Prevalence and molecular identification of *Cryptosporidium spp* in cattle in Baghdad province, Iraq

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Abstract

The objective of this study was to record the prevalence rate of *Cryptosporidium* and to determine the *Cryptosporidium spp* in cattle in different parts of Baghdad province. A total number of 100 fecal samples from different age groups were collected. Conventional method using modified Ziehl-Neelsen for staining fecal smears and molecular techniques for detection the prevalence and determines the species of *Cryptosporidium* that cause infection. The overall prevalence of infection with *Cryptosporidium* was 21% (21/100) by conventional method; nested PCR was done that targeting 18S rRNA gene on the same samples in which *Cryptosporidium* DNA identified in 38 samples (38%). Four species of *Cryptosporidium* in cattle were detected for the first time in Baghdad province: *C. parvum* (6/10), *C. andersoni* (2/10), *C. bovis* (1/10) and *C. ryanae* (1/10). The determination and characterization of *Cryptosporidium spp* in cattle was very important to avoid the infection to other animals and handlers and for applying control programs.

نسبة الانتشار والتشخيص الجزيئي لطفيلي *Cryptosporidium spp* في الأبقار في محافظة بغداد، العراق

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الخلاصة

هدفت هذه الدراسة إلى تسجيل نسب انتشار طفيلي الابواغ الخبيئة وتحديد أنواعه *Cryptosporidium* species في الأبقار وفي مناطق مختلفة من محافظة بغداد. جمعت ١٠٠ عينة براز من فئات عمرية مختلفة. تم الكشف عن الطفيلي بالطرق المجهرية التقليدية باستخدام ملون زيل نيلسون المعدلة لتلوين مسحات البراز والتقنيات الجزيئية لتسجيل نسب انتشار الطفيلي وتحديد الأنواع التي تسبب العدوى. بلغت النسبة الكلية للإصابة بطفيلي الابواغ الخبيئة ٢١٪ (١٠٠/٢١) باستخدام الطريقة التقليدية، وأجري تحليل سلسلة البلمرة المتداخل nPCR والذي استهدف جين 18S rRNA على نفس العينات حيث حدد الحمض النووي منقوص الأوكسجين DNA للطفيلي في ٣٨٪ من العينات، تم الكشف عن أربعة أنواع لطفيلي الابواغ الخبيئة في الأبقار حيث سجلت لأول مرة في بغداد: *C. parvum* (٦/١٠)، *C. andersoni* (٢/١٠)، *C. bovis* (١/١٠) و *C. ryanae* (١/١٠). إن تحديد وتوصيف أنواع طفيلي الابواغ الخبيئة في الماشية مهما لتجنب الاصابة في الحيوانات الأخرى وكذلك العاملين واقتراح برامج السيطرة.

Introduction

Cryptosporidium spp. are obligate intracellular (extra-cytoplasmic) protozoan parasites that can infect a wide range of animals and human hosts worldwide (1). Cattle are considered to be the major zoonotic reservoir for humans (2). Importantly, cryptosporidiosis in cattle causes acute or chronic gastrointestinal disturbance which is recognized as one of the major causes of profuse watery diarrhea, resulting in weight loss and growth retardation, reduced

milk production, morbidity and, in severe cases, death, leading to considerable economic losses (3). Cryptosporidiosis is transmitted mainly by the fecal-oral route, mostly by food or water contaminated with oocysts, by contact to infected animals or accidentally in laboratories (4). The conventional methods for the diagnosis of infection relies on the microscopic detection of *Cryptosporidium* oocysts in fecal samples, but these methods are unable to distinguish the different *Cryptosporidium spp.* based on morphometric or other phenotypic characteristics, due to lack of differentiating morphological features (5). Therefore, the molecular diagnostic techniques based on PCR can detect and identify genotypes. The molecular tools can identify more precise definitions of host-adapted specificity, transmission pathways, and zoonotic possibility of *Cryptosporidium spp.* (6). Worldwide, cattle are commonly infected with 4 *Cryptosporidium spp.* which include *C. parvum, C. andersoni C. bovis* and *C. ryanae* (1,7). Given this diversity of *Cryptosporidium* species in cattle and differing zoonotic potential, it is important to be able to estimate their prevalence and distribution in particular geographic regions using molecular tools (8).

The objective of this study was to investigate the prevalence of *Cryptosporidium* infections and determination molecular characterization of *Cryptosporidium spp.* isolates from different ages of cattle in Baghdad province/Iraq.

Materials and methods

A total of one hundred fecal samples of cattle were collected from different age groups and both sexes from different parts of Baghdad province, during the period from the beginning of January 2018 to the end of September 2018, fecal smears were prepared from each sample, stained with modified Ziehl-Neelsen staining technique as primary diagnosis of *Cryptosporidium* oocysts (9).

Molecular diagnosis using Nested PCR (nPCR)

The nested PCR technique was performed for detection *Cryptosporidium spp.* based 18S ribosomal rRNA gene from cattle fecal samples. This method was carried out according to method described by (10), which included DNA extraction fecal samples by using AccuPrep® stool DNA Extraction Kit, (Bioneer, Korea). Primary PCR master mix preparation by using first primer pair: forward (5-AGACGGTAGGGTATTGGCCT -3) and reverse (5-TACGAATGCCCCCAACTGTC-3) (Maxime™ PCR PreMix Kit (*i*-Taq)), then placed in PCR Thermocycler (MyGene. Bioneer. Korea). Secondary PCR master mix was prepared by using second primer pair: forward (5-ATTGGAGGGCAAGTCTGGTG -3) and reverse (5 -TACGAATGCCCCCAACTGTC-3) (Maxime™ PCR PreMix Kit (*i*-Taq)), then placed in PCR Thermocycler (MyGene, Bioneer, Korea).

Sequence analysis

The genetic analysis done by phylogenetic tree analysis between local *Cryptosporidium spp.* cattle isolates and NCBI-Blast submission *Cryptosporidium* species. Then the identification species isolates were submitted into of NCBI-GenBank. The DNA sequencing analysis was conducted by using Molecular Evolutionary Genetics Analysis version 6.0 (Mega 6.0) and Multiple sequence alignment analysis of the partial small subunit ribosomal rRNA gene based ClustalW alignment analysis and the evolutionary distances were computed using the Maximum Composite Likelihood method by phylogenetic tree UPGMA method (11).

Statistical analysis

Statistical analyses were computer assisted using SPSS, variables were assessed by Yat's Chi-square test (12).

Results

Total infection rate of *Cryptosporidium*

Conventional microscopic (modified Ziehl-Neelsen staining) method recorded the overall prevalence rate of *Cryptosporidium* infection in cattle was 21% (21/100). On the other hand, molecular (PCR) analysis identified *Cryptosporidium spp.* infection in 38% (38/100) (Table 1). All microscopy positive specimens were also found positive by PCR. The prevalence of *Cryptosporidium* detected by the two methods was significantly different (P≤0.05).

Table 1: Total infection rate of *Cryptosporidium* infection by conventional microscopic (modified Ziehl-Neelsen staining) method and molecular (Nested PCR) techniques in cattle

Host	Fecal samples	Conventional microscopy		Molecular (Nested PCR)	
Cattle	Total No. examined	No. of positive	%	No. of positive	%
	100	21	21% [B]	38	38% [A]

Variation in large horizontal letters refer to significant differences at a level of P≤0.05.

Nested PCR

Genomic DNA samples obtained from cattle fecal samples were subjected to molecular analysis by nested PCR using 18S rRNA gene specific primers in order to identify the species of *Cryptosporidium*. Nested PCR of all 100 samples employed in the study exhibited distinct band of 318 bp on agarose gel confirming the presence of *Cryptosporidium spp* (Figure 1).

Figure 1: Agarose gel electrophoresis image that show the Nested PCR product analysis of small subunit ribosomal RNA gene in *Cryptosporidium spp.* from Cattle fecal

samples. Where M: Marker 2000-100 bp, lane 1-12 showed some positive *Cryptosporidium spp.* samples at 318 bp Nested PCR product size.

Cryptosporidium **species in cattle**

The entire 10 samples which were positive by nested PCR amplification of 18S rRNA gene of *Cryptosporidium* were successfully sequenced. The results revealed the presence of four *Cryptosporidium spp.*, namely *C. parvum*, *C. andersoni*, *C. bovis* and *C. ryanae*, all of them have identity 100%. *C. parvum* was the most prevalent species being detected in 6/10, followed by *C. andersoni* 2/ 10, *C. bovis* and *C. ryanae* had the same infection rate 1/10 of each one (Table 2 and Figure 2).

Table 2: NCBI-BLAST Homology sequence identity between local *Cryptosporidium spp.* cattle isolates and NCBI BLAST *Cryptosporidium spp.* Isolates

Local cattle *Cryptosporidium spp.* No.	Gen-Bank accession No.	NCBI BLAST Homology sequence identity		
		NCBI BLAST *Cryptosporidium spp.*	Gen-Bank accession No.	Identity (%)
1	MH885559	*C. parvum*[a]	MH341586.1	100%
2	MH885560	*C. ryanae*[c]	MF671873.1	100%
3	MH885561	*C. parvum*[a]	MH341586.1	100%
4	MH885562	*C. parvum*[a]	MH341586.1	100%
5	MH885563	*C. andersoni*[b]	KX710086.1	100%
6	MH885564	*C. parvum*[a]	MH341586.1	100%
7	MH885565	*C. bovis*[c]	KC618591.1	100%
8	MH885566	*C. andersoni*[b]	KX710086.1	100%
9	MH885567	*C. parvum*[a]	MH341586.1	100%
10	MH885568	*C. parvum*[a]	MH341586.1	100%

Variation in small vertical letters refers to significant differences at a level of P≤0.05.

Discussion

The current study determines the prevalence and genotyping of *Cryptosporidium spp.* among cattle in different regions of Baghdad province. Fecal samples collected from cattle were screened by microscopy using modified Ziehl-Neelson staining technique and nested PCR. Although microscopy is cheaper to perform and only method to indicates active infection, higher prevalence of *Cryptosporidium* was recorded by the PCR in this study. The superior sensitivity of PCR in detecting *Cryptosporidium* infection has been shown earlier clinical trial and in patients from Northern India and South Africa (13,14).

The overall prevalence of bovine cryptosporidiosis in cattle in this study was 38%. This finding was comparable with the results of Benhouda *et al.* (15) whom recorded infection rate 40% in young calves in Algeria and the 35.5% were recorded in diarrheic calves in Sudan (16).

However, the prevalence was higher than the 14.4% infection prevalence reported from dairy farms in the Qinghai-Tibetan Plateau Area in China (7), 17.0% reported from cattle tested in Poland (17) and 18.6% in cattle in Ethiopia (18). On the other hand, the infection rate was lower than the 42.85% reported in calves in Kut city (19), 47.68% in pre-weaned dairy calves in Northeastern China (3) and 52.2% in neonatal calves in Algeria (20). The differences of infection ratios could be attributed to the differences in management systems, methods of rearing, age and breed of cattle, environmental conditions, the sampling method and sample size, as well as diagnostic techniques employed in different study localities.

The results of the current molecular study showed the presence of four major *Cryptosporidium spp.* in cattle. The most prevalent species in pre and post-weaned calves was *C. parvum*, followed by *C. andersoni, C. bovis* and *C. ryanae*. Our results are in agreement with the results that recorded by Silverlas (21) in Swedish cattle (22) in Indian

dairy calves, in calves in northeast China by Zhang *et al* (3), in Poland (17), in farm animals in Egypt (23), calves in Sudan (16) and in dairy cattle in northeast China (24) all of them recoded the four species of *Cryptosporidium*. On the other hand, only three species *C. andersoni, C. bovis* and *C. ryanae* were detected in the post-weaned calves, these results recorded in Malaysia by Muhid *et al.* (25) and in north-western China Qi *et al.* (26), while Silva *et al*; Shrestha (27,28) identified three *Cryptosporidium spp.* of

cattle, *C. parvum*, *C. bovis* and *C. andersoni*. In Mumbai region of India Hingole *et al.* (29) and in China Cai *et al.* (30) were detected three *Cryptosporidium* species also, *C. parvum*, *C. bovis* and *C. ryanae* in pre-weaned dairy calves. Yap *et al.* (11) detected only two species of *Cryptosporidium*: *C. ryanae* and *C. bovis* in cattle in Malaysia also Inpankaew *et al.* (31) detected two species of *Cryptosporidium*, *C. parvum* and *C. bovis* in dairy cows in Thailand.
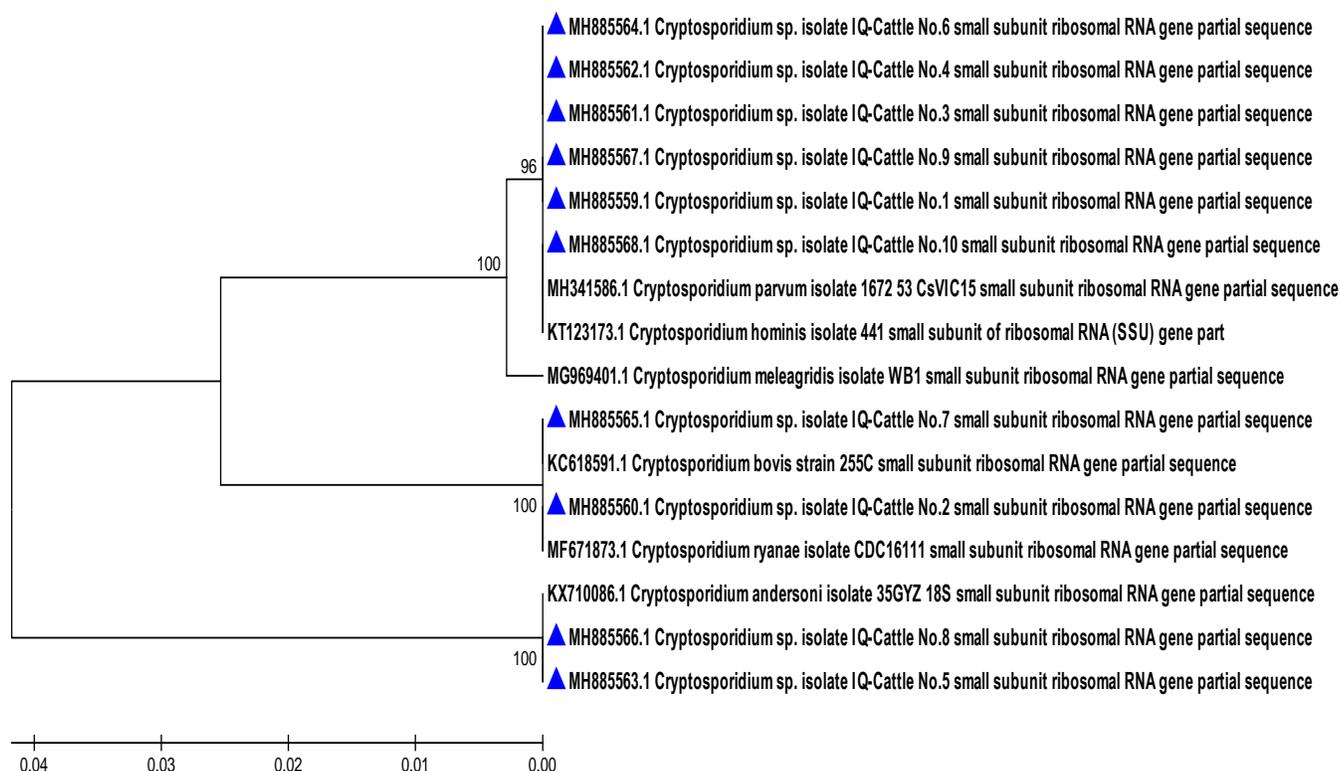

Figure 2: Phylogenetic tree analysis based on the partial sequence Small subunite rRNA gene in local *Cryptosporidium spp.* cattle isolates that used for *Cryptosporidium spp.* genetic identification analysis. The evolutionary distances were computed using the Maximum Composite Likelihood method by phylogenetic tree UPGMA (MEGA 6.0 version). The local *Cryptosporidium spp.* cattle isolates of No. 1, 3, 4, 6, 9 and 10 were showed closed related to NCBI-Blast *Cryptosporidium parvum* isolate (MH341586.1), The local *Cryptosporidium spp.* cattle isolates of No. 2 was showed closed related to NCBI-Blast *Cryptosporidium ryanae* isolate (MF671873.1), The local *Cryptosporidium spp.* cattle isolates of No. 5 and No.8 were showed closed related to NCBI-Blast *Cryptosporidium andersoni* isolate (KX710086.1), and the local *Cryptosporidium spp.* cattle isolates of no. 7 was showed closed related to NCBI-Blast *Cryptosporidium bovis* isolate (KC618591.1) at total genetic change (0.01-0.04%).

The species and genotypes of *Cryptosporidium* infecting cattle are known to vary according to the host age and geographical distribution (32). Given that this study focused more on calves aged <1 month to 6 months old, our study has included cattle with a broader age range <6 months to 3 years of age sampled from different regions of Baghdad province to provide a broader picture of

Cryptosporidium infections in cattle in Iraq. *C. andersoni*, *C. bovis* and *C. ryanae* are truly less pathogenic than *C. parvum*, resulting in low grade infection and lower oocyst output, which in turn reduce the infection pressure among infected animals (33). Our results are in agreement with many studies performed in other countries reported by Taha *et al*; Helmy; Thomson; Ng (33,34-35) and thus confirm a

similar age dependent pattern of infection for Sudan for the first time. *C. andersoni* infection usually occurs in older calves and adult cattle (16).

This study described the diversity of *Cryptosporidium* in cattle of all ages in Baghdad province / Iraq. Four species of *Cryptosporidium* were detected in this study; *C. parvum*, *C. bovis*, *C. ryanae* and *C. andersoni*. *Cryptosporidium* infection due to *C. parvum* and *C. andersoni* was more prevalent in cattle in the study area.

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