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MOLECULAR IDENTIFICATION *OF CRYPTOSPORIDIUM* SPP. FROM RABBITS IN BAGHDAD CITY, IRAQ

Israa N. Hussein Al-Dahhan and Zainab R. Zghair*
Department of Parasitology, Zoonosis unit, College of Veterinary Medicine, University of Baghdad, Iraq
*Corresponding author Email: zzghair@yahoo.com

Abstract

Molecular study was done to detection *Cryptosporidium* species *C. cuniculus* and *C. parvum* in 100 selected out of 180rabbits fecal samples. The results showed significant variation (P≤0.01) between infection rate by using molecular: 38% (38/100) and 26% (26/100) and microscopic methods26% (26/100) and 2.8% (5/100) in rabbits, respectively. There were significant differences in the rate of infection between age groups of rabbits, the highest rate of infection recorded in rabbits with age group of1-3 months 28.1%, and the lowest 25.9% recorded in 4-12 months age group. The rate of infection with *Cryptosporidium* in rabbits affected with sex, male and female recorded 21.4% and 29.09%, 41.6% and 40% respectively.

Keywords: Molecular identification, *Cryptosporidium spp.*, rabbits

Introduction

The apicomplexan protozoan parasite *Cryptosporidium* is mainly infect the gastrointestinal tract and, occasionally, the respiratory system of vertebrates (Xiao *et al.,* 2004), including mammals, reptiles, birds and fish (Chen *et al.*, 2002). *Cryptosporidium spp.* is important zoonotic protozoa, infecting at least 260 species of vertebrate hosts, including humans (Fayer, 2010). *Cryptosporidium* is the worldwide disease may have been the first to observe a species in 1895 by Edward Ernst Tyzzer who described spores lying upon the gastric epithelium of mice (Tyzzer, 1910). At present, the genes *Cryptosporidium* has about 30 species formally described and more than 60 genotypes and subtypes (Xiao and Fayer, 2008). *Cryptosporidium* spp. due to their resistance to common disinfection methods such as chlorination, itis a major threat to potable water systems (Jiang *et al.,* 2005). *Cryptosporidium* oocysts can remain infective in salt and freshwater for months, and the oocysts can also survive for months outside its host (Sunnotel *et al.,* 2006). Cryptosporidiosis can be acquired by direct contact with infected individuals or animals or contaminated fomites or by ingestion of contaminated food and water. (Fayer, 2010). Molecular study was conducted for detection and differentiated *Cryptosporidium* spp. infections in rabbits have been used Polymerase Chain Reaction technique (PCR), Various polymerase chain reaction-based methods have been developed and have the advantage of both is rapid, highly sensitive detection and specific identification (Xiao *et al.,* 2004).

Materials and Methods

Animals and samples collection

The (180) fecal samples were collected in clean plastic containers, and were tightly closed. The study involved from different location of Baghdad city, then the samples were transported in cool boxand divided into two parts for traditional examination to the Parasitology Laboratory, College of Veterinary Medicine, University of Baghdad, and samples stored in refrigerator in -20°C till use for DNA extraction.

Polymerase chain reaction (PCR)

The PCR technique was performed for detection *Cryptosporidium cuniculus* and *Cryptosporidium parvum* based 18S ribosomal rRNA gene from rabbit stool samples. This method was carried out according to method described by (Yu *et al.,* 2009).

PCR master mix preparation

PCR master mix was prepared by using (Maxime PCR PreMix Kit).And this master mix done according to company instructions as following table:

PCR Master mix	Volume
DNA template 5-50ng	5µL
18SrRNA Forward primer (10pmol)	1µL
18SrRNA Reverse primer (10pmol)	1µL
PCR water	13 µL
Total volume	20µL

After that these PCR master mix component that mentioned in table above placed in standard Maxime (PCR PreMix) that containing all other components which needed to PCR reaction such as: (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$, stabilizer, and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at (3000rpm for 3 minutes). Then placed in PCR Thermocycler.

PCR Thermocycler Conditions

PCR thermocycler conditions by using convential PCR thermocycler system as following table:

PCR step	Temp.	Time	Repeat
Initial Denaturation	95ºC	5min.	1
Denaturation	95 ºC	30sec.	30 cycle
Annealing	58 ºC	30sec	
Extension	72 ºC	1min.	
Final extension	72 ºC	5min.	1
Hold	4 ºC	Forever	-

Statistical Analysis:

The Statistical Analysis System- SAS (2012) program was used to detect the effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study

Results

1. Results molecular technique (Nested PCR) in rabbits

One hundred fecal samples were isolated from 180 samples collected from rabbits and screened for *Cryptosporidium* infection using traditional microscopic examination (sugar flotation and modified Ziehl-Neelsen staining method) resulted in 20% (20/100) were positive for *C. cuniculus* infection and3% (3/100) were positive for *C. parvum*. While by using molecular technique (Nested PCR) the results showed that the total infection rate of *Cryptosporidium cuniculus* in rabbits was 38% (38/100), *Cryptosporidium parvum* 26% (26/100) the statistical analysis showed high significant differences between these two techniques and its relation with sensitivity and specificity of each diagnostic technique (P≤0.01) and Chi-Square (χ^2) of *Cryptosporidium* infection with conventional and molecular techniques appeared as *C. cuniculus* 6.162 and *C. parvum* 8.027as in (Table 1).

Table 1 : Total prevalence of *Cryptosporidium* infection by conventional and molecular techniques (Nested PCR) in rabbits

Host	No. of samples examined		Traditional microscopy		Molecular (PCR)		Chi-Square (χ^2)
Rabbit	Total No. (PCR)	Total No. Traditional	No. of positive	%	No. of positive	%	
C. cuniculus	100	100	20	20	38	38	6.162 **
C. parvum	100	100	3	3	26	26	8.027 **
** (P≤0.01).							

2. Molecular detection of *Cryptosporidium* spp. in rabbits:

2.1. Nested PCR product analysis:

Genomic DNA samples obtained from rabbit's fecal samples were subjected to molecular analysis by nested PCR using small subunit ribosomal RNA gene specific primers in order to identify the species of *Cryptosporidium*. Nested PCR of all 100 samples employed in the study exhibited distinct, the PCR product analysis of 18S rRNA gene in *Cryptosporidium* where marker (1500-100bp) and Lane (1-10) some positive *Cryptosporidium cuniculus* were showed at (581bp) PCR product while marker (1500-100bp) and Lane (1-10) some positive *Cryptosporidium parvum* were showed at (540bp) PCR product (Fig. 1 and Fig. 2).

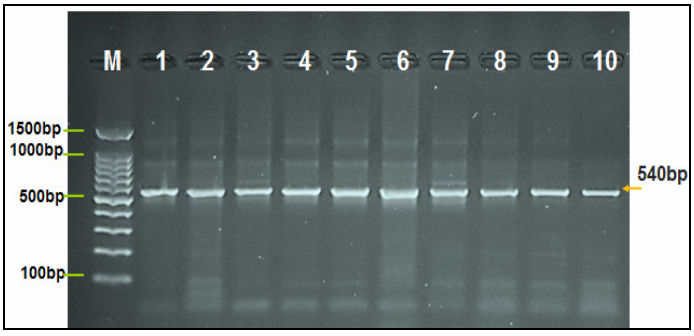

Fig. 1 : Agarose gel electrophoresis image that showed the PCR product analysis of 18S rRNA gene in *Cryptosporidium parvum* from rabbit feces samples. Where M: marker (1500-100bp) and Lane (1-10) some positive *Cryptosporidium parvum* were showed at (540bp) PCR product.

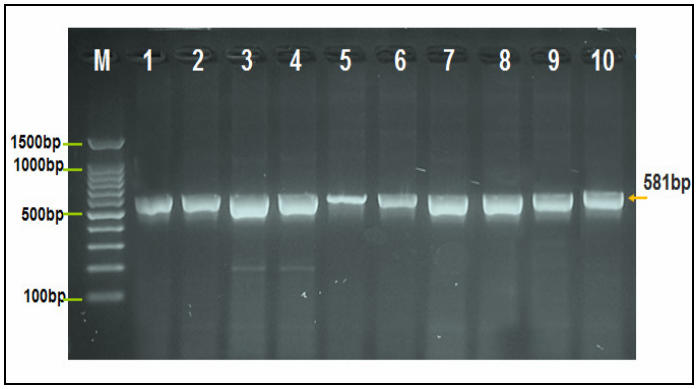

Fig. 2 : Agarose Gel electrophoresis image that showed the PCR product analysis of 18S rRNA gene in *Cryptosporidium cuniculus* from rabbit feces samples. Where M: marker (1500-100bp) and Lane (1-10) some positive *Cryptosporidium cuniculus* were showed at (581bp) PCR product.

3- Molecular prevalence of *Cryptosporidium* by using (nested PCR):

3.1. Molecular prevalence in relation to sex

The results of nested PCR, in 13rabbits male were found infected with *Cryptosporidium* out of 36 examined with a prevalence rate(36%). While recorded 25 rabbits female were found infected with *Cryptosporidium* out of 64examined with a prevalence rate (39%). Statistically, between male and female there was non-significant differences existed with prevalence of *Cryptosporidium* infection by nested PCR as in (Table 2).

Table 2 : Prevalence of *Cryptosporidium* infection by nested PCR in relation to sex groups of rabbits

Sex of rabbit	No. of samples examined	Positive samples	
		No.	%
Male	36	13	36
Female	64	25	39
Total	100	38	38
Chi-Square (χ^2)	---	---	NS:0.963
NS: Non-Significant.			

3.2. Molecular prevalence in relation to age groups

The results in rabbits showed a significant difference (P≤0.05)in the prevalence rates among different age groups. The highest infection rate was observed at 6-12 Months age group 40% (32/80), but the lowest at age group 1 – 6months which showed 30% (6/20)and Chi-Square (χ^2)4.371 as in (Table 3).

Table 3 : Prevalence of *Cryptosporidium* infection in relation to age groups by nested PCR

Age groups of rabbit	No. of samples examined	Positive samples	
		No.	%
1- 6 Months	20	6	30
6-12 Months	80	32	40
Total	100	38	38
Chi-Square (χ^2)	---	---	4.371 *
* (P≤0.05).			

Discussion

Cryptosporidium cuniculus (previously rabbit genotype) was first described in rabbits by Inman and Takeuchi (1979), who described the microscopic detection and ultra-structure of endogenous Cryptosporidium parasites in the ileum of an asymptomatic female rabbit. The rabbit genotype was first identified in rabbits from the Czech Republic (Ryan *et al*., 2003) and *C. cuniculus* was formally re-described as a species in 2010 (Robinson *et al.,* 2010). *Cryptosporidium cuniculus* oocysts were infectious for weanling rabbits, immunosuppressed Mongolian gerbils and immunosuppressed Porton mice but not neonatal mice (Robinson *et al*., 2010). *Cryptosporidium cuniculus* has a close genetic relationship with *C. hominis* with limited differences at the 18S rRNA, HSP70 and actin genes (0.51 %, 0.25 % and 0.12 %, difference, respectively) and is known to infect humans. In 2008, it was responsible for a human cryptosporidiosis outbreak in the UK (Chalmers *et al.,* 2009), which has raised considerable awareness about the importance of investigating rabbits in drinking water catchments as a source of Cryptosporidium transmissible to humans. In the UK reported that *C. cuniculus* was the third most commonly identified *Cryptosporidium species* in patients with diarrhea (Chalmers *et al.,* 2011). *Cryptosporidium cuniculus* has also been identified in a human patient in France and in children in Nigeria (Anon, 2010). The *Cryptosporidium* rabbit genotype is a common parasite infecting farmed rabbits in Henan, China. Although there has not been any reported human infection with the parasite in China, its genetic similarity to *C. hominis* and the recent finding of the parasite in humans in the United Kingdom indicate that rabbits can be a potential reservoir of zoonotic cryptosporidiosis. More systematic biologic characterizations of the parasite are needed to understand the taxonomic status of the *Cryptosporidium* rabbit genotype and its public health significance (Al-Biaty, 2002;Al Neaimi, 2019 and Ke Shi *et al.,* 2010).

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