

Molecular detection of *Cryptosporidium parvum* in chicken in Al-Diwaniya province

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Abstract

This study were conducted in Al-Diwaniya province, in south Iraq during the period from February to July 2019 to determine the rate of infection of *Cryptosporidium parvum* in domestic chicken, study the effect of some epidemiological factors such as sex and months on the rate of infection, addition to the molecular identification of *Cryptococcus parvum* by amplification HSP70 gene by conventional PCR. Number of collected fecal sample was 210 from domestic chicken and stained by Ziehl-Neelsen stain. The results of the microscopic examination showed that 108(51.4%) out of 210 fecal samples were infected with *Cryptosporidium spp.* The statistical analysis founded no marked difference in prevalence of infection between sexes. Significant difference was recorded between infection rate during the months of the study and higher prevalence of infection rate was observed in March 11.9%, while lowest infection rate was observed in July 5.23% . and June 5.23% . Genomic DNA was extracted from 108 fecal samples and HSP70 gene for *C. parvum* was amplified by PCR. PCR technique is showed that out of 108 fecal samples 21.3% were positive for *Cryptosporidium parvum*.

Keywords: *Cryptosporidium parvum*, Domestic chicken, PCR, Al-Diwaniya

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التشخيص الجزيئي لطفيلي الأبواغ الخبيثة في الدجاج في محافظة الديوانية

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الخلاصة

كان الهدف من هذه الدراسة التي أجريت في محافظة الديوانية، في جنوب العراق خلال الفترة من شهر شباط إلى شهر تموز ٢٠١٩ هو تحديد مدى انتشار الأبواغ الخبيثة في الدجاج المنزلي ودراسة تأثير بعض العوامل الوبائية مثل الجنس والأشهر على معدل الإصابة، بالإضافة إلى ذلك التشخيص الجزيئي لطفيلي الأبواغ الخبيثة من نوع بارفم من خلال تضخيم جين بروتين الصدمة الحرارية ٧٠ بوساطة تفاعل سلسلة البلمرة التقليدية. تم جمع مائتين وعشرة عينات من براز الدجاج المنزلي ولونت باستخدام ملون زيهيل نيلسن. كانت نتائج البحث المجهرية ١٠٨ (٥١,٤%) من أصل ٢١٠ عينة برازية مصابة بأنواع الأبواغ الخبيثة. أوضح التحليل الإحصائي للبيانات انه لا يوجد فرق معنوي في نسبة الإصابة بين الجنسين. تم تسجيل فرق معنوي بين معدل الإصابة خلال شهور الدراسة ولوحظ ارتفاع معدل انتشار العدوى في شهر آذار ١١,٩%، بينما لوحظ انخفاض معدل الإصابة في حزيران ٥,٢٣% وشهر تموز ٥,٢٣%. تم استخلاص الحمض النووي الجيني من ١٠٨ عينة براز وتم تضخيم جين بروتين الصدمة الحرارية ٧٠ لطفيلي الأبواغ الخبيثة من النوع بارفم بوساطة تفاعل سلسلة البلمرة. أظهرت نتائج تفاعل سلسلة البلمرة أنه من أصل ١٠٨ عينة برازية كانت ٢١,٢% منها موجبة لطفيلي الأبواغ الخبيثة من النوع بارفم.

Introduction

Cryptosporidiosis is an infection caused by parasite which belongs to genus *Cryptosporidium* that parasitizes fish, amphibians, reptiles, birds and mammals (1). *Cryptosporidium* spp is parasite belonging to phylum Protozoa, Apicomplexa class Sporozoea subclass, Coccidia order, and family Cryptosporidiidae (1). *Cryptosporidium* spp have one life cycle which is completed in gut canal in one host. Several unique properties of *Cryptosporidium* are including lack specificity of infection of organs and hosts, it could resist antiparasitic drugs and it has high ability for autoinfection of the host cell (2). Life cycle of protozoan is done in the epithelial cells in the respiratory and gut canal especially in Bursa of Fabricius, but it less common in remain organs (3,4). *Cryptosporidium parvum* is a coccidian parasite which invaded the intestinal of animals and humans, the diarrhea is most common clinical sign that occur due to *C. parvum* infection, and the infection could be chronic, and dangerous disease especially in immune compromised individuals (5,6). Transmission of the parasite is done by faecal-oral way by taking contaminated water and food, it can infect young individuals (humans and animals); older animals can infect and shed the oocysts in the faeces which transfer the infection to others animals. The susceptibility and infectivity are varying among the hosts depending on many factors (7,8). Infection of *Cryptosporidium* spp. causes inflammation in the mucosal tissues, associated with great numbers of macrophages and neutrophils in the infected tissue (9). Diagnosis of *Cryptosporidium* spp. is difficult due to their oocysts size when compared with another coccidian, therefore is difficult to observe, and the resemblance same to spores of yeast and fungi (10). Many of laboratory methods for diagnosis parasite infection have many limitations as acid fast stain or microscopic examination. The false-negative data are common in number of oocysts samples due to low sensitivity of the staining methods (11). Immunological methods used in the diagnosis of *C. parvum* like latex test (12), ELISA (13), IFAT (14) flow cytometry (15). Histological examination of intestinal biopsy with different stain is like eosin and hematoxylin, acid-fast stain and safranin-methylene blue also used in the diagnosis (16). Recently molecular technique used in the diagnosis, Polymerase chain reaction is modern technique can detection of *Cryptosporidium* spp. in high selectivity in all types of samples, as well as it used for genotyping by amplify suspect DNA thousands of times (17). The goal of present study was to determination what are more sensitive methods PCR methods or microscopic methods in diagnosis of *Cryptosporidium parvum* is done by using heat shock protein 70 (HSP70) gene that prepared from *Cryptosporidium parvum*.

Materials and methods

Samples collections

Fresh Two hundred and ten of domestic chicken (*Gallus gallus domesticus*) faecal samples were collected directly from rectum from several regions collected from several regions in Al-Diwanyia and kept in clean container labeled for date, animal age and sex, and then transferred to the Laboratory of parasite in College of Veterinary Medicine, Al-Qadisiya University, and to laboratory for genomic DNA extraction. Thirty-five faecal samples were processed and examined monthly.

Microscopic examination by Ziehl-Neelsen stain

Small quantity of fecal sample was taken and placed on a glass slide and mixed with adequate little physiological solution then left to dry for 11 minutes. All slides were fixed by immersing them in the concentrated methanol solution five minutes and left to dry at 25°C. The slides were flooded with Carpal Fuchsin, was placed in oven at 61 degree for 11 - 15 minutes and rinsed off in tap water. Then slides were stained with a blue methanol dye for 2 minutes, washed with water and left to dry, after that examined by light microscope under oil immersion (18). The positive preserved at -20 c° for molecular study.

Extraction of DNA

The genomic material was prepared by extraction and purifying from samples by kit called (stool DNA extraction), Bioneer Company, made in Korea). The extraction methods were done depend on company directions by using lysis protocol and Proteinase K. then, DNA was tested by Nanodrop apparatus and keep at -20°C.

Polymerase Chain Reaction (PCR)

The PCR reaction is done by using designed primer for *Cryptosporidium parvum* HSP70 gene, (GenBank: KC885897.1). The HSP70 forward primer AGG GTG AGA GAG CCA TGA CT and HSP70 reverse primer CAG TTT GGT TGT GCT CGA GC (Bioneer Company, Korea) with at 429 bp product size. The master mix was mixed by kit (AccuPower® PCR PreMix, Bioneer, Korea). The premix tube consists from dNTPs 250 µM, Taq polymerase enzyme, KCl 30 mM, Tris-HCl 10mM, stabilizer, MgCl₂ 1.5 mM and ethidium bromide stain. The master mix was prepared depend on kit directions which consist from DNA sample 5uL, and forward primer 01.5ul, and reverse primer 1.5ul, and completing contains by water (20ul) and then mixed by vortex. All time of reaction was done in thermocycler device (Mygene Bioneer Company, Korea) and setting the apparatus as the initial denaturation 95°C for five minute; second stage (denaturation) 30 cycles at 95°C for thirty second, the annealing stage 60°C for thirty seconds, the extension stage 72°C for sixty seconds, the final extension

72°C for 5 minutes, the products were tested by electrophoresis and then the sample is stained with ethidium bromide and the UV lighter is used for watching the bands.

Results

Microscopic result

The infection with *cryptosporidium spp* was detected in 108 (51.4%) out of 210 faecal samples examined using light microscopic, the infection rate was 42%. The oocyst is round in shape, length 7 -5.2 µm, width 4.5-4.8 µm (Figure 1).



Figure 1: Oocyst of *Cryptosporidium spp*.

Infection rate of *Cryptosporidium spp*. in relation to sex

In regard to sex, no marked effect in total prevalence between males 25.23% and females 26.19 % (Table 1). No significant differences were found between prevalence according to sex.

Table 1: The prevalence of *Cryptosporidium spp*. according to sex

Sex	Total No.	Positive	Percentage (%)
Males	98	53	25.23
Females	112	55	26.19
Total	210	108	51.4
X ²		0.518	
P-value		0.47169	

Infection rate of *Cryptosporidium spp* in relation to months

Monthly examination of 35 faecal sample from February to July 2019 is shown in (Table 2). The high prevalence rate was in March 11.9% and February 11.4% while lower prevalence was in June 5.23% and July 5.23%. significant differences were found between prevalence according to months.

Table 2: Infection rate of *Cryptosporidium spp* according to months

Sex	Total No.	Positive	Percentage (%)
February	35	24	11.4
March	35	25	11.9
April	35	21	10
May	35	16	7.61
June	35	11	5.23
July	35	11	5.23
Total	210	108	51.4
X ²		22.418	
P-value		0.000435	

Molecular result

The overall prevalence of *Cryptosporidium parvum* was 21.3% out of 108 samples was as estimated using HSP70 gene . Figure(2) .

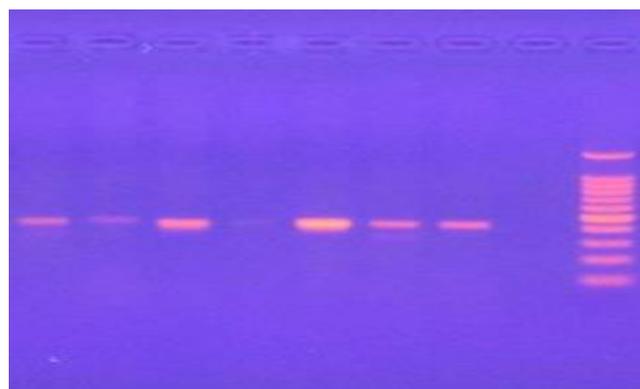


Figure 2: Bands under UV lighter which showed the gene HSP70 product, Line indicate to DNA marker, the Lanes 1-8 indicate to the positive and negative samples at 429 bp after use electrophoresis.

Discussion

In Al-Diwaniya province, south Iraq few reports are done about this subject in domestic chicken. The total percentage of *Cryptosporidium spp*. infection was 51.4% in chicken by staining with acid-fast staining. Our results were in resemblance with Al- Zubaidi (19) in Al-diwaniya and Baroudi (20) in Algeria, while were less than that in Brazil by Bomfim (21). The result of this study is disagreement with some of studies which recorded a low prevalence rate as Wang (22) which recorded 1.6% in China and Kichou (23) who found the infection of 24% in chickens in Morocco. The differences in the rates may be occurring due to using of many different methods and the sample taken from different area, differences in management practices, different species of birds and hygienic conditions. Concerning the sex, our

results showed that the difference was not statistically significant. A similar result was recorded in previous studies by Al-Zubaidi (24-26). They confirmed that both sexes are equally affected by the factors lead to infection and that the sex of the host does not play an important role in the infection with this disease. Other studies have been conducted with other host recorded no statistically significant between males and females as study by Casemore (27). In general, it could be said that these differences in the relationship of the host sex with parasite is relative since it varies female or the opposite, but it cannot confirm the existence of sex-related factors affect the parasitic infection and make it tends to infect a specific host sex without the other, we could not neglect the increase of infection during poor immune status during the egg laying period in females.

According to the months, the present study reveals significant differences between prevalence of infection. The high prevalence rate was in March 11.90% and February 11.42% and lower prevalence was in June 5.23% and July 5.23%. The cold months prolong the survival of the oocyst in the soil, due to optimum temperature and humidity for development of parasites oocyst. Our result was similar to the study in Al-Diwaniya province conducted by Al-Zubaidi (19) who recorded the higher prevalence was in March 87.5%, while lowest infection rate was observed in July 27.8%. The current study disagrees with result of study by Bamaïyi (28) which recorded the high rate in dry months because the spread of oocysts with dust particles.

The molecular characterization of animal could be used to investigate the possible zoonosis of *Cryptosporidium* spp. and genotyping (29). In this study, PCR with HSP70 target gene s was used for the identification of *C. parvum* isolated form 210 faecal samples of domestic chicken in Al-Diwaniya province. HSP70 structure in humans is likely to 72% with *drosophilae* (30), HSP70 of the chicken is 71% similar to *drosophila* and 80% similar to the gene in the human (31).

The results revealed that 23 (21.3%) of 210 faecal samples were contain *C. parvum* infection. the result was in agreement with other study in Al-Diwaniya province conducted by AL-Zubaidi (19) which recorded *C. parvum* in domestic chicken using 18S rRNA gene for molecular detection of this parasite in domestic chicken. *C. parvum* rate in the present study was higher than that reported in Germany 3.2% by Helmy (32) out of 158 samples. The result of this study was in agreement with other study conducted by Berrilli (33) in Italy.

Conclusion

For diagnosis of *C. parvum*, this study clearly indicated that a PCR method using the sequences of HSP70 gene to diagnosis of *C. parvum*.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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