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PREVALENCE AND SPECIES IDENTIFICATION OF *Cryptosporidium* FROM CATTLE IN KUANTAN PAHANG, MALAYSIA

BY

NUR HAZIRAH BINTI HISAMUDDIN

A thesis submitted in fulfillment of the requirement for the degree of Master of Health Sciences (Biomedical Science)

Kulliyyah of Allied Health Sciences
International Islamic University Malaysia

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ABSTRACT

Cryptosporidium spp. is a protozoan parasite that can cause gastrointestinal infection known as cryptosporidiosis. It is known since 1980s that cattle have been identified as significant hosts of zoonotic *Cryptosporidium.* This research was conducted to determine the prevalence of *Cryptosporidium* from cattle in Kuantan, Pahang and to identify the species of *Cryptosporidium* by using 18S rRNA gene. Besides, this study aims to investigate the association between *Cryptosporidium* infection and the age of cattle. Three hundred and seventy five cattle faecal samples were collected directly from five farms in Kuantan, Pahang. The samples were processed by using formal-ether concentration technique and stained by modified Ziehl-Neelsen. *Cryptosporidium* spp. was identified by nested PCR amplification of 18S rRNA gene. Based on microscopic examination, 16.3% (61/375) cattle were positive for *Cryptosporidium* infection. This research has shown that the highest prevalence of *Cryptosporidium* was recorded in calves with the percentage of 17.4% (12/69), followed by adult cattle and yearling with the percentage of 16.1% (29/180) and 15.9% (20/126), respectively. The findings demonstrated that there was no significant difference ($p > 0.05$) in *Cryptosporidium* infection rates by age and presence of diarrhea. From 61 positive samples detected by microscopic examination, one sample was successfully amplified by nested PCR. The species found was highly similar to *C. ryanae.* The present study suggested that good management practices and proper hygiene management must be practiced by farmers to control the *Cryptosporidium* infection. It is highly important to control the infection since cattle infected with parasites may serve as potential reservoirs of the infection to other animals and humans. Veterinary health agencies should organize health education program for farmers to give information about the risk of the zoonotic diseases. It is recommended that future studies should investigate the subtyping of *Cryptosporidium* for better understanding of sources of infection and the dynamics of cryptosporidiosis transmission.

خلاصة البحث

الكريبتوسبوريديوم هي نوع من الطفيليات الأولية التي تسبب العدوى المعوية المعروفة باسم كربتوسبوريديوسيس أو داء خفيات الأبواغ. من المعروف منذ ثمانينات القرن الماضي أن الماشية معتبرة كأكبر مضيف للكريبتوسبوريديوم الحيواني المصدر. تم إجراء هذا البحث لتحديد مدى انتشار الكريبتوسبيريديوم من الماشية في مدينة كوانتان، باهانج، وللتعرف على نوع الكريبتوسبيريديوم باستخدام جين 18S الريبوسومي. إلى جانب ذلك، هدفت هذه الدراسة إلى التعرف على العلاقة بين عدوى داء خفيات الأبواغ وعمر الماشية. ثلاث مئة وسبعون عينة من براز الماشية تم جمعها من خمس مزارع في كوانتان باهانج. تم تجهيز العينات باستخدام طريقة الفورمول والأثير لتركيز الطفيليات وصبغها بطريقة زيل نلسن المعدلة. تم حفظ العينات المجهرية الإيجابية في 2.5٪ ثاني كرومات البوتاسيوم في درجة حرارة 4 مئوية قبل استخراج الحمض النووي. تم التعرف على الكريبتوسبوريديوم بتفاعل سلسلة البوليميرات (PCR) المضخمة المتداخلة للحمض النووي الريبوزي الريبوسومي 18S. بناء على الفحص المجهري، 16.3% (375/61) من الماشية كانت إيجابية لداء خفيات الأبواغ. أظهرت هذه الدراسة أن أعلى نسبة لانتشار الكريبتوسبوريديوم تم تسجيله في العجول بنسبة 17.4٪ (69/12)، تليها الماشية البالغة بنسبة 16.1٪ (180/29) و الشياه الرضيعة بنسبة 15.9٪ (126/20). أظهرت النتائج أنه لا توجد فروق كبيرة ($P> 0.05$) في معدلات الإصابة بالكريبتوسبوريديوم حسب العمر ووجود الإسهال. من واحد وستين عينة تم اختبارها إيجابيا بالمجهر، تم تضخيم عينة واحدة بنجاح باستعمال تفاعل البوليميراز المتسلسل المتداخل. النوع التي تم العثور يشبه إلى حد كبير إلى سي. رياني (*C. ryanae*). أشارت الدراسة إلى أن الممارسة الإدارية الجيدة والإدارة الصحية المناسبة يجب أن تطبق من قبل المزارعين للسيطرة على عدوى داء خفيات الأبواغ، ومن المهم للغاية السيطرة على العدوى لأن الماشية المصابة قد تكون ناقلا محتملا للعدوى للحيوانات الأخرى وللبشر. من المستحسن أن تحقق الدراسات المستقبلية في التصنيف الفرعي للكريبتوسبوريديوم لفهم أفضل لمصادر العدوى وديناميكية انتقال داء خفيات الأبواغ.

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 dissertation for the Master of Health Sciences (Biomedical Science).

……..
Afzan Mat Yusof
Supervisor

……..
Ridhwan Abdul Wahab
Co- Supervisor

……..
Mardhiah Mohammad
Co- Supervisor

I certify that I have 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 dissertation for the Master of Health Sciences (Biomedical Science).

……..
Hanani Ahmad Yusof @ Hanafi
Examiner 1

……..
Mohamed Kamel Abdul Ghani
Examiner 2

This thesis was submitted to the Department of Biomedical Science and is accepted as a partial fulfilment of the requirements for the Master of Health Sciences (Biomedical Science).

……..
Ibrahim Adham Taib
Head, Department of
Biomedical Science

This thesis was submitted to the Kulliyyah of Allied Health Sciences and is accepted as partial fulfilment of the requirements for the Master of Health Sciences (Biomedical Science).

……..
Wan Azdie Mohd Abu Bakar
Dean, Kulliyyah of
Allied Health Sciences

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.

Nur Hazirah Hisamuddin

Signature ..... Date .....

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Affirmed by Nur Hazirah Hisamuddin

…….……. ……..

Signature Date

This thesis is dedicated to my mother, Nik Mahni Binti Nik Man and my father, Hisamuddin Bin Mohamed for laying the foundation of what I turned out to be in life.

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LIST OF SYMBOLS

~	Approximately
%	Percentage
°C	Degree Celsius
µL	Microliter
µm	Micrometer
µM	Micromolar
bp	Base pair
g	Gram
g	Gravitational Force
hr	Hour
KB	Kilobase
mg	Milligram
min	Minute
mL	Milliliter
mM	Millimolar
ng	Nanogram
pmol	Picomole
rpm	Revolutions per Minute
s	Second

LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
COWP	*Cryptosporidium* oocyst wall protein
DHFR	Dihydropholate reductase
dNTPs	Deoxynucleotide nucleoside triphosphates
DVS	Department of Veterinary Services
et al.	Et alia
HIV	Human immunodeficiency virus
HSP70	Heat shock protein 70
$MgCl_2$	Magnesium chloride
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
polyT	Polythreonine repeat
rRNA	Ribosomal ribonucleic acid
spp.	Species
TBE	Tris/Borate/EDTA
TRAP-C1	Thrombospondin-related adhesive protein 1
TRAP-C2	Thrombospondin-related adhesive protein 2

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Cryptosporidium spp. are apicomplexan protozoan parasites that cause gastrointestinal infection known as cryptosporidiosis. Since its discovery in 1907, the disease has spread in a broad range of vertebrate hosts including human and livestock (Zhang et al., 2013). In human, cryptosporidiosis can cause life threatening disease especially in immunocompromised individuals such as HIV patients (Chalmers & Davies, 2010). The common clinical signs of human and animal cryptosporidiosis are diarrhea, dehydration, fever, nausea and anorexia (Cui et al., 2014). This parasite has been associated with enteric-related illness and disease outbreak in livestock animals (Gormley et al., 2011). In farm animals, cattle are recognized as the most common mammalian species that can be infected by *Cryptosporidium* (Zhang et al., 2013; Cui et al., 2014; Huang et al., 2014). The major *Cryptosporidium* species that infect calves is *Cryptosporidium parvum* (*C. parvum*) which caused severe watery, yellowish and foul-smelling acute diarrhea that may lead to morbidity and mortality (Lendner & Daugschies, 2014).

1.2 PROBLEM STATEMENT

Cattle are recognized as one of the most important livestock around the world. It supplies meat, milk and other dairy products serving approximately 1.5 billion heads globally resulting in a ratio of 1:4.7 (cattle/person) (Robertson et al., 2014). It is

known since 1980s that cattle have been recognized as significant hosts of zoonotic *Cryptosporidium* (Izadi et al., 2014). *Cryptosporidium parvum* (*C. parvum*) is the most important zoonotic species found in a number of animals, especially young farm animals (Xiao, 2010). Cryptosporidiosis may cause anorexia, depression, weight loss, diarrhea, dehydration, high morbidity and in severe cases, mortality due to dehydration (Sahinduran, 2012).

In Malaysia, cattle farming have become an important part of Malaysian agriculture. With 751,497 cattle in 2013, cattle were the second highest population after swine (Department of Veterinary Services, 2013). Consistently, the data of Consumption of Livestock Products reported by Department of Veterinary Services (2013) indicated that the consumption of beef was the second highest after pork for the period of 2004 to 2013 in Malaysia. This data indicates that beef is one of the most important livestock products in Malaysia and among the main source of protein among Malaysian people (Jamaludin et al., 2014). The demand for beef is growing every year due to population growth and increasing per capita consumption (Hashim, 2015). However, one of the challenges in cattle farming is parasitic infestation whereby it can cause morbidity, mortality and eventually economic losses.

Recently, United Nations' Food and Agriculture Organization (FAO) and World Health Organization (WHO) have listed *Cryptosporidium* spp. as one of the top ten foodborne parasites due to its global distribution, acute and chronic morbidity as well as its impact on economy (FAO, 2014). By 2050, FAO and WHO had estimated that food production will increase by approximately 50% to feed an estimated world population of 9 billion people (Innes et al., 2011). Since the occurrence of protozoa among livestock can cause serious diseases, livestock production will be affected which could lead to economic losses (Innes et al., 2011; Fletcher et al., 2012).

Some studies have found an association between age and the presence of *Cryptosporidium* infection (Santín et al., 2004; Silverlas et al., 2010; Maikai et al., 2011; Zhao et al., 2013). The most common mammalian species that can be infected by *Cryptosporidium* is calves which are considered as the main reservoir for zoonotic infections. Nevertheless, the consequences of cryptosporidiosis among these young animals were abandoned and ignored. To date, there are five publications about bovine cryptosporidiosis in five states in Malaysia which are Johor, Negeri Sembilan, Pahang, Perak and Selangor (Halim et al., 2008; Muhid et al., 2011; Yap et al., 2016). Based on these data, it is obviously showed that *Cryptosporidium* had affected cattle in Malaysia. However, many cattle producers overlooked the matter of parasitic infection that usually can infect cattle population. Although previous study has been conducted in Kuantan, Pahang (Yap et al., 2016), however the number of samples in the study is small. Therefore, this study was carried out in larger samples to provide more valuable information regarding cryptosporidiosis among Kuantan cattle.

1.3 RESEARCH OBJECTIVES

The study aimed to achieve the following objectives:

1- To identify the presence of *Cryptosporidium* in cattle from farms located in Kuantan, Pahang.

2- To investigate the relationship between *Cryptosporidium* and age of cattle.

3- To identify the species of *Cryptosporidium* by using 18S rRNA gene.

1.4 RESEARCH HYPOTHESES

The research hypotheses are:

1- There is an occurrence of *Cryptosporidium* infection in cattle in Kuantan area.

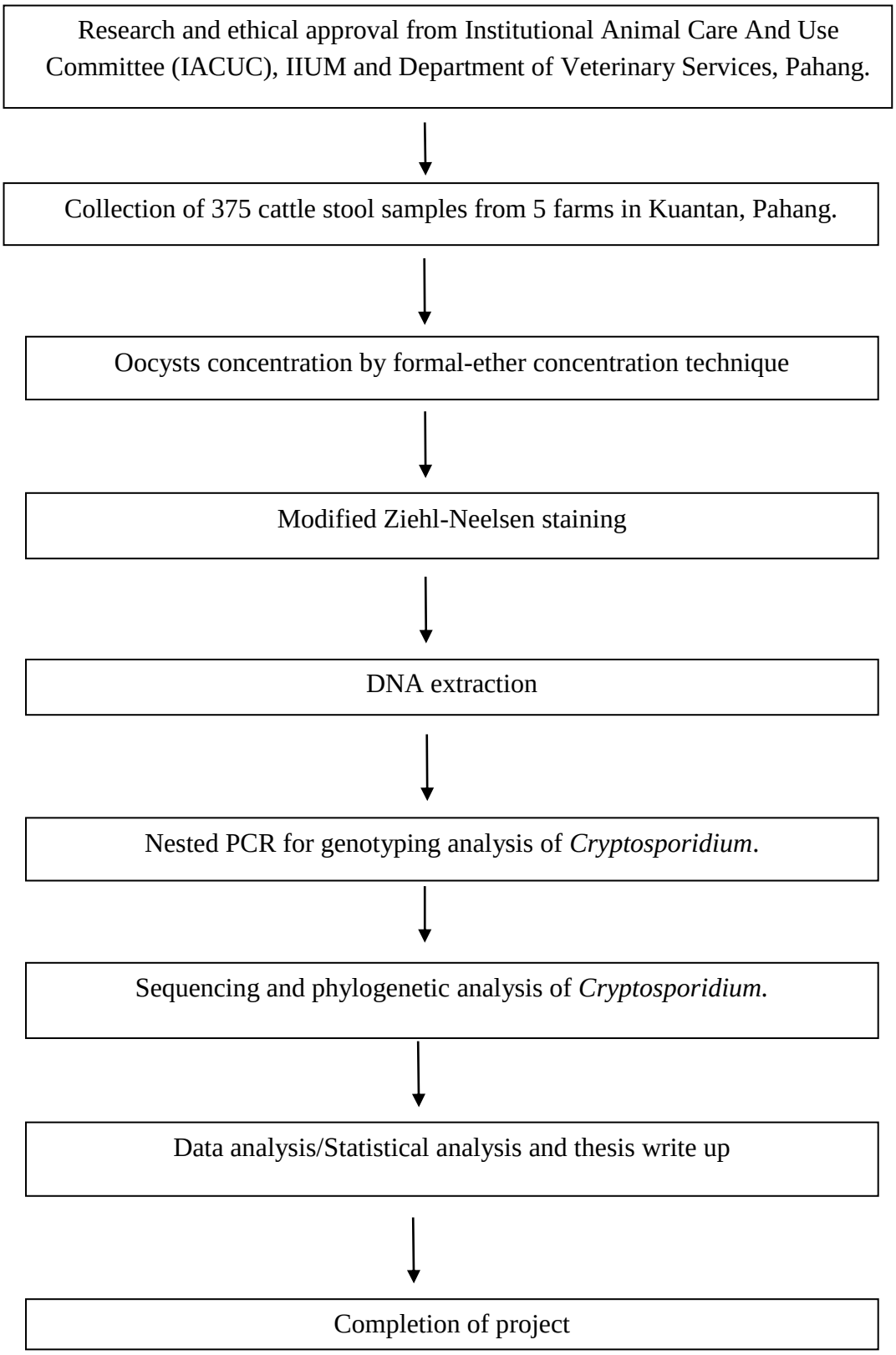
2- There is a significance difference between *Cryptosporidium* infection and age group of the cattle.

3- The identified spesies among the studied cattle are *C. parvum, C. bovis, C. andersoni* and *C. ryanae.*

1.5 SIGNIFICANCE OF STUDY

This study is carried out to provide new information especially for people who are in close contact with farm animals and to characterize the zoonotic potentials of *Cryptosporidium* infection. The results are hoped to supplement the existing information of *Cryptosporidium* infection at national and global region. It is vital to study the prevalence of cryptosporidiosis to understand the transmission of the pathogen and to design effective control measures. The results of this study will be useful to promote proper animal management practices in farms. The findings can be applied and implemented to formulate a better farm practice in order to reduce the infection among cattle and indirectly, preventing economic losses among farmers. Nonetheless, until now there was limited data available on cryptosporidiosis in cattle in Kuantan. Therefore, this research was carried out to determine the prevalence and molecular characterization of *Cryptosporidium* from cattle in Kuantan, Pahang.

1.6 THEORETICAL FRAMEWORK



1.7 CHAPTER SUMMARY

This chapter discussed the general information of cryptosporidiosis in cattle. As *Cryptosporidium* infection raised a significant public health issue, there was a strong justification in conducting this study. In addition, this chapter also outlined the statement of problem, objectives of the research and hypotheses. The significance of this study and theoretical framework of the research were also presented.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

This chapter deliberates the literature review of *Cryptosporidium* on its taxonomy, morphology, modes of transmission, life cycle and its pathogenesis. Besides, this literature review also discusses the epidemiology of *Cryptosporidium* in terms of global prevalence and the prevalence in Malaysia. Furthermore, the review will emphasize on the detection method of the parasites which consists of modified Ziehl-Neelsen staining, polymerase chain reaction and the genotyping of *Cryptosporidium*.

2.2 TAXONOMY

Cryptosporidium belongs to the family of Cryptosporiidae, order of Eucoccidiida, suborder of Eimeriina, class of Sporozoasida, subclass of Coccidia and phylum of Apicomplexa (Lim et al., 2013). Characteristics used to name *Cryptosporidium* species were based on host occurrence, morphology of the parasite, predilection host and the location of infection (Ghazy et al., 2015). Until now, 27 species of *Cryptosporidium* has been discovered worldwide (Ryan et al., 2015). Among those 27 species, the species that are pathogenic to human are *C. parvum* and *C. hominis* (Lim et al., 2013).

The three species in avian are *C. meleagridis, C. baileyi and C. galli*, 19 species in mammals are *C. parvum*, *C. hominis*, *C. wrairi*, *C. felis*, *C. muris*, *C. canis*, *C. suis*, *C. andersoni*, *C. bovis*, *C. xiaoi*, *C. fayeri*, *C. macropodum*, *C. ryanae*, *C.*

ubiquitum, *C. cuniculus*, *C. tyzzeri*, *C. viatorum*, *C. scrofarum* and *C. erinacei*, one species in amphibian is *C. fragile*, two species in reptiles are *C. serpentis* and *C. varanii*, and two species in fish are *C. molnari* and *C. huwi* (Ryan & Xiao, 2014; Ryan et al., 2014, 2015).

2.3 MORPHOLOGY OF *Cryptosporidium*

The morphological classification of *Cryptosporidium* by light microscope is difficult as the size of *Cryptosporidium* is small (Fall et al., 2003). In addition, the oocyst of *Cryptosporidium* is small with 4-6 µm in diameter (Ridley, 2012; Rossle & Latif, 2013). *Cryptosporidium* oocysts are smooth, colourless and spherical to slightly ovoid in shape (Smith, 2008). The sporulated oocyst contains four crescent shaped sporozoites surrounded by tough two-layered wall (Fayer et al., 2010). The contents of oocyst are hard to visualize by light microscope. To confirm the presence, 100x objective magnification must be used to measure the small size of oocysts (Smith, 2008).