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# Prevalence and strains of *Cryptosporidium* detected by microscopy and PCR in stools of cattle handlers.

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## ABSTRACT

*Obed Alseady HH, Kawan MH., Prevalence and strains of Cryptosporidium detected by microscopy and PCR in stools of cattle handlers, Onl J Vet Res., 22 (12):1095-1102, 2018.* Authors report prevalence and species of *Cryptosporidium* in stools of 100 volunteer cattle handlers aged <10 - to >40 years determined by microscopy and nPCR, collected during January to September 2018 in Iraq. We found oocysts in 17% by microscopy, but by PCR detected *Cryptosporidium* in 41% of stool samples. By PCR, children aged < 10 had a very high incidence of 69.9% ($P \leq 0.05$), 19-20 year old ~30%, and those over 40 years ~18%. There was no significant difference in presence of *Cryptosporidium* between genders. By PCR with 18S rRNA gene of 10 stool samples phylogenetic tree of our *C. parvum, C. hominis* and *C. andersoni* isolates referenced against Gen-Bank revealed 5 closely related to *Cryptosporidium andersoni* (KX710086.1) 3 to *Cryptosporidium hominis* (KT123173.1), and 2 to *Cryptosporidium parvum* isolates (MH341586.1) with a genetic difference of 0.01-0.04%. Datasets suggested strong genetic distinctiveness amongst species. *C. andersoni* has not been reported previously in this region in dairy/cattle handlers but *C. parvum* causes most infection.

**Key words:** *Cryptosporidium*, cattle/cow handlers, nested-PCR, 18S rRNA gen.

## INTRODUCTION

*Cryptosporidium* spp. is an intracellular extracytoplasmic coccidian-like protozoan parasite phylum Apicomplexa which causes diarrhea in humans and livestock worldwide (Zahedi *et al.,* 2016). *Cryptosporidium* was discovered in 1907, however it remained mostly unknown until 1976 when the first human cases of cryptosporidiosis were reported (Nime *et al.,* 1976; Tzipori and Widmer, 2008). The genus consists of multiple genetically distinct species and genotypes whose

identification relies on molecular methods since oocysts are morphologically indistinguishable. Thirty-four *Cryptosporidium* species and over seventy genotypes have been determined based on DNA polymorphisms of the small subunit (SSU) rRNA gene, although only two are responsible for most human infections, including the anthroponotic species *C. hominis* and the zoonotic species *C. parvum* (Ryan *et al.,* 2017; Zahedi *et al.,* 2017).

Human cryptosporidiosis is frequently accompanied by abdominal pain, fever, vomiting, malabsorption, general malaise, weakness, fatigue, loss of appetite, nausea, chills and sweats and diarrhea that may sometimes be profuse and prolonged (Chalmers and Davies, 2010). *Cryptosporidium* is well adapted to infect human beings and animals through zoonotic, waterborne, foodborne, and person-to-person routes of transmission (Helmy *et al.,* 2014). These routes enable *Cryptosporidium* to be endemic in many low-income countries and potentially epidemic in high-income countries. Feco-oral transmission between domestic animals and humans may be an important mode of infection and it is likely that both serve as reservoirs of the disease (Xiao and Fayer, 2008).

A diagnosis of cryptosporidiosis is based on the identification of *Cryptosporidium* spp. oocysts in the fecal sample by conventional and immunodiagnostic methods (Fayer and Xiao, 2008)Several conventional techniques such as flotation by Sheather's sugar/zinc sulfate solution, formal ether concentration method, formal ethyl acetate sedimentation technique, medefied Ziehl–Neelsen (mZN), and some of negative staining methods using Nigrosin, light green, malachite green and carbol fuchsin are used for the diagnosis of cryptosporidiosis (Current and Garcia, 1991). Molecular techniques (more sensitive and specific) like polymerase chain reaction (PCR) are widely used nowadays for the genotyping of cryptosporidiosis (Rekha *et al.,* 2016). Several studies agree on the higher sensitivity of PCR targeting the 18S rRNA gene. Nested PCR has been put forward as a means of improving the sensitivity of detection (Jothikumar *et al.,* 2008). We report prevalence of most important species of *Cryptosporidium* amongst cattle handlers in Baghdad province, Iraq by microscopy and nested PCR.

## MATERIALS AND METHODS

The study included one hundred stool samples of 5-10g each collected from cattle handlers of both sexes and different age groups in different regions of Baghdad province. The study was performed from the beginning of January 2018 to the end of September 2018. Fecal smears were prepared from each sample, and stained using the modified Ziehl–Neelsen staining technique for a primary diagnosis of *Cryptosporidium* oocysts (Current and Garcia, 1991).

Molecular diagnosis: A nested PCR was performed to detect *Cryptosporidium* spp. based on 18S ribosomal rRNA gene in volunteer stool samples as described by Ruecker *et al.,* (2013). Primary PCR master mix preparation by using (Maxime™ PCR PreMix Kit (*i*-Taq)) included the first primer pair: forward (5–AGACGGTAGGGTATTGGCCT –3) and reverse (5-TACGAATGCCCCCAACTGTC-3), then placed in PCR Thermocycler. Secondary PCR master mix was prepared by using (Maxime™ PCR PreMix Kit (*i*-Taq)) included second primer pair: forward (5-ATTGGAGGGCAAGTCTGGTG -3) and reverse (5 -TACGAATGCCCCCAACTGTC-3), then placed in PCR Thermocycler.

The two PCR rounds were done under the same conditions: Nested PCR mixtures contained 1x PCR buffer, 5 mM MgCl2, 200 lµ each deoxynucleoside triphosphate, 100 nM each primer and 1.25 U Hot Start Taq polymerase. Cycling conditions consisting of a hot start at 94˚ C for 5 min

followed by 30 cycles with de-naturation at 94˚ C for 30 seconds, annealing at 58˚ C for 30 seconds, and at 72˚ C for 30 seconds, and a final extension at 72˚ C for 5 minutes then holding at 4 ˚ C forever (Ruecker *et al.,* 2013).

We constructed a phylogenetic tree for our *Cryptosporidium* versus NCBI-Blast-GenBank. Positive PCR 18S rRNA gene were analyzed for DNA sequencing (Molecular Evolutionary Genetics Analysis version 6.0) and Multiple sequence alignment analysis (ClustalW). Evolutionary distances were computed by Maximum Composite Likelihood as described by Tamura *et al.,* (2013). Data was analyzed using SPSS version 17 and Chi-square test as described by Petrie and Watson, (2006).

## RESULTS

Results are shown in Tables 1-4 and Figures 1-3 below. By microscopy using Sheather's (sugar) flotation and modified Ziehl-Neelsen staining (Current and Garcia, 1991) we found *Cryptosporidium* spp. oocysts in 17% stool samples (17/100). However, by nested PCR we found *Cryptosporidium* infection in 41% (41/100). PCR confirmed microscopic findings in all cases. BY PCR all stool samples exhibited a distinct band of 318 bp on agarose gel for *Cryptosporidium* sppas depicted in Figure 1, below.

Table 1. *Cryptosporidium spp.* (%) by microscopy and Nested PCR in stools of 100 cattle handlers.

| Host | Stool samples | Conventional microscopy | | Molecular-Nested PCR | |
|---|---|---|---|---|---|
| Handlers | Total No. | No. | % | No. | % |
| | 100 | 17 | 17 % [B] | 41 | 41 % [A] |

Different letters in same row are significantly different ($P \leq 0.05$)

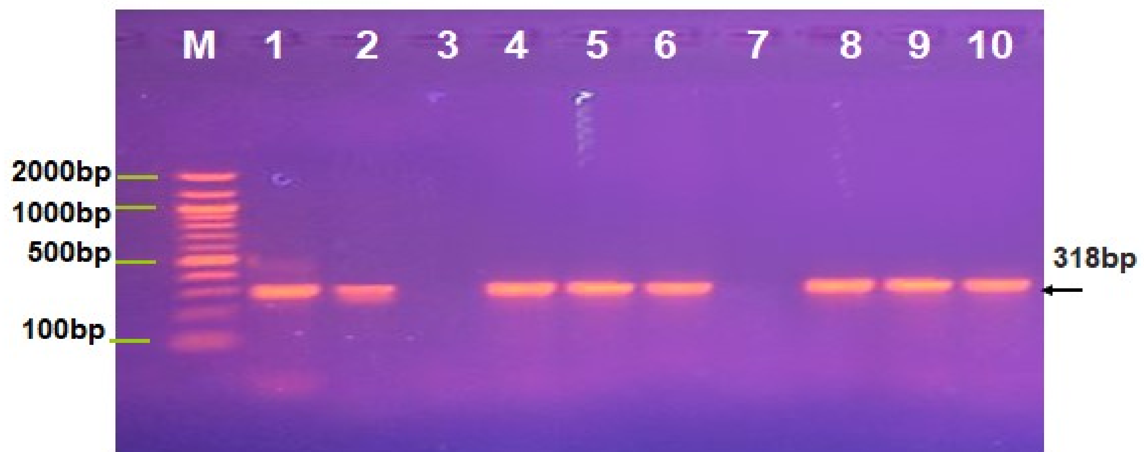


Figure 1. Agarose gel electrophoresis of a Nested PCR product subunit ribosomal RNA gene in *Cryptosporidium spp.* from 10 animal handlers stool samples. M: Marker (2000-100bp), lanes 1-10 show *Cryptosporidium* spp. at 318bp Nested PCR product size**.**

There was no difference between genders at 41.6% in males and 40% in females (Table 2).

Table (2): *Cryptosporidium* by nested PCR and gender.

| | Number | Positive | |
|---|---|---|---|
| | | No. | % |
| Male | 60 | 25 | 41.6 % [a] |
| Female | 40 | 16 | 40 % [a] |

Different letters in same row are significantly different ($P \leq 0.05$)

Results in Table 3 below show significantly (<0.05) higher (69.9%) incidence in children < 10 years old compared to handlers aged 10-19 and 20-29 years ~32%, 30-39 with ~18% and older than 40 ~15%.

Table (3): *Cryptosporidium* by nested PCR and age.

| Age groups | Number | Positive | |
|---|---|---|---|
| | | No. | % |
| <10 Years | 33 | 23 | 69.96 % [a] |
| 10-19 Years | 28 | 9 | 32.14 % [b] |
| 20-29 Years | 15 | 5 | 33.33 % [b] |
| 30-39 | 11 | 2 | 18.18 % [c] |
| 40 < | 13 | 2 | 15.38% [c] |

Different letters in same column are significantly different ($P \leq 0.05$)

Ten positive PCR stool samples with *Cryptosporidium* species strains were compared with gen-bank revealing *C. andersoni* in 5, *C. hominis* in 3 and *C. parvum* in 2 (Table 4).

(Table 4): *Cryptosporidium* strains in cattle handlers and NCBI BLAST homology sequences.

| Local Human *Cryptosporidium* sp. No. | Gen-Bank accession No. | NCBI BLAST Homology sequence identity | | |
|---|---|---|---|---|
| | | NCBI BLAST *Cryptosporidium* sp. | Gen-Bank accession No. | Identity (%) |
| 1 | MH885549 | *C. andersoni* [a] | KX710086.1 | 100% |
| 2 | MH885550 | *C. parvum* [c] | MH341586.1 | 100% |
| 3 | MH885551 | *C. andersoni* [a] | KX710086.1 | 100% |
| 4 | MH885552 | *C. andersoni* [a] | KX710086.1 | 98% |
| 5 | MH885553 | *C. hominis* [b] | KT123173.1 | 100% |
| 6 | MH885554 | *C. andersoni* [a] | KX710086.1 | 98% |
| 7 | MH885555 | *C. parvum* [c] | MH341586.1 | 100% |
| 8 | MH885556 | *C. hominis* [b] | KT123173.1 | 100% |
| 9 | MH885557 | *C. andersoni* [a] | KX710086.1 | 98% |
| 10 | MH885558 | *C. hominis* [b] | KT123173.1 | 100% |

Different letters in same column ($P \leq 0.05$)

Figure 2 shows a Phylogenetic tree of *C. parvum, C. hominis* and *C. andersoni* referenced against those of Gen-Bank which highlight differences by DNA STAR (Tamura *et al.,* 2013). *Cryptosporidium* spp. 1, 3, 4, 6 and 9 were closely related to NCBI-Blast *Cryptosporidium*

*andersoni* (KX710086.1) 5, 8 and 10 to NCBI-Blast *Cryptosporidium hominis* (KT123173.1), and 2 and 7 to NCBI-Blast *Cryptosporidium parvum* isolates (MH341586.1) with a genetic difference of 0.01-0.04%. Datasets suggest strong genetic distinctiveness amongst species.

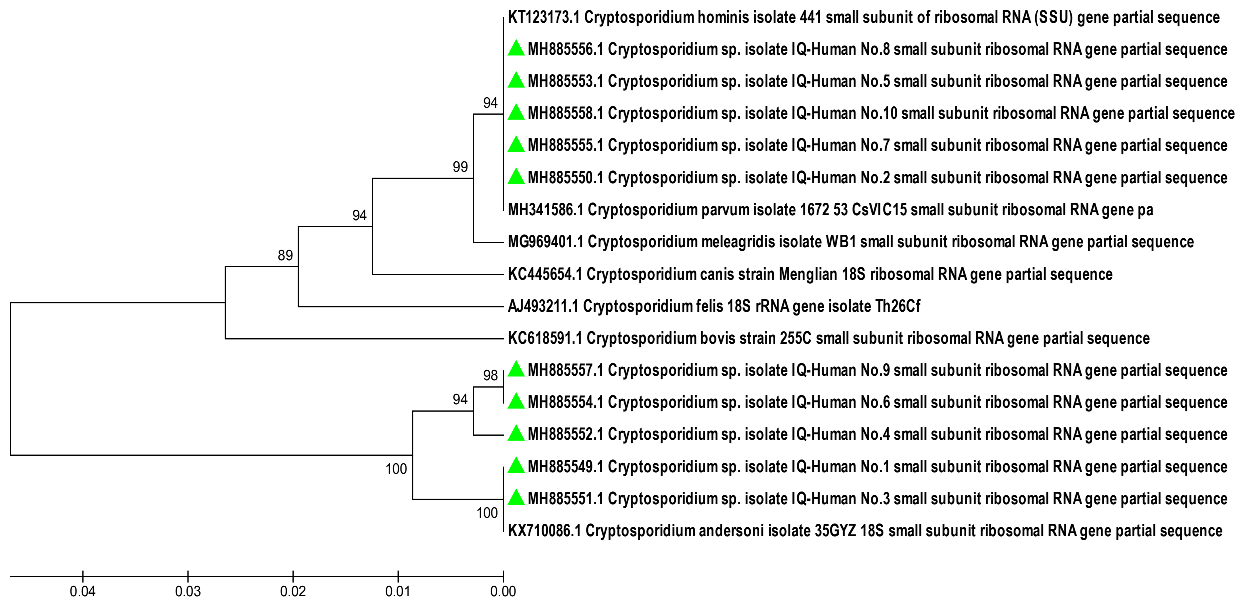


Figure 2: Phylogenetic tree analysis based on the partial sequence Small subunit rRNA gene in *Cryptosporidium* spp. isolated from stools of cattle handlers and *Cryptosporidium* species genetic identification analysis.

## DISCUSSION

Lal (2014) and Lal *et al.,* (2016) did a major survey with sheep, poultry, pig, deer and dairy cattle density and concluded that only dairy cattle density was associated with a risk of cryptosporidiosis for handlers. Water may be the major route of transmission for *C. hominis*, *C. parvum* and *C. andersoni* genotypes, which accounts for high incidence of *C. parvum* and *C. andersoni* during the rainy season (Zahedi *et al.,* 2018). We found a much higher incidence of *Cryptosporidium* by PCR (41%) compared with microscopy (17%). The superior sensitivity of PCR in detecting *Cryptosporidium* infection has been reported by Uppal *et al.,* 2014 and Omoruyi *et al.,* 2014 in India and South African handlers. We presume the high prevalence is due to direct contact with cattle, unsanitary conditions and lack of parasite treatments in cattle. Our results are similar to those of Altaee *et al.,* (2014) who recorded an infection rate by microscopy of 47.72% in animal handlers in Baghdad.

Wide variations in infectivity in cattle handlers may be due to hygiene, age, gender, rural or urban, environment, sampling and sample size, diagnostic techniques and/or direct contact with cattle. We found no difference in prevalence between males (41.6%) and females (40%). These results were in agreement with other studies conducted worldwide (Rahi and Raheem, 2013; Salman *et al.,* 2015) which indicates that both genders have equal chance of being infected. However because are more likely to be in contact with cattle/contaminants they may a higher change of infection (Tairsh *et al.* (2017) and Adler *et al.* (2017)

In our study lower age was correlated with higher prevalence. We found a very high rate of infection (69%) in children under 10, compared with previous findings (~10%) (Abdul Razak and Jasm, 2011; Sadek, 2014; Abdul-Sada, 2015; Salman *et al.,* 2015 and Tairsh *et al.,* (2017).

However, Saeed and Khair (2014) reported 39.76% in children at Al-Ressafa Baghdad and Lal et al (2016) found a prevalence of 59% in children under 5 years in areas with high dairy cattle densities in New Zealand. The high infection rate in children is probably due to immature immunity, contact with soil and/or contaminated materials, livestock and poor sanitary conditions as noted by Al-Omashi, (2014) and Al-Ward (2010). AL-Gelany, 2003; Adler *et al.*, 2017 and Kaminsky and Garcia, (2017) maintained that maximum infection rates were in age groups (<1-5) and (6-12) years, and Yoder and Beach, (2010) in 1–9 year olds.

For nested PCR we used Small Subunit rRNA gene as it covers the major region of interspecies/genotype variability in the gene, allowing identification of nearly all *Cryptosporidium* spp. and genotypes by sequence analysis and is highly conserved with the abundance of multi-copy hyper-variable regions (Coupé *et al.,* 2005). Using 10 PCR positive sasmples we detected *C. hominis, C. parvum* and *C. andersoni*. Guyot *et al.* (2001) found *C. parvum, C. meleagridis, C. felis* and *C. andersoni* (*C. muris*) in humans in France, whereas Leoni *et al.* (2006) detected *C. hominis, C. parvum, C. meleagridis, C. andersoni, C. felis, C. canis, C. suis* and *C. cervine* type in 2414 humans with diarrhea in England. Morse *et al.* (2007) reported *C. hominis, C. parvum, C. hominis/C. parvum, C. meleagridis* and *C. andersoni* in Malawi. Hijjawi *et al.* (2016) *C. hominis* and *C. parvum* in Jordan and Hussain *et al.* (2017) *C. parvum* and *C. andersoni* in India. Our findings are similar to those of Hussain *et al.* (2017) who reported *C. andersoni* in 6.97% and *C. parvum* in 1.7% in patients with diarrhea from India. While Leoni *et al.* (2006) and Morse *et al.* (2007) reported lower prevalence of *C. andersoni* than other *Cryptosporidium* species. We believe that to our best knowledge, this might be the first report on the prevalence of *C. andersoni* in Baghdad province/Iraq, using molecular techniques (Nested PCR and sequencing).

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