أن هذه الأنواع الفرعية كانت مرتبطة ارتباطًا وثيقًا، وكان بينها أسلافًا مشتركة مع متسلسلات متجانسة عالية للعلاقة الوراثية. أظهرت ST3، و ST5، و ST10 تباينا في نشاط إنزيم البروتياز الكمي داخل وخارج الفرع، والذي عبر بشكل رئيسي عن بروتياز السيستين وبروتياز السيرين جزئيا، والبروتياز الأسبارتي، والبروتياز المعدني في الأنواع الفرعية المذكورة. ختاما، فقد تم التعرف في هذه الدراسة على معدلات معتدلة من عدوى المتبرعمة الكيسية بستة أنواع فرعية مختلفة في الماشية، وكان لأنظمة إدارة المزرعة والتنظيف والصرف الصحي وكذلك حالة العزل أثر على انتشار المتبرعمة الكيسية العدوى المرتبطة بشكل كبير مع عمر الماشية وحالة المزرعة. بينما تمت ملاحظة نشاط البروتياز بشكل شائع في ST3 و ST4 و ST7، اكتشفت هذه الدراسة المتبرعمة الكيسية أيضا في ST3 و ST5 و ST10. يشير المتغير الملحوظ في نشاط إنزيم البروتياز إلى أن المعزولات من الأنواع الفرعية المختلفة قد تظهر حالات مرضية مختلفة عند إظهارها للمرض، ومع ذلك فإنه يقترح إجراء المزيد من الدراسات اللازمة في المستقبل. ومع ذلك فقد قدمت هذه الدراسة تحديثات على وجود المتبرعمات الكيسية في الماشية في ولاية باهانج في ماليزيا. هذه المعلومات مهمة في فهم العلاقة بين المضيف والطفيل المرتبطة بأمراض الجهاز الهضمي وتحديد عامل الضراوة المحتمل في المستقبل.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Health Science (Biomedical Science)

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DECLARATION

I hereby declare that this thesis 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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This thesis is dedicated to my family and friend for laying the foundation of what I turned out to be in life

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%	Percentage
cm	Centimetre
ml	Millilitre
µl	Microliter
bp	Base pair
°C	Celsius
rpm	Revolutions per Minute
min	Minutes
mg	Milligram
sec	Seconds
ng/µl	Nanogram per Microliter
kV	Kilovolt
mAH	Milli amp hour
m	Meter
mm	Millimetre
mM	milliMolar
°C/min	Celsius per Minutes
ml/min	Millilitre per Minutes
mg/ml	Milligram per Millilitre
gm	Gram
±	Plus or Minus
Igs	Immunoglobulins
y/o	years old

LIST OF ABBREVIATION

ASEAN	Association of Southeast Asian Nations
BLAST	Basic Local Alignment Search Tool
BSC	Body Score Condition
CDC	Centers for Disease Control
DF	Dilution Factor
DMEM	Dulbecco Modified Eagle Medium
DNA	Deoxyribonucleic acid
DVS	Department of Veterinary Service
EDTA	Ethylenediaminetetraacetic acid
et al.	(et alia): and others
FAO	Food and Agriculture Organization (United Nations)
FECT	Formalin-Ether Concentration Technique
FIA	Fluorescent Immunoassays
GDP	Gross Domestic Product
HIV/AIDS	Human Immunodeficiency Viruses and Acquired Immune Deficiency Syndrome
IA	Iodoacetamide
IBS	Irritable Bowel Syndrome
LE	Locke's Egg
MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum Likelihood
MLST	Multilocus Sequence Typing
MTZ	Metronidazole
NCBI	National Centre Biotechnology Information

NL	Neighboring Joining
NTZ	Nitazoxanide
OD	Optical Density
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
Pep A	Pepstatin A
PMSF	Phenylmethylsulphonyl fluoride
qPCR-HRM	Quantitative PCR High-Resolution Melting
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphisms
sp.	Species
SSU rDNA	Small Subunit Ribosomal DNA
SSU rRNA	Small Subunit Ribosomal Ribonucleic Acid
ST	Subtype
STS	Sequence-Tagged Site
TMP-SMX	Trimethoprim / Sulfamethoxazole
UK	United Kingdom
USA	United Stated of America
WHO	World Health Organization
XIVC	Xenic *In-Vitro* Culture

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

The protozoan parasitic infections are major public health concerns worldwide, especially in low income countries such as Africa and Brazil (Hotez et al., 2009). Neglected infections by parasites causes substantial illness for more than one billion people globally. In fact, almost 200 million people in ASEAN countries live in extreme poverty and are exposed to parasitic infection such as leishmaniasis, giardiasis, cryptosporidiosis, toxoplasmosis, malaria or blastocystosis (Hotez et al., 2015). These parasites have gained its attention by impairing human health as well as causing disruptive impact on productivity and stability of the economy (Hotez and Alibek, 2011).

Blastocystis sp. is an enteric protozoan which colonises in humans and animals. It exhibits a genetic diversity, but with low specificity. This parasite has infected more than 1 billion people worldwide (Scanlan and Stensvold., 2013), which infects mainly high risk population including immigrant (Piubelli et al., 2019), immunocompromised individuals such as cancer patients (Mohamed et al., 2017), patient with human immunodeficiency virus (HIV)/Acquired immunodeficiency syndrome (AIDS) (Piranshahi et al., 2017) and children (Rebolla et al., 2016). The infection may cause nonspecific gastrointestinal symptom (Ramirez et al., 2017) and chronic irritable bowel syndrome (IBS) (Ragavan et al., 2015b). Though numerous studies have been carried out in human, many studies has reported of its occurrence of in a wide range of

animals including non-human primates, mammals, amphibians, avian, insects and reptiles (Cian et al., 2017). Nonetheless, several studies in livestock animals revealed high incidence of infection. For example, 100% of rate of infection in chickens (Tanizaki et al., 2005), 95.0% in pigs (Abe et al., 2002) and 80% in cattle (Ramirez et al., 2014) and 58.0% in goats (Song et al., 2017b).

Today, livestock production is one of the fastest growing sector in developing countries. The agricultural ouput has contributed 40% of global value and supported the livelihoods and food security for almost 1.3 billion of people (FAO, 2014-2017). It was estimated that the demand would doubled over the next 20 years, driven by urbanization and population growth. This potentially lead to more infected animals, which became a major threat to global animal health and welfare (Tomley and Shirley, 2009). Some parasitic infection like *Trypanosoma*, *Theileria*, *Babesia*, and *Anaplasma* impacted livestock by affecting their health, growth and development (Maharana et al., 2016). Meanwhile, *Blastocystis* sp. infection that occurred persistently in carrier animals are vulnerable when being transmitted to human (Lee et al., 2018). Hence, surveillance system are required to control and eliminate the distribution of the *Blastocystis* sp. in the early stage.

Potential risks of zoonotic transmission possibly lead to the occupational diseases in agricultural industry. In Malaysia, agriculture sector contributed RM96 billion of the economic activity, which the livestock industry has contributed 11.4% in the year of 2017 (Department of Statistics Malaysia, 2018). Cattle is an important source of animal protein and consumed by more than 60% of the population (Ariff et al., 2015). In 2017, total population cattle has risen from 737,827 in 2016 to 744,174 in 2017 (Department of Statistics Malaysia, 2018) and these figures is expected to rise over the coming year (Abdulla et al., 2016; Lokman, 2018). Hence, the increase in the

cattle population may serve as potential reservoirs of parasitic diseases towards high risk industry workers such as farmers, veterinarians and slaughterhouse workers due to their close contact with the infected animals.

Proteases are known to play important roles in parasites' biology and host-pathogen interactions. Recently, there has been an increased interest in the proteases of *Blastocystis* sp., which proven to have important roles in host cell invasion, catabolism of host proteins, differentiation, cyto-adherence as well as evasion of host immune responses (Cifre et al., 208). Proteases from *Blastocystis hominis* B and WR1 involved in the degradation of immunoglobulin A (Ig A) giving rise to its ability to colonise in the intestine through immunological response alteration as well as induction of barrier dysfunction (Puthia et al., 2008ab; Mirza and Tan 2009). Though, the roles of these enzymes as virulence factor remain debatable, a study conducted on *Blastocystis* subtype 7 (ST7) revealed reliable information on predicted secretory proteins that contain putative activities that may induce host physiology including proteases, particularly cysteine protease (Denoeud et al., 2011) Wawrzyniak et al. (2012) indicated that this enzyme might have cytopathic effects, which disturbed the gut function and lead to intestinal disorders in infected human. For *Blastocystis*, studies have revealed inter-and intra-subtypes variation in protease activity between different isolates of ST4 and ST7, which may results in different cytopathic effects (Mirza and Tan, 2009). Clearly, it was important to determine proteases activity in different genotype of *Blastocystis* sp. and investigate the association with its pathogenicity. Therefore, our study would determine the occurrence and subtype distribution of *Blastocystis* in cattle from Pahang before inter- and intra-subtypes variation in proteases activity were evaluated.

1.2 PROBLEM STATEMENT

Blastocystis sp. has been studied since 1911, yet it still remain as a controversial protozoan especially in its pathogenicity as well as many other biological aspects of this protozoan still remain unexplored. Therefore, additional data on the morphology characteristic, reproductive and mode of transmission are valuable in understanding the biological complex of its pathogenic potential. According to the Centre for Disease Control and Prevention (CDC, 2016), *Blastocystis* sp. is a common parasite organism which inhabits the intestine and has been discovered globally. However, limited data was available on *Blastocystis* infection in cattle from Malaysia. Indeed, there were two studies of *Blastocystis* in cattle in Malaysia (Lim et al., 200; Hemalatha et al., 2014). Current situation, the cattle production has risen to meet the increasing demand of its products, which has lead us to investigate the distribution of *Blastocystis* sp. in the cattle (Department of Statistics Malaysia, 2018). Since, cattle may serve as potential reservoir as well as healthy carrier of *Blastocystis* sp., the rapid growth in the local cattle production may affect health and quality of the animal product as well as increased risk of infection toward human, who work closely with the animal.

Furthermore, the relationship between the occurrence of *Blastocystis* sp. in cattle and potential risk factor such as gender, age groups and types of farm management practices have not been thoroughly reviewed because most of the studies were mainly focused on the occurrence and/or selected risk factor. Given the lack of published data on farm managements and potential risk factor of the *Blastocystis* sp. in the cattle, the present study was undertaken to elucidate more satisfactory data.

In addition, molecular characterisation of *Blastocystis* sp. has never reported from cattle in Malaysia, which eight subtypes from eleven studies of worldwide

distribution have been reported (Zhu et al., 2017; Wang et al., 2018b; Lee et al., 2018; Udonsum et al., 2018). These studies suggested possibility of ST5, ST10 and ST14 that usually be found in mammals including cattle as zoonotic genotypes (Zhu et al., 2017; Cian et al., 2017). In addition, ST1 and ST3 were also suggested by Yoshikawa et al. (2004a) and Ramirez et al. (2014) due to mutual infection in human and the cattle. Though mixed subtypes in animals was uncommonly occur, the transmission might happen in domestic animals and farm personnel due to contaminated water and food. Therefore, the present study was the first to perform identification of genotypic subtype of *Blastocystis* sp. in large samples of cattle aimed at extracting more valuable information regarding distribution and possible source of transmission from three farms in Pahang, Malaysia.

Proteases are enzymes that break peptide bond in proteins and play vital role in the pathogenesis of protozoan parasites especially malaria, trypanosomiasis, and leishmaniasis (McKerrow et al., 2006), amebiasis (Serrano-Luna et al., 2013), toxoplasmosis and cryptosporidiosis (Siqueira-Neto et al., 2018). It has been suggested that by studying proteases activity among subtypes of *Blastocystis* may unraveled the understanding on the capability of this enzyme in causing diseases. Previously, protease activity was reported only in *Blastocystis* ST3, ST4 and ST7 while none on the other subtypes. A study conducted on the most predominant ST3 of human isolates showed higher protease activity in symptomatic than asymptomatic isolates (Abdel-Hameed and Hassanin, 2011). Additionally, *Blastocystis* ST4 isolated from rodents secreted mostly aspartic protease (Puthia et al., 2008a). However, study of protease activity in *Blastocystis* sp. was limited and has never been reported in Malaysia. *Blastocystis* sp. has indicated different level of quantitative inter- and intra-subtypes in protease activity (Mirza and Tan., 2009). Interestingly, it also exhibited

variation peak of protease activity within isolates of the same subtypes. Rajamanikam and Govind, (2013) observed that higher percentage of amoeboid forms in culture elevated the protease activity than culture that mainly consist of vacuolar and granular forms. So, by studying the variation of protease activity that could influence the subtype capability and cell size distribution would contribute towards understanding the host-parasite relationship as well as the pathogenicity of *Blastocystis* sp..

1.3 RESEARCH OBJECTIVE

The aims of this study was to characterise and subtype distribution of *Blastocystis* sp. in cattle regarding its association with potential risk factor and protease activity. The specific objectives were:

i To determine *Blastocystis* sp. occurrence based on location and cattle farm management practices and its cells biological characteristics using cultivation of faecal sample cattle and microscopic evaluation.

ii To investigate the relationship between potential risk factors such as gender, age groups and farm type as well as *Blastocystis* sp. distribution in the cattle.

iii To identify the genotypic subtypes of *Blastocystis* sp. and its phylogenetic relationship using deoxyribonucleic acid (DNA) sequencing analysis.

iv To evaluate the quantitative variation of inter and intra- subtype in protease activity of selected subtypes of *Blastocystis* sp. isolates using azocasein assay.