Molecular characterization of *Cryptosporidium* spp. in sheep and goat in Al-Qadisiyah province/ Iraq

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Summary

The present study was conducted during the period from September 2015 until February 2016. 100 fecal samples were collected from 60 sheep and 40 goats for diagnosis of *Cryptosporidium* parasite from diverse areas in Al-Qadisiyah province. The study amid to know the genetic characters of *Cryptosporidium* spp. parasite by using a molecular technique such as the nested polymerase chain reaction and DNA sequencing analyzed by phylogenetic tree to identify the parasite species. This study was done on the sheep and goat at first time in the middle region of Iraq and the identified species were recorded in NCBI-Genbank database. In sheep, the results of positive infected samples was (40%) while, in the goats were (32.5%), the DNA sequencing and phylogenetic analysis method based on small ribosomal RNA gene (18s rRNA) for *Cryptosporidium* species typing. The results were conducted by Neighbor-Joining phylogenetic tree analysis method and the 18s rRNA gene sequences were confirmed by using NCBI-BLAST data analysis in order to compare with NCBI submitted selected references isolates of (18s ribosomal RNA) gene in *Cryptosporidium* spp. parasites. Our finding in present study appeared to follow spp. (*C. parvum, C. hominis, C. andersoni, C. ubiquitum, C. xiaio* and *C. suis*). These identified species which primary affected sheep and goats as mentioned in previous studies when compared with newly Iraq isolates strains.

Keywords: *Cryptosporidium*, Phylogenetic, Polymerase Chain Reaction, Sheep, Goat.

Introduction

The apicomplexan protozoan parasite *Cryptosporidium* infects an extensive range of farm animals, primarily causing gastritis, diarrhea, and/or catarrhal respiratory signs (1 and ٢). The multiple spp. of *Cryptosporidium* parasite recognized in feces samples of sheep by molecular techniques are *C. parvum, C. xiaoi, C. ubquitum, C. fayeri, C.hominis*, and *C. andersoni*, whereas *C. parvum, C. hominis* and *C. xiaoi* may infect goats (3). Previous studies showed that *C. parvum* was the dominant *Cryptosporidium* spp., as well as *C. xiaio, C. hominis*, a goat genotype, and a new Cryptosporidium genotype have also been identified in goats (4 and 5). Two major species have been recognized in goat kids in European countries: *C. parvum* (6) and recently *C. xiaio* from two kids suffering from diarrhea minimum than 21 days old (7). Other species such as *C. ubiquitum*, and *C. andersoni* were also described in goats (8). The aim of this study was to evaluate the genetic characters of *Cryptosporidium* spp. in sheep and goat.

Materials and Methods

The nested PCR technique was performed for detection *Cryptosporidium* spp. based 18S ribosomal rRNA gene from sheep and goat fecal samples by multiple steps: The feces samples were subjected to nucleic acid extraction by using commercial Stool-DNA extraction kit from Bioneer-Corporation, Korea (Accu-Prep®stool-DNA Extraction kit). The extraction method was done according to the manufacturing instructions by using stool DNA Protocol extraction method by stool lysis buffer and 10 mg/ml Proteinase-K. The extracted Stool-DNA was estimated by nanodrop device at 260/280 nm, and then kept at deep freezer until used in PCR method. The PCR primers used in this study for detection *Cryptosporidium* spp based on 18s rRNA gene by using Nested PCR technique were designed by (9) and these primers were provided from Bioneer company, Korea as following (Table, 1).

PCR primary round was prepared using (Accu-Power®PCR-PreMix-Kit) master mix reagent and done depending on company instructions as following (Table, 2).

Table, 1: PCR primers for detection of *Cryptosporidium* spp. based on 18s rRNA gene

Primer		Sequence	PCR Size
18S rRNA first	F	GACATATCATTCAAGTTTCTGACC	763bp
	R	CTGAAGGAGTAAGGAACAACC	
18S rRNA second	F	CCTATCAGCTTTAGACGGTAGG	587bp
	R	TCTAAGAATTTCACCTCTGACTG	

Table, 2: Company instructions of PCR master mix.

PCR Master mix	Volume
DNA template	5µL
18SrRNA first Forward primer (10pmol)	1.5µL
18SrRNA first Reverse primer (10pmol)	1.5µL
PCR water	12 µL
Total volume	20µL

Primary thermocycler conditions round using PCR thermocycler protocol as following (Table, 3). Nested PCR secondary round was prepared using (Accu-Power®PCR-PreMix-Kit) master mix reagent and done depending on company instructions as following (Table, 4). Thermocycler conditions of second round using Nested-PCR thermocycler protocol as following (Table, 5).

Table, 3: PCR thermocycler conditions .

PCR step	Temp.	Time	Repeat
Initial	94˚C	5 min	1
Denaturation	94˚C	30 sec.	
Annealing	58˚C	30 sec	30 cycle
Extension	72˚C	30 sec	
Final extension	72˚C	5 min	1
Hold	4˚C	Forever	-

Table, 4: Company instructions of Secondary PCR master mix.

PCR Master mix	Volume
DNA template (PCR product)	5µL
18SrRNA second Forward primer (10pmol)	1.5µL
18SrRNA second Reverse primer (10pmol)	1.5µL
PCR water	12 µL
Total volume	20µL

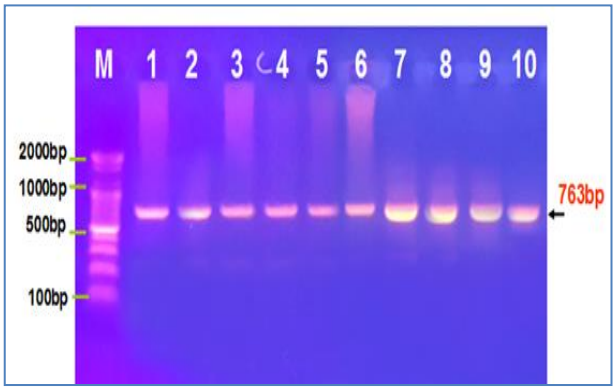
Table, 5: Nested- PCR thermocycler system.

PCR step	Temp.	Time	Repeat
Initial	94˚C	5 min	1
Denaturation	94˚C	30 sec.	
Annealing	58˚C	30 sec	30 cycle
Extension	72˚C	30 sec	
Final extension	72˚C	5 min	1
Hold	4˚C	Forever	-

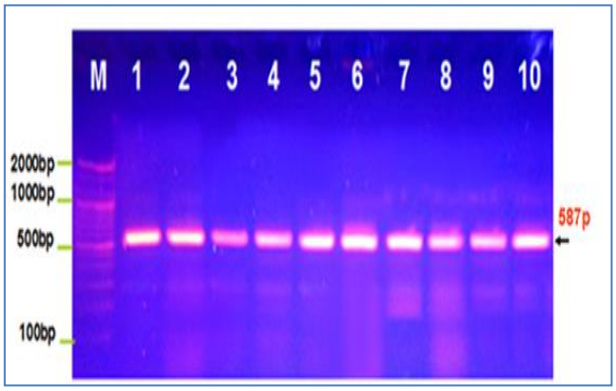
DNA sequencing method for *Cryptosporidium* spp. target gene (18S-ribosmal RNA gene) was done after positive amplification of a 578 bp nested PCR product purified from gel by using (Agrose gel purification Kit, Biobasic Corporation in Canada). After that, these purified 18SrRNA genes samples were sent to DNA sequence in Korea to perform the DNA sequencing and analyzed by using phylogenetic analysis (Mega 6.0 version) and NCBI-Local Basic Sequence Alignment data base.

Results and Discussion

The results of Nested-PCR investigation in stool-DNA samples of sheep were showed, among 60 sheep stool-DNA samples, 24 (40%) showed positive samples, on the other hand, among (40) goats stool-DNA samples, 13 (32.5%) showed positive samples. The statistical analysis has shown no significant differences at (P>0.05) level as (Fig.1 and 2).



Figure, 1: Agarose gel electrophoresis show the PCR product analysis of 18S rRNA gene in *cryptosporidium* spp. positive fecal samples. Where M: Marker (2000-100 bp), lane (1-5) positive sheep sample and lane (6-10) positive goat sample (763 bp) PCR product (First around).



Figure, 2: Agarose gel electrophoresis show the Nested PCR product analysis of 18S rRNA gene in *cryptosporidium* spp. positive fecal samples. Where M: Marker (2000-100 bp), lane (1-5) positive sheep samples and lane (6-10) positive goat sample (587 bp) PCR product (Second around).

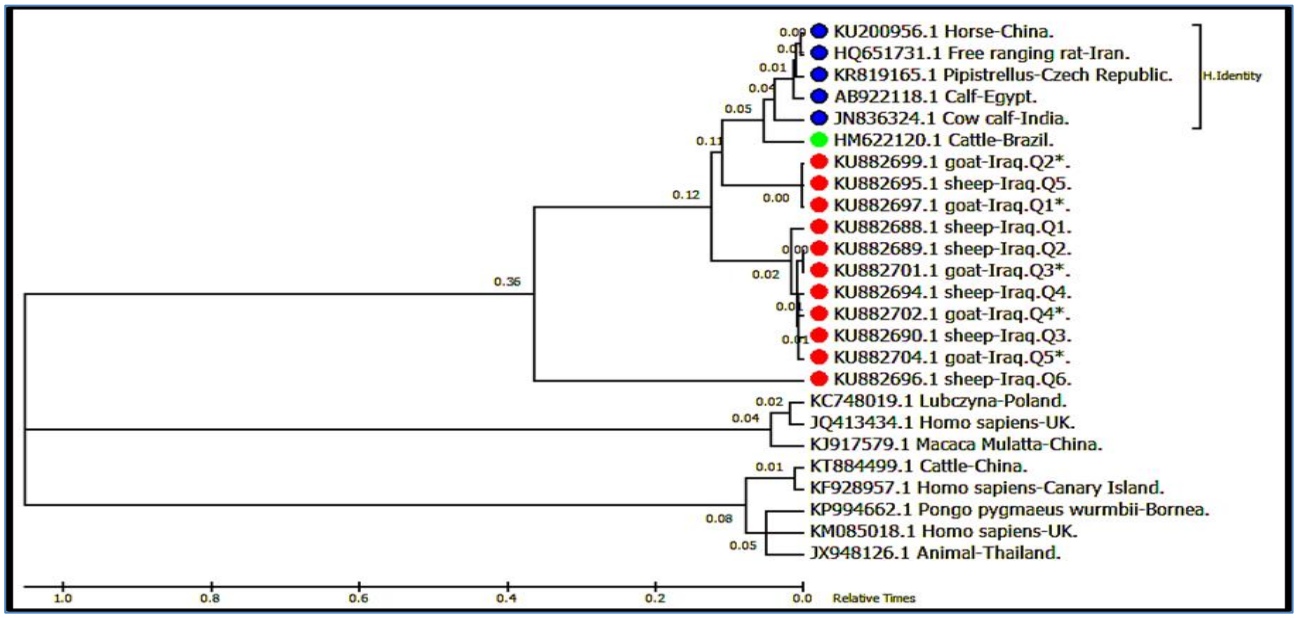
The results of this study were agreement with the pervious results that showed that

Cryptosporidium oocysts were detected in Serbia 42.1% of sheep and 31.8% in goat (10 and 11). *Cryptosporidium* prevalence peaked to 43.9% in sheep and (31.6) in goat from north-western Spain (12), and these evidences have shown less prevalence rates in sheep, when compared with a previous study in Egypt that up to show 51.11% prevalence rates (13). Whereas, in goat, the results of current study showed a rate near to (14) but in sheep the study showed a higher than (14). As well as in another study, the results showed a rate higher than the rate in our study (15). Increase the prevalence of *Cryptosporidium* infection especially in farm (sheep and goat) that up to (40% and 32.5%) respectively, it may cause a potential threat to the population of small ruminant (16), and in study of (12) where *C. parvum* for extreme, PCR-positive cases were investigated from both lambs (74.4 %) and of goat kids (93.8 %). Whereas the study of (17) the rate was 27 (76.4%) goat kids as well as the 83.3 % of the 54 examined goat herds , there is different rate in the world by using nested- PCR, Also 77.4% from the United States, 25% from Brazil and 24.5% from Australia were reported by (18-20), there was no relationship between prevalence and lamb age (21), however evidence suggests that *Cryptosporidium* prevalence is not highest in very young lambs, For example, previous studies in west Australia have reported prevalence's by PCR of 26% for slaughter age lambs (2) and 24.5% for pre-weaned lambs (aged 1–8 weeks) in Australia (20). Further longitudinal research is require to better understand the association between the prevalence of *Cryptosporidium* and lamb age and in goat our study show low rate of infection that agree with (11), the prevalence of infection reduced with older age, with the highest rate, 62.7%, being investigated in goat kids while the lowest rate of infection, 22.5%, was recorded in adult that result agree with (22).

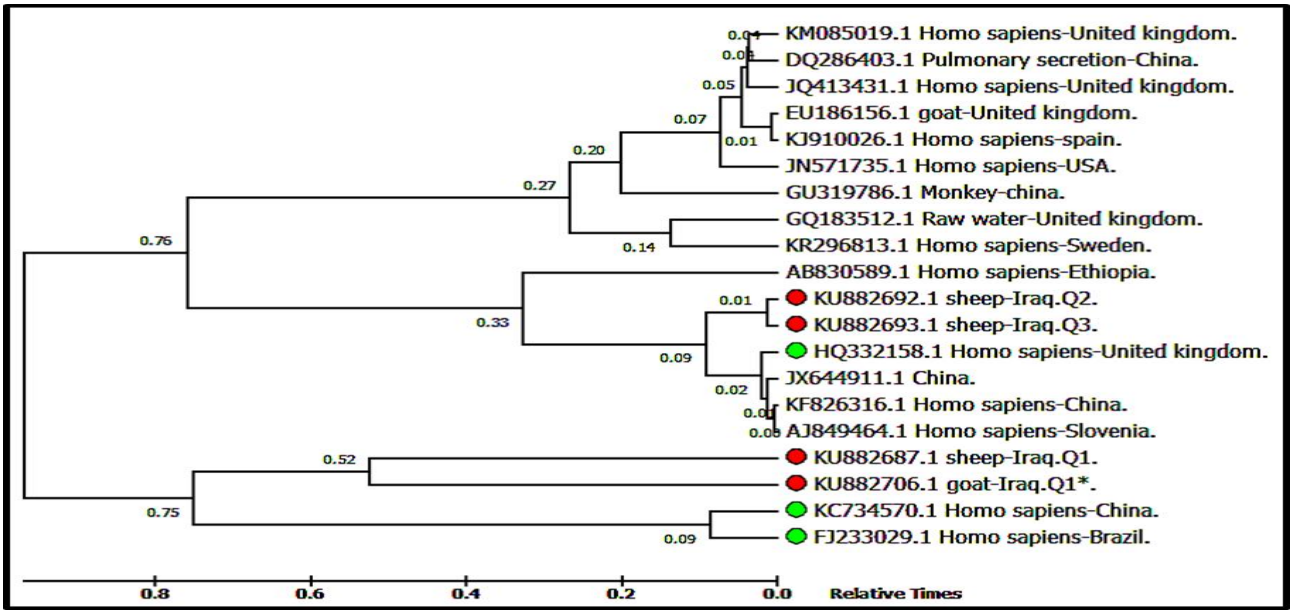
The DNA sequencing results at first were used to confirmation of Nested-PCR product results of *Cryptosporidium* spp. based on 18S-ribosmal RNA gene. Where, the nucleotides sequence was analyzed by using the NCBI (BLAST analysis data base) that aligned the nucleotide query with 18s ribosomal RNA references of gene of *Cryptosporidium* parasite that include *C. parvum, C. hominis, C. andersoni, C. ubiquitum, C. xiaio* and *C. suis* gene sequences that recorded in Gen Bank as well as the out references groups to investigated the degrees of total identity and total similarity score of the 18s ribosomal RNA gene of *cryptosporidium* spp. which generally effected sheep and goats and compared with our Iraqi isolates. The results of local *Cryptosporidium* spp. that includes (6 sample of sheep and 5 sample of goat) were showed closed related to reference *C. parvum* isolates, the total percent identity score ranged (97.70-100%), 3 sample from sheep and 1 sample from goat was showed neighboring related to NCBI-BlAST *C. hominis*, the percentage of identity score ranged from (97.71-100%), 1 sample from goat was showed closed related to NCBI-Blast *C. andersoni*, the percentage of identity score (100%), 2 sample from goat was showed neighboring related to NCBI-BlAST *C. ubiquitum*, the percentage of identity score (100%), 1 sample from goat were showed neighboring related to NCBI-BlAST *C. xiaio*, the percentage of identity score (100%) and 1 sample from sheep were showed neighboring related to NCBI-BlAST *C. suis*. The phylogenetic tree analysis was used for *Cryptosporidium* species typing detection, the 11 Iraqi *C. parvum* isolates of the present study as in (Fig. 3). The phylogenetic tree analysis was used for *Cryptosporidium* species typing detection, the 4 Iraqi *C. hominis* isolates of this study as in (Fig. 4).

The phylogenetic tree analysis was used for *Cryptosporidium* species typing detection, including the (1) Iraqi *Cryptosporidium andersoni* isolates of this study as in (Fig. 5). The phylogenetic tree analysis was used for *Cryptosporidium* species typing detection, including the 1 Iraqi *Cryptosporidium xiaio* isolates of this study as in (Fig. 6).

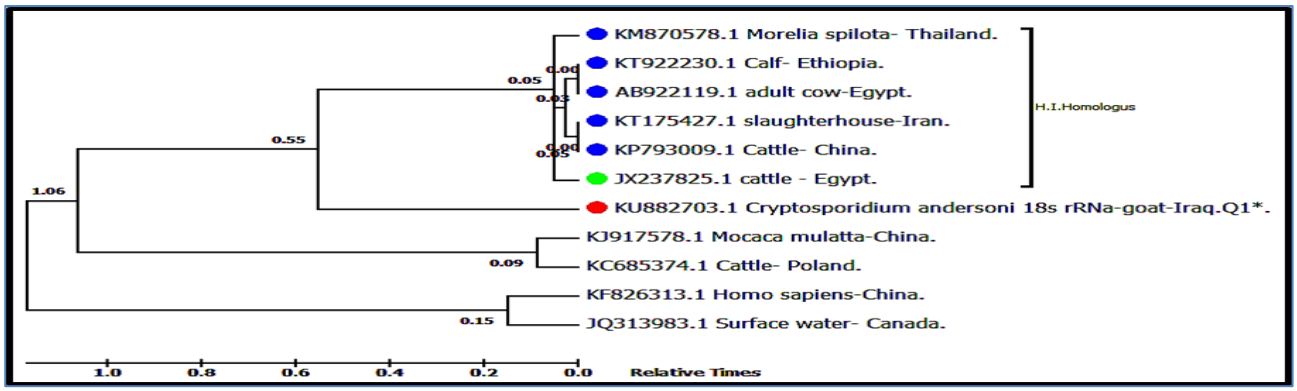
The phylogenetic tree analysis was used for *Cryptosporidium* species typing detection, including the 2 Iraqi *C. ubiquitum* isolates of the this study as in (Fig. 7). The phylogenetic tree analysis was used for *Cryptosporidium* species typing detection, including the 1 Iraqi *C. suis isolates* of the this study as in (Fig. 8).



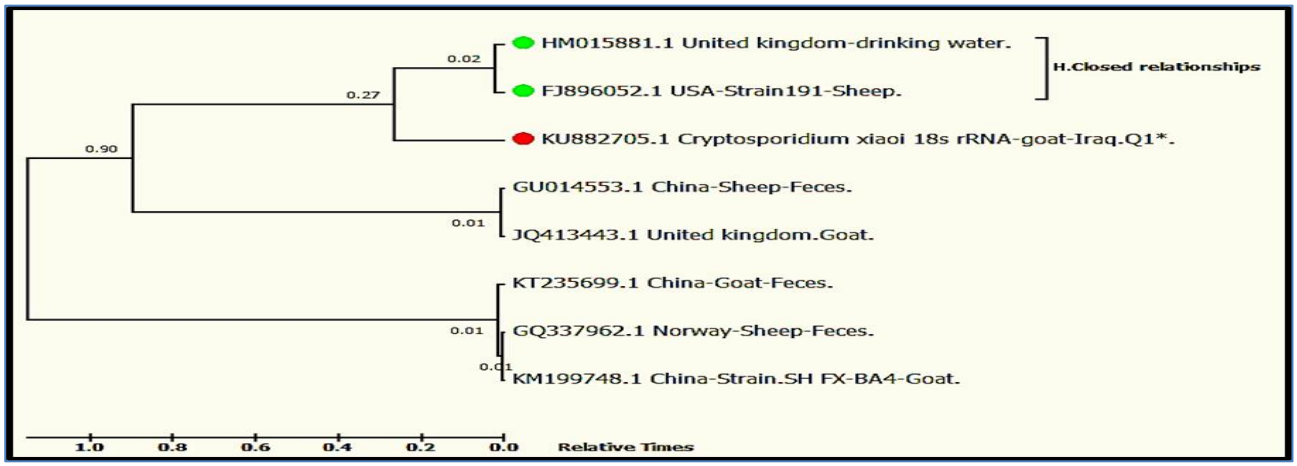
Figure, 3: Phylogenetic tree depend on the Neighbor joining tree method.The phylogenetic distances analyses were conducted by MEGA6. *Red spot: *C. parvum* strains isolated from Iraq. *Green spot: Strain high homologous with Iraqi –*C. parvum* isolated from sheep and goat. *Blue spot: Strains highly identity with Iraqi –*C. parvum* isolated from sheep and goat.



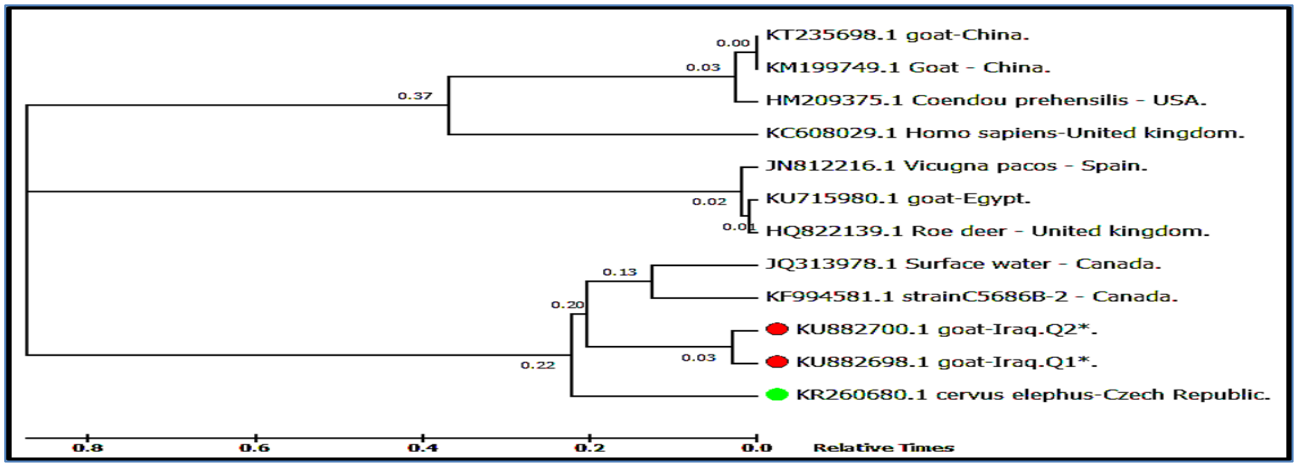
Figure, 4: Phylogenetic tree using the Neighbor joining method. The evolutionary distances analyses were conducted in MEGA6.*Red spot: *C. hominis* strain isolated from Iraq.*Green spot: Strain high homologous with Iraqi –*C. hominis* isolated from sheep 1*, 2*.



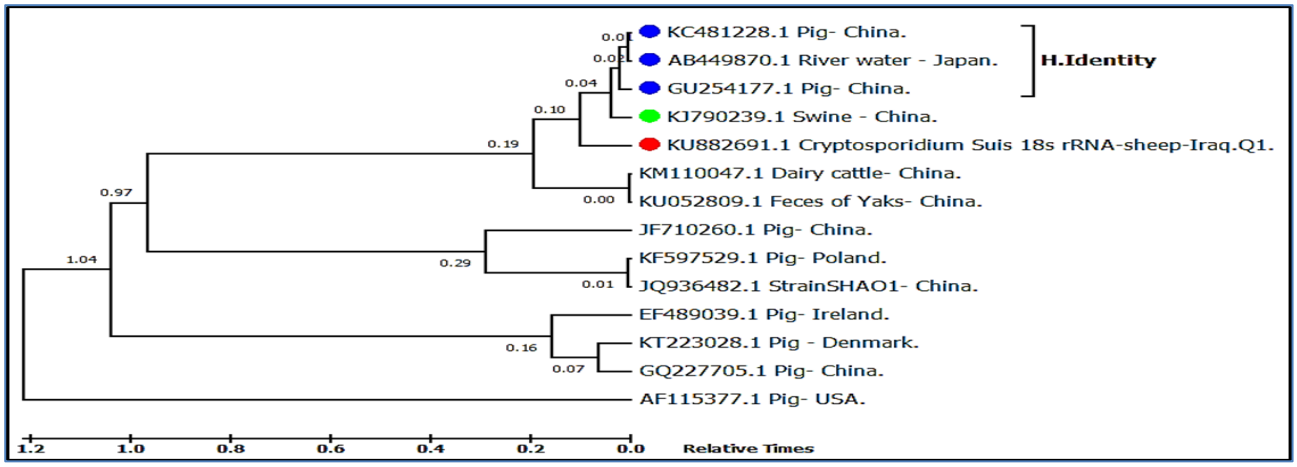
Figure, 5: Phylogenetic tree depend on the Neighbor joining tree method. The phylogenetic distances analyses were conducted by MEGA6.*Red spot: *C. andersoni* strain isolated from Iraq.*Green spot: Strain high homologous with Iraqi –*C. andersoni* isolated from goat. *Blue spot: Strains highly identity with Iraqi –*C. andersoni* isolated from goat.



Figure, 6: Phylogenetic tree depend on the Neighbor joining tree method. The phylogenetic distances analyses were conducted by MEGA6.*Red spot: *C. Xiaio* strain isolated from Iraq.*Green spot: Strain high homologous with Iraqi –*C. Xiaio* isolated from goat.



Figure, 7: Phylogenetic tree depend on the Neighbor joining tree method. The phylogenetic distances analyses were conducted by MEGA6. *Red spot: *C. ubiquitum* strains isolated from Iraq.*Green spot: Strain high homologous with Iraqi – *C. ubiquitum* isolated from goat 1*, 2*.



Figure, 8: Phylogenetic tree depend on the Neighbor joining tree method. The phylogenetic distances analyses were conducted by MEGA6.*Red spot: *C. suis* strain isolated from Iraq. *Green spot: Strain high homologous with Iraqi – *C. Suis* isolated from sheep.*Blue spot: Strains highly identity with Iraqi –*C. suis* isolated from sheep.

Sequencing study and phylogenetic analysis: The DNA sequencing study reflected the chief *Cryptosporidium* spp which were *C .parvum*, *C. hominis* and *C. suis*. While in another study, the genotypes have been investigated in sheep feces: *C. parvum*, *C. suis*, *C. hominis*, *C. bovis*, new sheep genotype (23). The sample of goat the results were

recorded five spp of *Cryptosporidium* spp *C. parvum, C. hominis, C. andersoni, C. ubiquitum* and *C. xiaio* noted in other study with (24) including *Cryptosporidium ubiquitum* (24 from 44) in Henan and Chongqing, and *Cryptosporidium andersoni* (16 from 44) and *Cryptosporidium xiai*o (4 from 44) in Henan and *Cryptosporidium* species/genotypes have been recognized in goats thus far, including *C. parvum, C. xiaio, C. hominis*, a goat genotype, and a new *Cryptosporidium* genotype (5). *C. parvum* is the main *Cryptosporidium* species, which has been established from goats in Italy, Spain, Belgium, Czech Republic, the holland, France, India, Sri Lanka, Zambia, and Egypt (25) and *C. ubiquitum and C. andersoni* represent the first investigated *Cryptosporidium* species in goats, which have once upon a time been create in sheep in Henan, China (26).

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التشخيص الجزيئي للأبواغ الخبيئة في الأغنام والماعز في محافظة القادسية/ العراق

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الخلاصة

أجريت الدراسة الحالية خلال المدة من شهر أيلول ٢٠١٥ ولغاية شهر شباط ٢٠١٦ حيث جُمعت ١٠٠ عينة براز وبواقع ٦٠ عينة من الأغنام و٤٠ عينة من الماعز للتحري عن طفيلي الأبواغ الخبيئة من مختلف مناطق محافظة القادسية، صُممت هذه الدراسة لمعرفة صفات الطفيلي باستعمال بعض التقنيات الجزيئية والتي تضمنت تفاعل السلسلة المتبلمرة المتداخل كذلك استعمال طريقة تحليل ترتيب النيوكليتيدات. وحُدّدِت العلاقات الوراثية التطورية (التحليل الشكلي) للأنواع السائدة وللمرة الأولى في المنطقة الوسطى من العراق وتسجيلها عالميا" في بنك الجينات العالمي، في هذه الدراسة كانت نسبة الإصابة في الأغنام والماعز ٤٠% و٣٢,٥% على التوالي باستعمال تفاعل السلسلة المتبلمرة المتداخل. كذلك استعملت الدراسة الحالية تحليل وقراءة ترتيب النيوكليتيدات عشرين نموذج DNA بعد استخلاصها وتنقيتها من هلام الأكاروز للحصول على الترتيب النيوكلوتيدي لجين (18s rRNA) وقد تم مطابقة نتائج تحليل الترتيب النيوكلوتيدي عن طريق الاتحاد الدولي للتقنيات الاحيائية عبر الانترنيت وفُحِصَت وأكِدَت مع عتر الطفيلي المسجلة عالميا" في بنك المورثات العالمي والتي تضمنت أنواع طفيلي الأبواغ الخبيئة وهي *C.parvum*، *C.hominis*، *C. andersoni*، *C.ubiquitum*، *C.xiaio* و *C.suis*.

الكلمات المفتاحية: داء الابواغ الخبيئة، الشجرة الوراثية، تفاعل تسلسل البلمرة، الماعز، الأغنام.