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EVALUATION OF MIR-21 AND MIR-145 EXPRESSION IN *CRYPTOSPORIDIUM PARVUM* INFECTED HCT-8 AND HT-29 CELL LINES

BY

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ABSTRACT

Cryptosporidium parvum is a widespread pathogenic parasite which causes cryptosporidiosis in human. The host cell-*C. parvum* interaction causes alteration of expression of a series of microRNAs or miRNAs within the host cells due to activation of defense mechanism. In immunocompromised host cells, miRNAs play a key role in post-transcriptional gene regulation. The regulation of miRNAs in the infected cells may be identified as possible biomarkers in cancer onset and progression. Upregulation of oncomicroRNAs in the host epithelial cells due to the *C. parvum* infection may lead to colorectal cancer initiation and progression in human. HCT-8 and HT-29 were grown in different L-glutamine concentration and pH to observe the relative growth. The study was aimed to delineate the host epithelial colorectal cancer cells-*C. parvum* interaction and its associated miRNAs involvement in the immunocompromised host cells *in vitro*. The differential expression level of oncomiRNA, miR-21 and tumor suppressor miRNA, miR-145 in host cells upon *C. parvum* infection was observed in order to establish a relationship between *C. parvum* infection and colorectal cancer in humans. As *Cryptosporidium* mainly infects the epithelial cells of the colorectal region, the host cells of interest in this study are HCT-8 and HT-29 cell lines. The direct immunofluorescent staining method has been applied using fluorescein conjugated *Vicia Villosa* Lection (VVL) to observe the *C. parvum* infection sites on HCT-8 and HT-29 cell lines after 2.5 hours of infection period. In addition, scanning electron microscopy has been used to observe the surface micrographs of different life stages of *C. parvum* adhered to the cell lines. The real time quantitative PCR (RT-qPCR) was performed to observe the relative expression of miR-21 and miR-145 in infected HCT-8 and HT-29 cell lines. Data normalization of the miRNA expression was carried out using reference gene RNU44. The result showed that, the cell growth was optimum for cell lines in higher L-glutamine concentration and higher pH. In the immunofluorescent staining assay, the micrographs show multiple sites of *C. parvum* infection on both HCT-8 and HT-29 cell lines. Meanwhile, scanning electron micrographs demonstrated the adherence of *C. parvum* oocysts in both HCT-8 and HT-29 cell lines after 24 hours of inoculation. Along with the surface meront I, several numbers of early trophozoites in their developmental stages were confined to the apical surfaces of HCT-8 and HT-29 cell lines. Besides, a few sporozoites have been observed to be attached on the HCT-8 and HT-29 cell lines. The normalized expression quantification data obtained from RT-qPCR has shown upregulation of miR-21 expression and significant downregulation of miR-145 expression in HCT-8 and HT-29 cell lines upon *C. parvum* infection. The miR-21 expression showed significantly higher upregulation in infected HCT-8 than in infected HT-29. On the other hand, miR-145 downregulation was significantly lower in HT-29 cells than in HCT-8 cells upon *C. parvum* infection. In this study, immunofluorescent micrographs and scanning electron micrographs indicated that *C. parvum* effective infects human colorectal cancer cell lines. The upregulation of oncomiRNA and downregulation of tumor suppressor miRNA in infected HCT-8 and HT-29 cells possibly indicates the influence of *C. parvum* in cancer progression. However, the findings are less sufficient to confirm the *C. parvum* as a pathogenic parasite for cancer progression. Future studies should be aimed to observe more targeted genomic and transcriptomic alterations, relative expression of sets of targeted miRNAs to absolutely verify the progression of colorectal cancer by *C. parvum* infection.

خلاصة البحث

طفيل كريبتوسبوريديوم برفيم هو طفيل ممرض واسع الانتشار يسبب داء خفيات الأبواغ للإنسان. الخلية المضيفة للطفيل
) داخل الخلايا المضيفة بسبب تفعيل miRNAsتتفاعل معه فيسبب تغير في التعبير الجيني لسلسلة ميكرو اران اي (
آلية الدفاع. في خلايا المضيف التي تعاني من نقص المناعة، تلعب الميكرو اران اي دورًا رئيسيًا في معالجة الحمض
النووري الريبوزي. ان الميكرو اران اي في الخلايا المصابة يمكن أن يستعمل كمؤشر حيوي محتمل على بداية السرطان
وتطوره. يمكن أن يؤدي النشاط المفرط لميكرو اران اي إلى سرطان القولون والمستقيم. في هذه التجربة تم تمنية خلايا
ودرجات حموضة مختلفة لمراقبة النمو. هذه الدراسة هدفت L-glutamine في تراكيز HT-29 و HCT-8
إلى تحديد الخلية المضيفة لطفيل كريبتوسبوريديوم برفيم المسبب لسرطان المستقيم وتفاعلات الميكرو اران اي المرتبطة بها
في الخلية المضيفة miR-145 و miRNAفي الخلايا المضيفة في المختبر. وقد لوحظ مستوى تعبير كلا من
للطفيل ووجود علاقة بين الإصابة بالطفيل وبين سرطان القولون في الإنسان. وبما أن الطفيل يصيب بشكل أساسي
الخلايا الطلائية في منطقة القولون والمستقيم ، فإن الخلايا المضيفة التي في محل اهتمامنا في هذه الدراسة هي خلايا
السرطانية. تم إجراء اختبار طريقة الصبغ الفلورية المناعيه باستخدام الفلورسين مع فيشيا HT-29 وHCT-8
بعد ساعتين ونصف من الإصابة HT-29 و HCT-8) لمراقبة مواقع الطفيل على خلايا VVLفيلوسا لاكتين (
بالعدوى. بالإضافة إلى ذلك ، تم استخدام المجهر الإلكتروني لمتابعة دورة حياة الطفيل في الخلايا المزروعة. تم إجراء
و miR-21) لمراقبة التعبير النسبي لـ RT-qPCRكذلك اختبار تفاعل البوليميراز المتسلسل اللحظي (
المصابة. تم التأكد من البيانات باستخدام الجينات المرجعية HT-29 و HCT-8 في خلايا miR-145
ودرجة L. كما أظهرت النتائج أن أوساط النمو المناسبة للخلايا كانت في تراكيز مرتفعة للجلوتامين RNU44
كما HT-29 و .HCT-8الحموضة. في اختبار الصبغ الفلوري، تظهر مواقع متعددة للطفيل على كلاً من
بعد 24 ساعة HT-29 و HCT-8أظهر المسح الميكروسكوبي التصاق جرثومات الطفيل في كل من خلايا
،كان بعضاً من النواشط الطفيليه في مراحل تطورها تقتصر على السطوح القمية meront Iمن التلقيح. بجانب
HT- و HCT-8 ولوحظ وجود عدد قليل من البويضات الطفيلية على خلاياHT-29 و .HCT-8من
وانخفاض كبير في miR-21 زيادة تنظيم تعبير RT-qPCR. أظهرت البيانات التي تم الحصول عليها من 29
ارتفاعا عالياً miR-21 في الخلايا المصابة بالطفيل. أظهر تعبير HT-29 وHCT-8 في miR-145تعبير
أقل بكثير miR-145. من ناحية أخرى ، كان انخفاض تنظيم HT-29 المصابة منه أكثر من HCT-8في
. في هذه الدراسة ، أشار المسح المناعي ومسح ميكروسكوب الإلكترون HCT-8 من خلايا HT-29في خلايا
HT- و HCT-8 في miRNAإلى أن الطفيل يؤثر في خلايا سرطان القولون والمستقيم. نستنتج من اختبارات
إلى وجود تأثير فعال للطفيل في الإصابة بسرطان القولون وتطوره. النتائج غير كفاية للتأكد من أن طفيل29
كريبتوسبوريديوم برفيم كطفيل ممرض يمكن أن يسبب السرطان. يجب أن تهدف الدراسات المستقبلية إلى مراقبة التغيرات
الجينومية والتعبيريه ، والتعبير النسبي لميكرو رنا للتحقق بشكل كافي من علاقة الطفيل بتطور سرطان القولون والمستقيم

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 Sciences.

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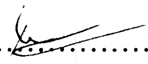

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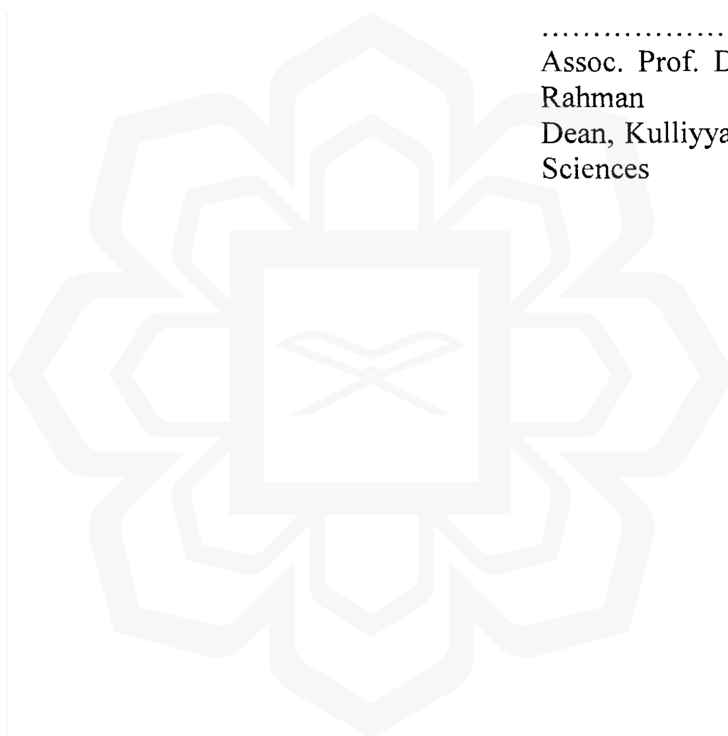

DECLARATION

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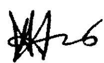

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LIST OF SYMBOLS

~	Approximately
˚C	Degree celsius
%	Percent
<	Less than
>	Greater than
µg	Microgram
µL	Microliter
µm	Micrometer
CO_2	Carbon dioxide
g	Gravitational force
h	Hour
HCL	Hydrochloric acid
mL	Milliliter
mM	Millimole
NaOH	Sodium hydroxide
ng	Nanogram

LIST OF ABBREVIATIONS

Ago2	Argonaute-2
ATP	Adenosine Triphosphate
B7-H1	B7 homolog 1
BTG2	B-cell translocation gene 2
CCL20	C-C motif chemokine ligand 20
cDNA	complimentary deoxyribonucleic acid
CIS	Cytokine-inducible src homology 2 protein
DGCR8	DiGeorge Syndrome Critical Region 8
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-mesenchymal transition
FBS	Fetal bovine serum
GalNAc	N-acetyl-D-galactosamine residues
GTP	Guanosine Tri-phosphate
HBSS	Hank's Balanced Salt Solution
HDACs	Histone deacetylases
HDMS	Hexamethyldisilazane
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HS	Horse serum
ICAM-1	Intercellular adhesion molecule-1

ICZN	International Code of Zoological Nomenclature
IFA	Immunofluorescent assay
IFN-γ	Interferon gamma
IGF	Insulin-like growth factor
IGF-IR	Insulin-like growth factor receptor and insulin receptor
IL	Interleukin
IRS-1	Insulin receptor substrate-1
KSRP	KH-type splicing regulatory protein
MAPK	Mitogen-activated protein kinase
miRNA	microRNA
mRNA	messenger RNA
MYD88	Myeloid differentiation primary response 88
NAD	Nicotinamide Adenine Dinucleotide
NF-κB	Nuclear factor kappa B
NLRs	Nucleotide binding oligomerization domain-like receptors
PACT	Protein activator of PKR
PBS	Phosphate buffer saline
PDCD4	Programmed cell death 4 gene
PGE2	Prostaglandin E2
PRRs	Pattern recognition receptors
PTEN	Phosphate and tensin homolog
pre-miRNA	Precursor miRNA
pri-miRNA	Primary microRNA
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid

RPMI-1640	Roswell Park Memorial Institute- 1640
RREB	Ras-responsive element-binding protein
RT-qPCR	Real time (quantitative) polymerase chain reaction
SCID	Severe combined immunodeficient
SEM	Standard error mean
SEM	Scanning electron microscopy
SIRT1	Sirtuin-1
SOCS	Suppressors of cytokine signaling
STAT1	Signal transducer and activator of transcription 1
TLR4	Toll like receptor 4
TLRs	Toll-like receptors
TMEM49	Transmembrane protein 49
TNF-α	Tumor necrosis factor-α
TRBP	TAR RNA binding protein
UV	Ultraviolet
VGEF	vascular endothelial growth factor
VVL	*Vicia villosa* lectin
YES	Yamaguchi sarcoma viral oncogene

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Cryptosporidium parvum (*C. parvum*) is a species of genus *Cryptosporidium*, commonly known waterborne and food borne parasite (Ortega & Sterling, 2018; Osman et al., 2018). Even though the parasite is ubiquitously found in water, livestock and even in vegetables planted in wastewater, the harmful effect of *Cryptosporidium* is often being neglected (Domenech et al., 2018). It has been reported that *Cryptosporidium* can potentially cause cryptosporidiosis in human and animal (Sharifuzzaman et al., 2015; Yusof & Isa, 2017; Xiao & Cama, 2018). Commonly, cryptosporidiosis is gastrointestinal abnormalities aroused after *Cryptosporidium* infection which is characterized by short-term or long-term diarrhea with abdominal pain (Rossle et al., 2013; Benschop et al., 2017).

In general, the *Cryptosporidium* oocysts are hardy cysts which can survive in the relatively extreme environmental conditions (Young et al., 2015). *C. parvum* is a pathogenic *Cryptosporidium* species in human, generally ingested orally via water or contaminated food and once ingested, the oocysts move to the small intestine and release sporozoites which infect at the intestinal microvilli, especially in the area of jejunum and ileum (Ryan & Hijjawi, 2015). Several *Cryptosporidium* species including *C. parvum* remain in the lower intestinal tract in human for up to five weeks (Omoruyi et al., 2014).

Colorectal parasitosis is the parasitic invasions in the colon that exhibits consequences of bowel diseases (Hechenbleikner & McQuade, 2015; Tin &

Wiwanitkit, 2015). The apicomplexan parasite *Cryptosporidium* can potentially infect the gastrointestinal tract of both normal and immunocompromised individuals (Varughese et al., 2014). In immunocompetent individuals, *Cryptosporidium* invasion leads to diarrhoea but such infection could be life threatening for immunocompromised patients (Dehkordy et al., 2010; Rossle & Latif, 2013).

Apart from causing diarrhoeal disease, *Cryptosporidium*'s ability as pathogen that could initiate and progress colon cancer was not taken seriously until the beginning of the twentieth century. In 2007, for the first time, *C. parvum* was found to be a potential pathogen to initiate colorectal cancer (CRC) in severe combined immunodeficient (SCID) mice gut (Certad et al., 2007). The study observed that *C. parvum* infection has led to the formation of adenocarcinoma lesions and polyps in the gut of dexamethasone-treated SCID mice (Certad et al., 2007). Later on, *in vivo* study on mice model delineated that *Cryptosporidium* infection can induce intestinal dysplasia (Abdou et al., 2013), gastrointestinal neoplasia (Certad et al., 2010, Benamrouz et al., 2012), digestive adenocarcinoma (Certad et al., 2010) and ileocaecal adenocarcinoma (Benamrouz et al., 2014).

Colorectal cancer is the third most widespread malignancy in the world (Siegel et al., 2014; Arnold et al., 2017). According to report, every year there are more than 1.4 million new cases of colorectal cancer around the world and causes about 700,000 deaths (Arnold et al., 2017). According to the Malaysian National Cancer Registry Report 2007-2011, colorectal cancer is the second most common cancer in Malaysia with 13,693 reported cases, accounting for 13.2% of the total cancer incidents (Azizah et al., 2016).

In colorectal cancer patients, the susceptibility of *Cryptosporidium* infection is higher than in normal individuals (Sulżyc-Bielicka et al., 2012; Osman et al., 2017). A

recent study documented a high prevalence (23%) of *Cryptosporidium* infection in the stool samples of 108 colorectal cancer patients in a medical university in Poland (Sulżyc-Bielicka et al., 2018). However, there is still a lack of understanding on the role of *Cryptosporidium* in cancer progression. In this context, it is important to observe the infectivity of *C. parvum* in colorectal cancer cell lines. The progression or initiation of colorectal cancer requires multiple genetic and epigenetic alteration within the cells (Sulżyc-Bielicka et al., 2012). Dysregulation of certain genes expression causes the transformation of normal cells to cancerous cells, or the cancerous cell starts to rapid progression (Kastan & Bartek, 2004).

A potential biomarker for colorectal cancer is microRNAs (miRNAs), which refers to a group of small noncoding RNAs (Luo et al., 2011; Thomas et al., 2015; Carter el al., 2017). In the human body miRNAs are only 1-3% of the total genes, but interestingly, this small fraction synchronizes more than 30% of all human genes (Slaby et al., 2008). The miRNAs control multiple gene expressions and consequently influence in the cancer regulation in human and animals (Hayes et al., 2014; Gambari et al., 2016). Meanwhile, the oncogenic microRNAs (oncomiRNAs) refer to the oncogenes that promotes tumor growth while the tumor suppressor miRNAs act as tumor suppressor genes to inhibit the oncogenic function of the cells (Ariff et al., 2017; Gambari et al., 2016).

The infection of *C. parvum* on the human epithelial cell lines causes alteration of a wide range of microRNAs expression profile (Zhou et al., 2012). The most common infection site of the *C. parvum* is the human gut, especially the ileum of the small intestine. However, the consequences of *C. parvum* infection is life-threatening in immunocompromised patients especially colorectal cancer patients (Dehkordy et al.,

2010; Rossle & Latif, 2013). Hence, it is important to understand the miRNAs regulation in colorectal cell lines upon *C. parvum* infection.

There are several microscopic detection methods available to identify the host-parasite interaction. Immunofluorescent assay is the most popular and convenient technique to detect the host-parasite interaction. On the other hand, scanning electron microscopy assay provides the surface images that can be helpful to understand the different stages of life cycles of *C. parvum* after certain period of inoculation to the cell lines.

1.2 STATEMENT OF THE PROBLEM

In an immunocompetent host, apicomplexan parasite *C. parvum* may constitute self-limited diarrheal disease (Ali et al., 2014). In this regard, an incompetent immunity profile is not able to defend an immunocompromised host from the parasite infection. Consequently, *C. parvum* infection could be life threating in immunocompromised individuals such as HIV and cancer patients (Benamrouz et al., 2014).

The most common site of *Cryptosporidium* infection is the cells in the gastrointestinal tract epithelium in vertebrates including humans (Sulżyc-Bielicka et al., 2018). However, *Cryptosporidium* has ability to travel from intestine to extra-intestinal area such as lung and liver especially in immunocompromised patients (O'Hara et al., 2011). Hence, beside intestinal cryptosporidiosis, few cases of extra-intestinal cryptosporidiosis have also been reported in immunocompromised patients (Reina et al., 2016; Gupta et al., 2018). A recent clinical study reported a 14-years old girl with nephrotic syndrome who was diagnosed with *Cryptosporidium* in the gall bladder (Gupta et al., 2018). Moreover, *Cryptosporidium* can travel from the intestine to the respiratory tract and infect the tract (Sponseller et al., 2014), and at a hospital in Uganda,

HIV infected children were diagnosed with *Cryptosporidium* in their saliva and cough (Mor et al., 2010). However, compared to the cases of intestinal cryptosporidiosis, cases of hepato-biliary and respiratory cryptosporidiosis are very rare.

Parasitic infection in the epithelial cell lines triggers numerous genetic and epigenetic changes within the cells (Schmid Hempel, 2011). In a cancerous cell line, the infection may cause rapid progression of cancer. Hence, observation of the changes in regulation of genes in cancer cells after *C. parvum* infection would help to understand the relative changes in multiple cellular functions. The miRNAs are the potential controller in the synchronization of numerous genes and regulate disease biology (Zhang, 2008; Li & Kowdley, 2012), and the regulation of oncomiRNAs and tumor suppressor miRNAs effectively influence in development of chronic diseases including cancer (Adams et al., 2017).

In most previous *in vitro* experiments, hepatic or lung cancer cell lines were used to observe the miRNA expression profile (Zhou et al., 2009; Hu et al., 2010; Gong et al., 2011; Zhou et al., 2012; Zhou et al., 2013; Guesdon et al., 2015). However, the primary infection site of the *Cryptosporidium* is colorectal region of the human and the colorectal cancer patients are more frequently infected by this parasite (Sulżyc-Bielicka et al., 2018). Consequently, more *in vitro* studies should be performed using colorectal cancer cell lines for *C. parvum* infection model and the observation of regulation of oncomiRNAs and tumor suppressor miRNAs is important to justify the role of *C. parvum* in the progression of cancer.

Based on what discussed above, the current study was designed to quantify the regulation of an oncomiRNA (miR-21) and a tumor suppressor miRNA (miR-145) upon *C. parvum* infection in two colorectal cancer cell lines (HCT-8 and HT-29). Moreover, to support the infection effectivity of *C. parvum* in host colorectal cell lines and to

observe the different developmental stages of life cycle of *C. parvum* the immunofluorescent assay and the scanning electron microscope assay have been performed in this study. Additionally, cancer cells are rapidly growing cells with higher energy demand and L-glutamine is a potential energy supplier in the *in vitro* cell growth media. As being cancer cell lines, HCT-8 and HT-29 are dependent on L-glutamine concentration to meet energy demand in the form of adenosine triphosphate by L-glutamine metabolism. In addition, the cells undergo aerobic glycolysis which releases huge lectic acid that effects the extracellular pH condition largely (Huang et al., 2013). Hence, cancer cell growth in lower pH will reduce the level beyond the minimum range i.e. pH 5.6 (Tannock & Rotin, 1989; Griffiths, 1991) and affect the cell growth. Therefore, in this current study the growth of the HCT-8 and HT-29 cells in different concentration of L-Glutamine and pH was determined.

1.3 RESEARCH HYPOTHESIS

Colorectal cancer cells are in the class of rapidly growing cells and require high energy to continue cell growth. Hence the cell growth will be higher in higher concentration of L-glutamine in the media. As being a potential pathogenic and monoxenous parasite, *C. parvum* should be appeared to infect in the immunofluorescent and scanning electron microscopy. The *C. parvum* infection leads to inflammation on the epithelial cells. Therefore, it was hypothesized that, the oncogenic microRNA miR-21 would be upregulated while miR-145 would be downregulated in both HCT-8 and HT-29 upon infection.

1.4 OBJECTIVES OF THE STUDY

1.4.1 General Objective

The general objective of this research is to quantify the changes in miRNA regulation upon *C. parvum* infection in the colorectal cancer cell lines which will help to understand the fatal consequences of *C. parvum* infection in the colorectal cancer patients.

1.4.2 Specific Objectives

This study is aimed to achieve the following specific objectives:

1. To observe the growth of HCT-8 and HT-29 cell lines in different L-glutamine concentration at different pH.
2. To detect the *C. parvum* infection in HCT-8 and HT-29 cell lines via immunofluorescent microscopy and to observe different developmental stages of *C. parvum* infection on cell lines using scanning electron microscopy.
3. To observe miR-21 and miR-145 expression of HCT-8 and HT-29 colorectal cancer cell lines infected with *C. parvum* by using SYBR Green real-time quantitative polymerase chain reaction (RT-qPCR).
4. To compare expression of miR-21 and miR-145 in between HT-29 cell line and HCT-8 infected with *C. parvum*.

1.5 SIGNIFICANCE OF THE STUDY

C. parvum is a very common pathogen which causes infection in the human and animal intestine. It has been established that *C. parvum* produce cryptosporidiosis in humans and some recent studies have also suggested the role of *C. parvum* in the progression of