GENOTYPE OF *Cryptosporidium spp.* ISOLATED FROM BOVINE OF AL-QADISIYAH PROVINCE /IRAQ.

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ABSTRACT

The current research included examination of 100 fecal sample from bovine was collected from AL-Qadisiyah province, from September 2018 until February 2019.The Microscopically result showed that oval or spherical shaped with dark pink color or red oocyst on blue ground and 30(30%) positive sample out 100 case . It was recorded that the maximum rate 41.66% (5/12) was seen in November, but the lowest rate 18.75% (3/16) was seen in the month of February with no significant differences at level ($p<0.05$.) According to age the maximum rate of incidence 40%(14/35)was found in the age group lower than a month, but the lowest incidence was seen in the group(>1 years). There is no significant differences at $p<0.05$.between male and female. In the currently study the N-PCR in molecular investigation were used ,the positive sample was18 (60%) out of 30 fecal sample. Sequencing of a fragment of the(18s rRNA) gene (834 bp) that separated from many distinct area in AL- Qadisiyah government recorded (50%)6/12 sample related to NCBI – Blast *Cryptosporidium parvum* ,(33.33%) 4/12 sample display deep related to NCBI –Blast *Cryptosporidium bovis (*this first study reported *C. bovis* in Iraq *)* , (16.66%) 2/12 sample showed closed related to NCBI –Blast *Cryptosporidiumandersoni.*

INTRODUCTION

Protozoan *Cryptosporidium* are highly important parasites, which can cause parasitic diarrhea in animals. It is also widely spread in different countries, whether developing or developed countries, Where it affects most of the host, such as humans ,wild and domestic animals(1). Cryptosporidosis in cattle is characterized by multiple symptom such as diarrhea, vomiting, weight loss, abdominal pain and other signs ,but these signs do not lead to the mortality of the animal(2). There are many of *Cryptosporidium* spp. that include *C. meleagridis*, *C. canis* , *C. felis* and *C. parvum* that have zoonotic effect(3),but *C. parvum* most important species which infected both human being and animals particularly cattle (4). The *Cryptosporidium parasite* resistance oocysts are transmitted by oral-fecal rout, *C.hominis, C.parvum* is among the eight widely distribution species of *Cryptosporidium*(5). The shizonts were founded in the intestinal tissue of the host demonstrates and confirms the presence of the *Cryptosporidum parasite* in the host (6).The Zeihl Neelsen stain was used in the detection of *Cryptosporidium*, to separate and concentrate the oocysts The Sheather's sugar floatation methods is used(7). There have been many tests to eliminate *Cryptosporidium parasites* over the years but with limited success, including the use of halofuginon lactate is useful but does not work on completely prevention or cure of symptoms of the disease (8).

MATERIAL AND METHODS

- **Collection of specimens**

100 sample feces were collected from bovine of different ages , the age groups of cattle were divided into four groups (less than one month) (1-6)months, (6-12)months and from both sexes during the period from September 2018 until the end of February 2019 , Includes different areas of Qadisiyah province .these samples were taken directly from the animals rectum , the samples are placed in sterilized containers marked with information such as age and sex and clinical symptom of the animals .The sample were transferred to the laboratory of the Veterinary Medicine College in University of Al - Qadissiyah for the necessary tests.

- **Microscopic Examination of *Cryptosporidium* oocyst**

The diagnosis of *Cryptosporidium* oocysts depends on the microscopic examination of the in the fecal smear and usually uses modified acid fast stain protocols , such as Ziehl-Neelsen(acid-fast)
stain, and the microscopic examination of the oocysts is shown as red-stained sphericals. This is the best way to examine the oocysts because it is uncomplicated , fast and cheap price (9).

- **DNA isolation and molecular analysis**

1. DNA was extracted from 30 *Cryptosporidium* positive fecal samples by using fecal DNA kit (Accu Prep® stool DNA Extraction Kit , Bio neer. Korea) , Where we followed the protocol of the manufacturer. The DNA is preserved in -20c until it is used in PCR.

- **Nested - PCR .**

polymerase chain reaction was used to amplify of 18s r RNA the detection of *Cryptosporidium spp*. (10)with some modifications. In the first step, partial 18S rRNA of *Cryptosporidium* was amplified in a 25 µl reaction mixture having (20) pmol of every primer (CRP-DIAG1 Forward: 5' TTC TAG AGC TAATAC ATG CG 3' and CRP-DIAG1 Reverse: 5' CAT TTC CTT CGA AAC AGG A 3'), in the first round of PCR employing the following thermal cycling protocol: one cycle of initial denaturation at 94 °C for 5 min, sequences by 35 cycles each of de naturation at 94°Cfor 1 min, annealing at 56°C for 1 min and extension at 72°Cfor 1 min. This was sequence by last extension for 10 min. 72°C.in the second around 1µl of the first PCR product was employed as a template and 20 pmol of primers (CRP-DIAG2 Forward: 5' GGA AGG GTT GTA TTT ATT AGA TAA AG 3' and CRP-DIAG2 Reverse: 5' AAG GAG TAA GGA ACA ACC TCC A 3') were used in 50 µl reaction mixture. The PCR reaction and cycling condition were same to the environment employed for primary PCR, but the annealing temperature was at 60°C for 1 min .

- **Sequencing**

Nested polymerase chain reaction products were sent to Macrogen Co./ Korea where they were subjected to direct sequencing. *Cryptosporidium spp.* and subtypes were recognized by employing the BLAST search against the GenBank database.

- **Statistical analysis:**

All statistical calculations were done by employing Statistical Package of Social Sciences (SPSS), version 23 (Inc., Chicago, IL, USA) computer software. Variation between different groups were analyzed using chi-square test (X2) . The level of statistical significance was set at alpha equal to 0.05 (a = 0.05). A value of $P < 0.05$ was reflected on statistically significant.(11).

RESULT

- **Diagnostic Description of *Cryptosporidium spp.***

By using (MZN) stain the *Cryptosporidium* spp. oocyst was identified in bovine faeces when they were examined under microscope 100x as in figure (1) identified as spherical -shaped or oval objects with dark pink or red color on blue ground .

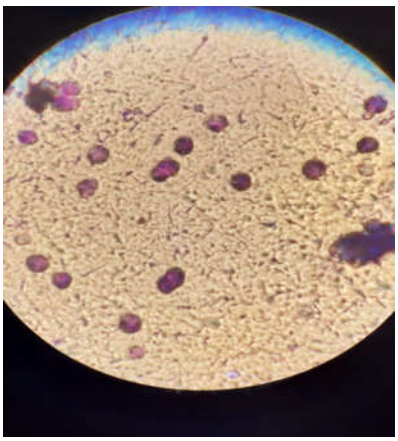

Figure (1).show *Cryptosporidium spp.* oocyst stained with Ziehl-Neelsen stain 100x

- **Results of microscopic examination:**

In this study, 100 samples of cattle faeces were examined microscopically usingZiehle –Neelsen stain ,where 30 (30%) samples gave positive results.

- **Infection rate of *Cryptosporidium spp.* in bovine depended on the months of study**

Based on the effect of the month on incidence rate, These results display that the highest rate of *Cryptosporidium* infect in bovine (41.66)5/12 was detected in November , but the lowest rate of incidence (18.75%)3/16 was observed in the month of February .

Table (1) Infection rate *Cryptosporidium spp*. in bovine depended on month of research .

Month s	Examination No.	Positive No.	Percentage %
September	**8**	**2**	**25**
October	**25**	**9**	**36**
November	**12**	**5**	**41.66**
December	**15**	**3**	**30**
January	**24**	**8**	**33.33**
February	**16**	**3**	**18.75**
Total	**100**	**30**	**30**
X^2	**3.107(NS)**		
P value	**0.683**		

NS: Non-significant differences at p<0.05.

- **The rate Infection of *Cryptosporidium spp.* in bovine depended on the age:**

In this research , the age groups of cattle were divided into four groups (less than one month) (1-6)months, (6-12)months , and older than one years . where the highest rate 40%(14/35)was found in the age group less than a month, but the lowest rate was observed in the group older than one years

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Table (2) The rate of Infection of *Cryptosporidium spp.* in bovine depended on the animals age.

Age group month	Examination No.	Positive No.	Percentage %
<1	35	**14**	**40**
(1-6)	25	**7**	**28**
(6-12)	22	**6**	**27.27**
>1 years	18	**3**	**16.66**
Total	100	**30**	**30**
X^2	**3.316(NS)**		
P value	**0.345**		

NS:Non-significant differences at p<0.05.

- **Infection rate of *Cryptosporidium spp.* in bovine depended on sex**

In this research , we observed that the maximum rate of infection 30.90%(17/55) was in female, but the minimum rate 28.88(13/45) in male .

Table (3) Infection rate of *Cryptosporidium spp*. in bovine depended on sex.

Sex	Examination No.	Positive No,	Percentage %
Male	45	13	28.88
Female	55	17	30.90
Total	100	30	30
X^2	0.048(NS)		
P value	0.826		

ns: non-significant differences at p<0.05.

- **molecular result**

Depended on Nested –PCR examination of bovine DNA sample , the result showed that between (30) bovine faecal samples positive by microscopic while 18/30(60%)positive sample by N-PCR. As in figure (2)

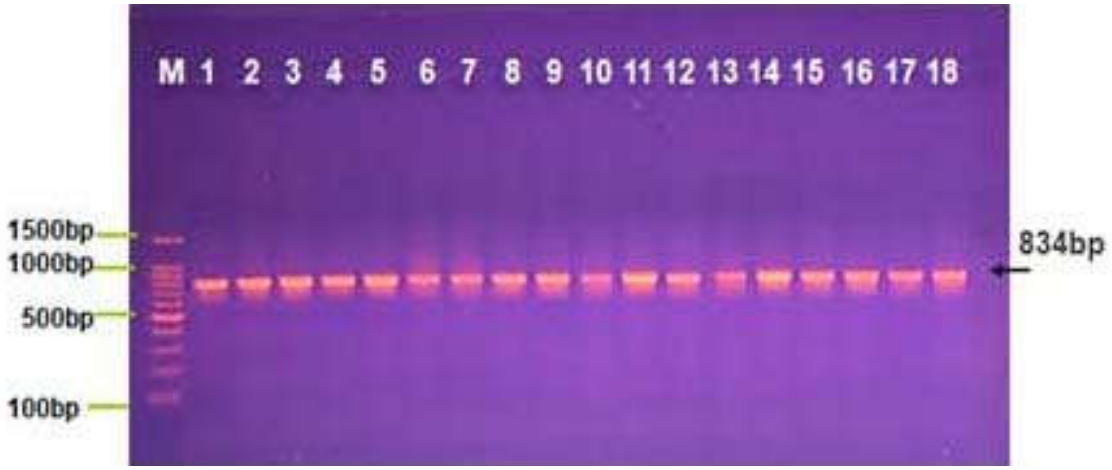

Figure (2): Agarose gel electrophoresis picture that showed the Nested - PCR product analysis of (18S rRNA gene) in *Cryptosporidium spp.* positive samples. Where M: marker (1500-100bp) and lane (1-18) positive *Cryptosporidium* spp. were showed at (834bp) PCR product

The result of sequencing

The nucleotides sequenc results of this research proved and examined by employing the NCBI – Basic Local Alignment Search Tool (BLAST analysis) by employed nucleotide information within nucleotide query program online. Sequences verification and investigation were proved by employing references of 18s rRNA gene of *Cryptosporidium* that involved *Cryptosporidium parvum* , *C. hominis* , *Cryptosporidium bovis* , *C. andersoni* gene sequences data information that reported in Gene Bank and the out groups to discovered the degrees of identity and similitude score of the 18s rRNA gene of *Cryptosporidium spp.* commonly that effected the animals and comparsion with current isolates strains . The results of present local *Cryptosporidium* spp. (50%)6/12 sample from bovine were showed deep related to NCBI – Blast *Cryptosporidium parvum* isolates , The identity score percentage range from (99.62-100%) , (33.33%)4/12 sample from bovine were display deeply related to NCBI –Blast *Cryptosporidium bovis* The identity score percentage ranged from(94.61-100%),(16.66%) 2/12 sample from bovine were deep related to NCBI –Blast *Cryptosporidium andersoni* The identity score percentage ranged from(99.87-100%) as in figure (3). There were no significant differences in bovine species at $p<0.05$, as in table (8)

Table (4) genotyping of *Cryptosporidium species* in bovine in Al-Qadisiyah Province.

Species	No. of strain and %
C. parvum	**6(50)**
C.bovis	**4(33.33)**
C.andersoni	**2(16.66)**
Total	**12(100)**
X^2	**3(NS)**
P value	**0.223**

NS: Non-significant differences at p<0.05

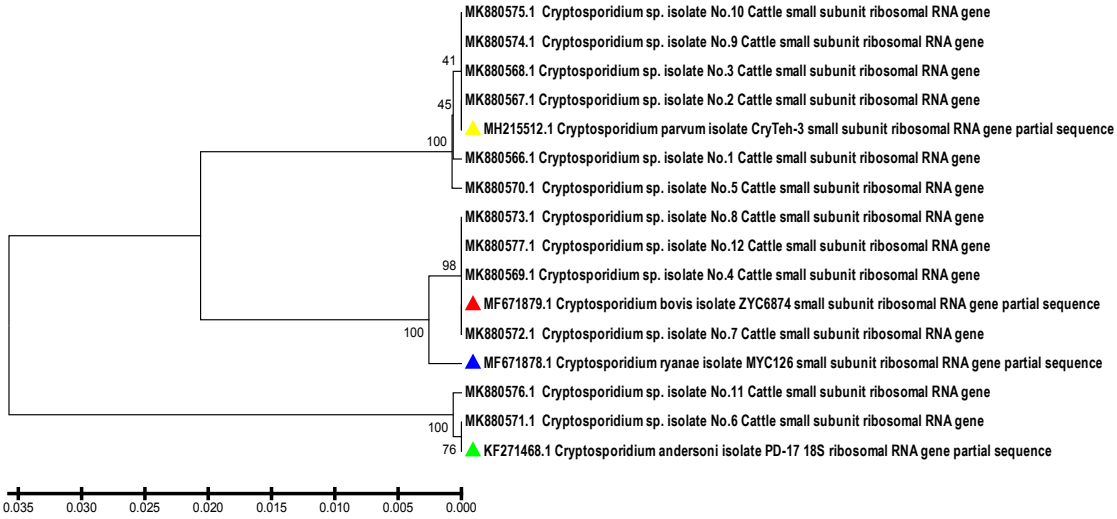

Figure (3): analysis Phylo genetic tree depended on small subunit ribosomal RNA gene partial sequence in local "*Cryptosporidium sp"*. cattle isolates that employ for genetic *Cryptosporidium* species identification . The phylo genetic tree was created utilizing Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local *Cryptosporidium strain* No.1, No.2 , No.3 , No.5 , No.9 and No.10 were showed deep related to NCBI-BLAST *Cryptosporidium parvum* isolate (MH215512.1). The local *Cryptosporidium* strain No.4, No.7 , No.8 and No.12 were display deep related to NCBI-BLAST *Cryptosporidium bovis* isolate (MF671879.1). Wherase the local *Cryptosporidium* isolate No.6 and No.11 were showed deep related to (NCBI-BLAST) *Cryptosporidium andersoni* strain (KF271468.1).at total genetic changes (0.035-0.005%).

DISCUSSION

In the current study ,100 fecal samples from bovine in different age were collected from different areas in Al-Qadisiyah province, There is 30(30%) of the samples were present positively for oocysts of *Cryptosporidium spp.* The results of our study were close to the results of the study conducted by (12) where it was recorded that 34%of calve in Baghdad were infected with *Cryptosporidium spp*., but differ from the results of the study conducted by(13) in Qadisiyah province ,The dissimilarity in was predominant in many countries should be attributed to the criteria used in selecting the study community, and different geographical location and reports may reveal differentiation in the level of manger calf practice used at the farming level and the calves nursing condition (14).The study revealed the relationship between parasitic prevalence rates and the seasons. The results showed that the rates increased in the Autumn and recorded the maximum rate of incidence in November, where as the incidence rate was 41.66%,This agree with the results of studies in Iraq(15) and(16), And in the results of global study (17). This may be due to the climatic conditions of autumn in the survival of oocysts in the environment . The highest rate of incidence in the <1 month age group was 40% (14 positive samples out of a total of 35), and the rate of infection gradually decreased with age more than 1years 16.66 % (3 positive samples out of 18 samples) . The severity of infection in young animals and the inverse relationship between the proportion of infection and age is a fact confirmed by most previous research in this area(18). The current study an agreement with(19) ,They recorded that high infection rate in pre-weaned calves (1-8 week of age)than post -weaned calves 3-12 month of age). This occurs for two main reasons. The first is the inefficiency of the immune system of these newborns and the second is exposed to large numbers of oocysts raised with newborn cattle faces(20). In this study it was recorded that the highest rate of infection of *Cryptosporidium* parasites is in calves less than one year fallowed by yearling and adult this agreement with(19) ,but our result disagree with the study conducted by(21)they recorded there is no difference in happening of Cryptosporidiosis between calve and cows, also our result less than result conducted by (22)in Denmark. According to sex, The results showed a similarity between males and females, with 28.88% and 30.90% respectively . There was no significant difference between this ratios, These results

agreement with those of (23) in calves. This is due to the reality that both sexes are obviously exposed to the same environmental conditions and sources of contamination , as there is no specific factor in the males or females that increases the animal's preparedness or conributes to the resistance . In our current study we have relied on molecular techniques in the detection of *Cryptosporidium* parasites in bovine of different ages, depended on Nested –PCR examination of bovine DNA sample , the result showed that from (30) bovine sample positive microscopically ,there is 18(60%) positive sample by N-PCR. Attempts failed to identify of DNA fragment in the rest of the microscopic examination positive samples because faecal samples may contain a low density of oocysts or inadequate DNA template quality , also, *Cryptosporidium* parasites may be morphologically similar to some organism such as yeast , and sometimes ruminant feces containing PCR inhibitors give false negative PCR result (24). The result of our study agree with (25) recorded that 52.3% of PCR samples from calves have diarrhea in Nigeria were positive for *Cryptosporidiosis* , but less than former study conducted on the calve which display predominant rate 82.1% in Brazil (26). In Europe ,broad range of *Cryptosporidium* pre valence (6.2-52%) has been recorded in young calve (27).

Results of the N-PCR and the analysis of the sequences of the 18S rDNA gene showed *C. parvum* as the widely distribution *Cryptosporidium spp.* in the bovine w ith a rate of 6/12(50%), followed by *C. bovis* 4/12(33.33%), and *C. andersoni* 2/12(16.66%), This result disagree with(28),who re showed *C. andersoni* was recognized in 23 (85.1%), *C. bovis* in 3 (11.1%), and the zoonotic *C. parvum* in one (3.7%), While (29) reported that *C. bovis* having maximum incidence rate (37.8%) than *C. parvum* with infection rate (31.4%). The results of the current research are agree with the results of researches in North America, Australia, New Zealand and Europe, where *C. parvum* is the a frequent cause of the infection in pre-weaned calves but *C. andersoni* is widespread in old calves and bovine aged more than 2 years and *C. bovis* is common in the age of 3 months to 2 years(30,31).In conclusion ,the current study concluded that phylogenetic tree and homology sequences identity give a clear differentiation of *Cryptosporidium species* that can be isolated at high rate from domestic cattle in AL-Diwaniya province ,which may lead to an outbreak of Cryptosporidiosis in livestock .

التشخيص الجزيئي لطفيلي الابواغ الخبيئه في الابقار في محافظة القادسية ،العراق

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الخلاصة

تضمنت الدراسة الحالية فحص ١٠٠ عينة براز جمعت من الابقار بمختلف الاعمار وذلك للكشف عن انواع طفيلي الابواغ الخبيئة في محافظة القادسية للمدة مابين شهر ايلول وشباط وعند الفحص المجهري كانت النتيجة ٣٠ عينه موجبه، ولوحظ ان اعلى معدل للإصابة(٤١.٦٦%) كان في شهر تشرين الثاني بينما كانت اقل نسبه(١٨.٧٥%) في شهر شباط .اما في ما يخص تأثير العمر كانت اعلى نسبه للإ صابه(٤٠%) في سن اقل من شهر ولم يكن هناك تأثير للجنس في اصابة الابقار حيث كانت نسبة الاصابه ٢٨.٨٨% و٣٠.٩٠%في الذكور والاناث على التوالي وايضا خلال الدراسة الحالية استخدم(Nested- PCR) وكانت النتيجة ١٨ عينه موجبه من اصل ٣٠ عينه موجبه بطريقة الفحص المجهري. وكذلك تناولت الدراسة تحليل وقراءة الترتيب النيوكلوتيدي للجين الرايبوسومي للاثني عشر عينة وتم مطابقة النتائج مع عترهذا الطفيلي التي سجلت عالميا في بنك المورثات العالمي وكانت النتيجة (٦)عينات من نوع *C.parvum* اظهرت تشابهها للسلالة المعزولة من استراليا والبرازيل و(٤)عينات من نوع *C.bovis* والتي درست للأول مره في العراق ، اظهرت تشابهها للسلالة المعزولة من الصين و(٢)عينات من نوع *C.andersoni*اظهرت تشابهها للسلالة المعزولة من الصين.استنتجت الدراسة الحالية بأن تحليل الشجرة الوراثية وفحص التطابق بين القواعد النيتروجينية يعطي تفريق واضح لأنواع طفيلي الكريبتوسبوريديوم *Cryptosporidium*والتي يمكن ان تعزل بنسب عالية من الابقار المحلية في محافظة الديوانية والتي من المحتمل يؤدي الى حدوث تفشي لمرض *Cryptosporidiosis* في المواشي

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