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Molecular subtype analysis of Cryptosporidium spp. in sheep of Babylon province, middle Iraq

Akeel M. Kadim[1], Abdul H. Habash[2], Mansoor J. Ali[3]

[1]Department of Parasitology, College of Veterinary Medicine, University of Al-Qasim Green, Babylon, Iraq
[2]Department of Biology, College of Education for Pure Sciences, University of Basrah, Basrah, Iraq
[3]Department of Parasitology, College of Veterinary Medicine, University of Qadisiyah, Qadisiyah, Iraq

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ABSTRACT

The aim of this study was to investigate the zoonotic potential and genetic diversity of Cryptosporidium species and subtype of C. parvum & C. hominis in sheep. A total of 180 fecal samples were collected from four sites in Babylon province, tested by modified Ziehl-Neelsen stain and 50 positive samples were exposed to the Nested PCR reaction. Four species (C. parvum, C. hominis, C. ubiquitum and C. andersoni) were identified based on sequence analysis of the heat shock protein 70 (Hsp70). Molecular phylogenetic analysis of the gp60 genetic sequence of 10 C. parvum, and 10 C. hominis isolates revealed diagnosis of four subtype included IIdA17G1a, IIdA17G1, IIdA18G1, IIdA21G1 and two subtypes of C. hominis, (IbA21G2 and IbA13G3). The result suggest that the sheep may be considered as reservoir host of IId (C. parvum) and Ib (C. hominis) subtypes to other animals and human.

1. Introduction

Cryptosporidiosis is global problem caused by coccidian protozoan parasite Cryptosporidium species in human and animals [1, 2]. This parasite is particularly important in domestic ruminants since it is often involved in neonatal diarrhoeal outbreaks, leading to significant economic losses [3]. Cryptosporidium spp. transport to human or animals by regular contact with worker or animals [4]. In sheep the most dominant species of Cryptosporidium are C. xiaoi, C. ubiquitum and C. parvum [5]. Other Cryptosporidium spp. that have also been found in sheep include C. hominis, C. bovis, C. baileyi, C. suis, C. andersoni, C. fayeri, C. ryanae, C. canis and C. scrofarum C. canis [6, 7, 8]. The determination of genotypes of Cryptosporidium spp. can be established by several molecular markers including the 70 kDa heat shock protein (HSP70), 18S rRNA and COWP 9. HSP70 gene belongs to a multigene family that is highly conserved across the prokaryotes and eukaryotes. Sequence analysis of (60 kDa) glycoprotein gp60 gene was used for subtyping the C. parvum and C. hominis [10], which allows to diagnose the subtype families of C. hominis and C. parvum, as well as some subtypes in each family [11].

*Corresponding author email : akeelmohammed@vet.uoqasim.edu.iq

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Materials and methods

1.1.Samples collection

One hundred and eighty feces samples from diarrheal and uninfected sheep of all ages were collected through the period extended from January to July 2021, the collection sites involved local farms in Babylon province (Hilla city, Al-Hashmiyah, Mahaweel and Al-Musayyib districts). Feces samples were collected directly from the rectum using gloves and plastic containers. Fresh feces were examined for the presence of Cryptosporidium oocysts using modified Ziehl–Neelsen stain (Syrbio Syria company) . After confirming the presence of Cryptosporidium oocyst, 50 samples were sent for the molecular examination.

1.2. DNA Extraction

After collecting feces samples and detection the infection by staining with modified Ziehl–Neelsen stain and microscopic examination. Flotation method was used, DNA extraction kit of ADDBIO, Korea were performed according to the manufacturer instructions. then, DNA stored at -20ºC until used in downstream processes 12.

1.3. Molecular diagnosis using Nested PCR (nPCR)

The reaction mixture of neseted PCR consist of (Taq DNA polymerase (0.2) µl, dNTPs (2) µl, MgCl2 (2) µl, 10X buffer (2) µl, DNA (2) µl, PCR water 9.8 µl, Forward primer (1) µl and Reverse primer (1) µl. The reaction condition of PCR is denaturation temperature 94 C° for 30 second and Annealing as mentioned in the primer's table and Extension 72 C° for 60 second. Agarose powder (1 g) was dissolved in 1x TBE buffer (100 ml) and heated till reach 95 C°, left to cool down at 60 C. Then 7 µl of safe gel dye stain was added to the melted agarose. After using Nested PCR for of Cryptosporidium species and C. parvum and C. hominis and appearance of band on the agarose, they were sent to the sequencing to know the Cryptosporidium spp. and C. hominis and C. parvum subtype in sheep [12]. 13 PCR products for Cryptosporidium spp and 20 products for (subtypes identification) were sent for DNA sequencing and phylogeny. The results were analysed then deposited in the gene bank with provided accession numbers and compared with other different world strains (Phylogenetic tree analysis) using Molecular Evolutionary Genetics Analysis version 10 (Mega x) and multiple sequence alignment analysis based on Clustal W alignment analysis. The identified species typing analysis was done by phylogenetic tree analysis in comparison with NCBI-Blast known sequences [12]. The nested PCR reaction was used to diagnose Cryptosporidium species by targeting heat shock protein 70 kDa (HSP70) gene, and the same reaction was used to diagnose the subtypes of C. parvum & C. hominis by targeting (gp60) gene 11. Three primers were used for each process, as shown in Table 1 [13, 14, 15].

Table 1. Oligonucleotide primer sequences used for Cryptosporidium species PCR methods.

Gene	Target	PCR	Oligonucleotide primer 5'- 3'	Expected size of PCR product (bp)	Program (C°*)	Ref.
HSP-70	Universal	conventional	F:GGTGGTGGTACTTTTGATGTAT R: GCCTGAACCTTTGGAATACG	448	52	[13]
		nested	F: GCTGSTGATACTCACTTGGGTGG R:CTCTTGTCCATACCAGCATCC	325	52	[13]
GP60	*C. parvum*	conventional	F: ATAGTCTCCGCTGTATTC R: GGAAGGAACGATGTATCT	1400	50	[14]
		nested	F: TCCGCTGTATTCTCAGCC R: GCAGAGGAACCAGCATC	800	51	[14]
GP60	*C. hominis*	conventional	F: ATAGTCTCGCTGTATTC R: GCAGAGGAACCAGCATC	1400	50	[15]
		nested	F: TCCGCTGTATTCTCAGCC R: GAGATATATCTTGGTGCG	800	53	[15]

2. Result

2.1. Prevalence of Genotyping of Cryptosporidium spp. in sheep of Babylon province

The nested PCR products of Cryptosporidium spp. Fig.1. The were sent to Korea Seoul for the sequencing to know the Cryptosporidium spp. in sheep. The sequencing results for Cryptosporidium spp. showed different ratios between the species, which is the most common. The total percentage of C. parvum in sheep was 46.15% of the samples sent, with the Accession number (MZ787768, MZ787769, MZ787770. MZ787771. MZ787772 & MZ787773). The total percentage of C. ubiquitum was 23.07%, with the Accession number (MZ787776, MZ787777, MZ787775). The lowest common is C. hominis and C. andersoni which were 15.38% with the Accession number (MZ787774, & MZ787775, MZ787779, and MZ787780)e.

3.2. Prevalence of subtypes of C.parvum and C.hominis in sheep of Babylon province

The results of nested PCR of C.parvum and C.hominis subtype are shown in Fig. 2 and the sequence analysis of C. parvum isolates were grouped in one family: IId. Extra sub-classification led to five different subtypes. The subtype IIdA17G1a MZ787795, MZ787796, MZ787797, & MZ787798) was the most common (40.00%), while the subtype IIdA17G1 (MZ787799, MZ787800 & MZ787801) recorded 30.00%. The Subtype IIdA21G1 (MZ787803 & MZ787804) recorded 20.00% and the subtype IIdA18G1 (MZ787802) represented 10.00% of the total subtypes.C. hominis alignments with reference sequences classify all isolates into one family: Ib. Extra sub-classification led to two subtypes identified with subtype IbA21G2 being the most common, with rate 80.00% with accession number (MZ787805, MZ787806, MZ787807, MZ787808, MZ787809, MZ787810, MZ787811& MZ787812). Subtype IbA13G3 identified with rate 20.00% with accession number (MZ787813 & MZ787814).

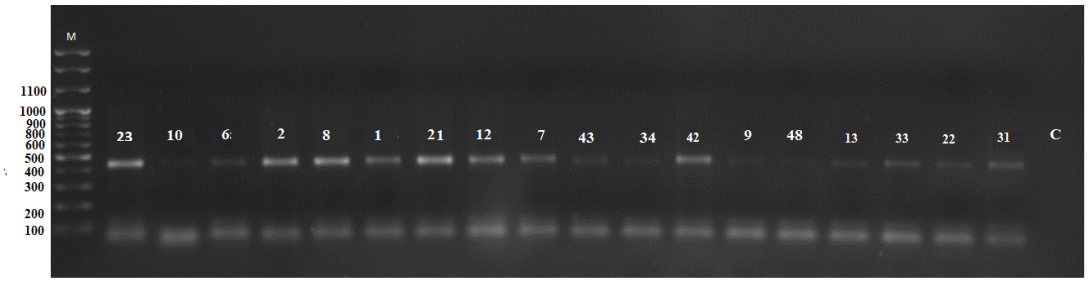

Fig. 1. Gel electrophoresis image (1 %) *agarose* (1-48) shows positive PCR sheep Cryptosporidium samples (size= 448 bp) universal primers (first round) targeting (HSP 70). M is molecular marker (100- 1100 bp); C is control negative in which master mix and primers and H2O was added instead of template DNA.

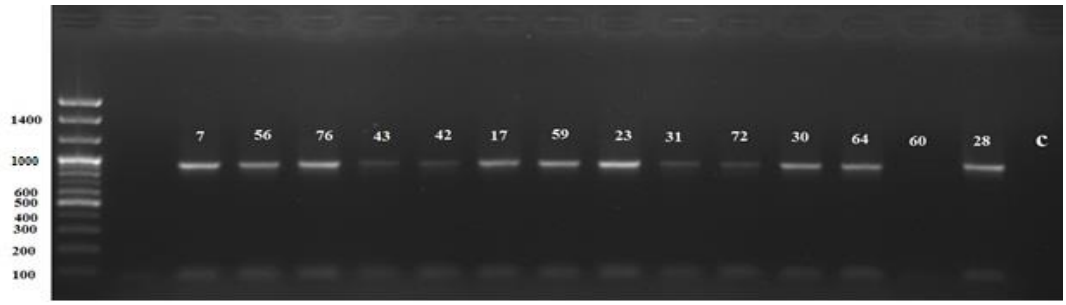

Fig. 2. Gel electrophoresis image (1 %) *agarose* (1-76) shows positive PCR sheep Cryptosporidium samples (size= 800 bp) for the subtypes of Cryptosporidium hominis (second round) targeting (gp60 gene). M is molecular marker (100- 1100 bp) from (ADDBIO, Korea); C is control negative in which master mix and primers and H2O was added instead of template DNA.

3.3. Phylogenetic tree

A neighbor-joining tree was constructed using MEGA version 6 for all 14 Cryptosporidium isolates to assess the relationship among various Cryptosporidium species in sheep. six isolates of C. parvum. three isolates for C. ubiquitum . Both C. hominis and C. andersoni in two isolated have been consigned in the GenBank database under accession no. MZ787774 to MZ787775 for C. hominis and MZ787779 to MZ787779 for C. andersoni . There is good relation between C. parvum and C. hominis and C. ubiquitum .The C. parvum isolate was matched to C. parvum isolate from the Netherlands under accession no ABV82086.1 and the C. hominis isolate showed high similarity to the C. hominis isolate from Brazil under accession no AMR08234.1. While the C. ubiquitum and C. andersoni isolates were identical to C. ubiquitum isolate from UK under accession no ADX53321.1 and C. andersoni isolate from China under accession no CAD99402.1 respectively Fig. 3.

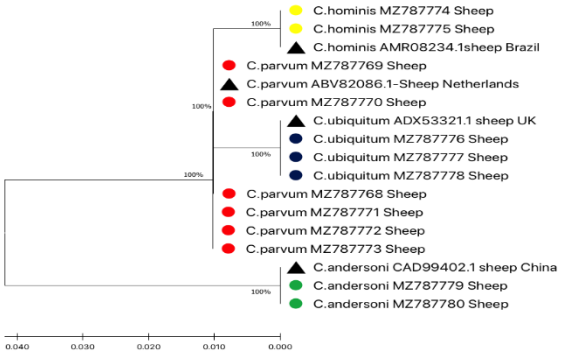

Fig. 3. Phylogenetic tree analysis based on the partial sequence the Hsp70 gene of sheep isolates used for local Cryptosporidium species (referred as circular) compared with global Cryptosporidium species (referred as triangle).

Ten isolates of C. parvum subtype, have been deposited in the NCBI GenBank database e (Korea) under accession no. MZ787795 to MZ787804 and ten isolates for C. hominis subtype have been deposited in the NCBI GenBank database under accession no. MZ787805 to MZ787814. There is close relation between the subtype IIdA17G1a, IIdA17G1, IIdA18G1 and IIdA21G1. The IIdA21G1 subtype C. parvum isolate showed high similarity to IIdA21G1 C. parvum isolate from the Portugal under accession no ABB89137.1 Also IIdA21G1 C. parvum was matched to C. parvum isolate from the Turkey under accession no ANS57191.1. The C. parvum IIdA17G1a subtype isolate showed high similarity to the IIdA17G C. parvum isolate from China under accession no AMW87772.1. In case of subtype C. hominis, there is good relation between the subtype IbA21G2 and IbA13G3 .The C. hominis IbA13G3 subtype isolate was the same as to C. hominis isolate from the Peru under accession no ABK32087. Fig. 4.

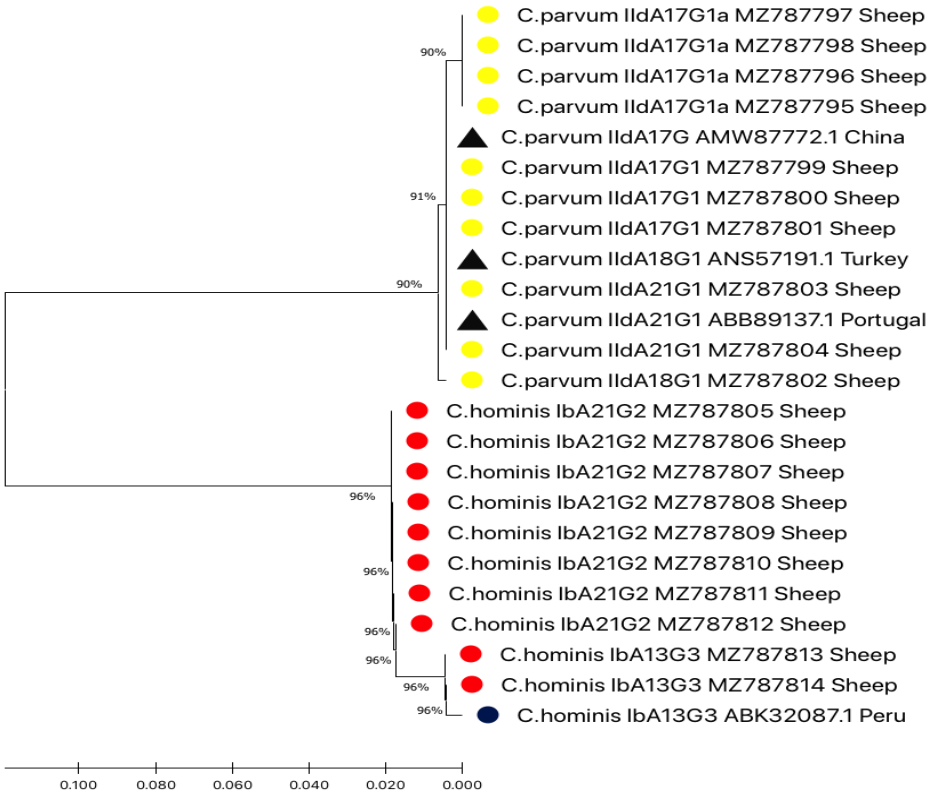

Fig. 4. Phylogenetic tree analysis based on the partial sequence the gp60 gene of sheep isolates that used for local C. parvum and *hominis* subtype (referred as circular) compared with global C. parvum and *hominis* subtype (referred as triangle).

3. Discussion

The results of molecular study revealed that C. parvum has the highest infection rate in sheep. Similar results was found by [16, 17] on sheep of Al-Qadisiyah province.The current study does not agree with [18] in lamb in Algeria and [19] in sheep in China, which showed that C. xiaoi was the dominant species in sheep. The high percentage of C. parvum compared to the other species of Cryptosporidium may be attributed to the fact that C. parvum is not specific to a host, and it is the most prevalent species in other animals and the second most prevalent after C. hominis in humans [20, 21] . Ten GP60 sequences belonging to C. parvum indicate that one subtype families is IId, in sheep with four subtypes IIdA17G1a, IIdA17G1, IIdA18G1 and IIdA21G1. The IIdA21G1 and IIdA17G1a subtypes were previously recorded in Iraq [17], while in this study the IIdA17G1 subtype was recorded for the first time in Iraq. The first C. parvum subtype IIdA21G1 is isolates from sheep and has been found by [22] in Portugal isolated from HIV-infected patients and sheep. In Spain, [23]

studied the Cryptosporidium species and subtype in goat kids and lambs and found IIdA21G1 in lambs. In China, the presence of the C. parvum subtype IIdA21G1 found in dairy cattle [24]. In Denmark, [25] searched for Cryptosporidium Species and subtype and found subtype IIdA21G1 from human stool samples. In Italy [26] reported IIdA21G1 subtype for the first time in horses. In Aragón (North-Eastern Spain) 27 recorded the subtype IIdA21G1 in wastewater sample. In Al-Diwaniyah province [17] found IIdA21G1 in sheep. The second subtype of C. parvum is IIdA17G1a found only in sheep. In Al-Diwaniyah province 17 found the same subtype in sheep. The same subtype was also isolated form goat kids and lambs in Spain [23]. The third subtypes of C. parvum subtype IIdA17G1 found in sheep. In Portugal, it was isolated from water [28]. In Ethiopia, the subtype IIdA17G1 had been isolated form HIV-infected patients [29]. In China, subtype IIdA17G1 was found in cattle 30. In Qatar, detected the subtype IIdA17G1 in human [31]. In British, [32] noticed the subtype IIdA17G1 in (Erinaceus europaeus) European hedgehogs. The four subtype of C. parvum (IIdA18G1) found only in sheep. In Kuwait, [33] found the same subtype in children. Belgrade, Serbia and Montenegro isolated the same subtype from calves [34]. In Turkey [35] noticed the subtype IIdA18G1 in calves. In Spain IIdA18G1 detected in lambs [23]. In Iran, [36] noticed the same subtype in humans. In Wales and England, [37] isolated the subtype from human. In Iran, 38 found the subtype in the river water. In Denmark, 25 showed the subtype in human stool samples. In Qatar, [31] isolated the subtype form human. In Sudan, [39] found the subtype calves. Two subtypes were achieved by the GenBank database, they are IbA21G2 & IbA13G3. One subtypes (IbA21G2) were previously recorded and the subtypes (IbA13G3) 17 were recorded for the first time in Iraq. The first C. hominis subtype IbA21G2 sequences is isolates from sheep in current study. The same subtype isolated form China water [40]. In Al-Diwaniyah province IbA21G2 found in sheep 17. The second C. hominis subtype (IbA13G3) is isolates from sheep, and this subtype has been found in many studies, in Peru, the same subtype isolated from human [41]. In Ghana, [42] found the subtype in children. In Swedish, and France the same subtype was found in patients and calves [43, 44]. In Spain, the same subtype was detected in human [45]. The Ib subtype family of C. hominis is wide spread and that may cause infection in human and animals especially sheep. This agrees with [46, 47] who revealed that animals may be considered as a source for C. hominis.

4. Conclusion

These results indicate a common occurrence of four species of Cryptosporidium in sheep Four species (C. parvum, C. hominis, C. ubiquitum and C. andersoni) and four subtype of C. parvum IIdA17G1a, IIdA17G1, IIdA18G1 , IIdA21G1 and Three subtypes of C.hominis (IbA21G2 and IbA13G3).

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تحليل الانواع الفرعية لـطفيلي الابواغ الخبيئة في الاغنام/ محافظة بابل وسط العراق

عقيل محمد كاظم الموسوي*[١]، عبد الحسين حبش عواد*[٢]، منصور جدعان علي الخالد[٣]

[١] قسم الطفيليات، كلية الطب البيطري، جامعة القاسم الخضراء، بابل، العراق.
[٢] قسم علوم الحياة، كلية التربية للعلوم الصرفة، جامعة البصرة، البصرة، العراق.
[٣] قسم الطفيليات، كلية الطب البيطري، جامعة القادسية، القادسية، العراق.

معلومات البحث

الاستلام ٢٥ شباط ٢٠٢٢
القبول ٦ حزيران ٢٠٢٢
النشر ٣١ تموز ٢٠٢٢

الكلمات المفتاحية

انواع الابواغ الخبيئة ، التنميط الجيني، الانواع الفرعيه، الاغنام، العراق.

الملخص

تهدف الدراسة الحالية الى التحقق من امكانية انتقال طفيلي خفية الأبواغ من الحيوان الى الانسان ودراسة التنوع الجيني لأنواع ونويعات طفيلي C. parvum وC. hominis لاغنام محافظة بابل. تم جمع ١٨٠ عينة براز من أربعة مواقع في محافظة بابل. تم اختباره بواسطة صبغة Ziehl-Neelsen المعدلة، تم أخذ ١٠٠ عينة إيجابية لتفاعل البوليميراز المتسلسل المتداخل. تم تحديد اربعة أنواع (C. parvum وC. hominis وC. ubiquitum وC. andersoni) بناءً على تحليل تسلسل بروتين الصدمة الحرارية ٧٠ (Hsp70). اظهر التحليل الوراثي الجزيئي للجين gp60 وجود ١٠ عزلات من C. parvum و ١٠ عزلات من C. Chominis سجل اربعة انواع فرعيه هي IIdA17G1a و IIdA17G1 وIIdA21G1 وIIdA18G1 ينتميان إلى C. parvum. ونوعين فرعين من C. hominis هما: IbA21G2 وIbA13G3 يمكن القول بأن الأغنام يمكن اعتبارها مضيفة خازنة لـ C. parvum وC. hominis والانواع الفرعية IId وIb للحيوانات الاخرى والبشر.

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***Corresponding author email :** akeelmohammed@vet.uoqasim.edu.iq

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