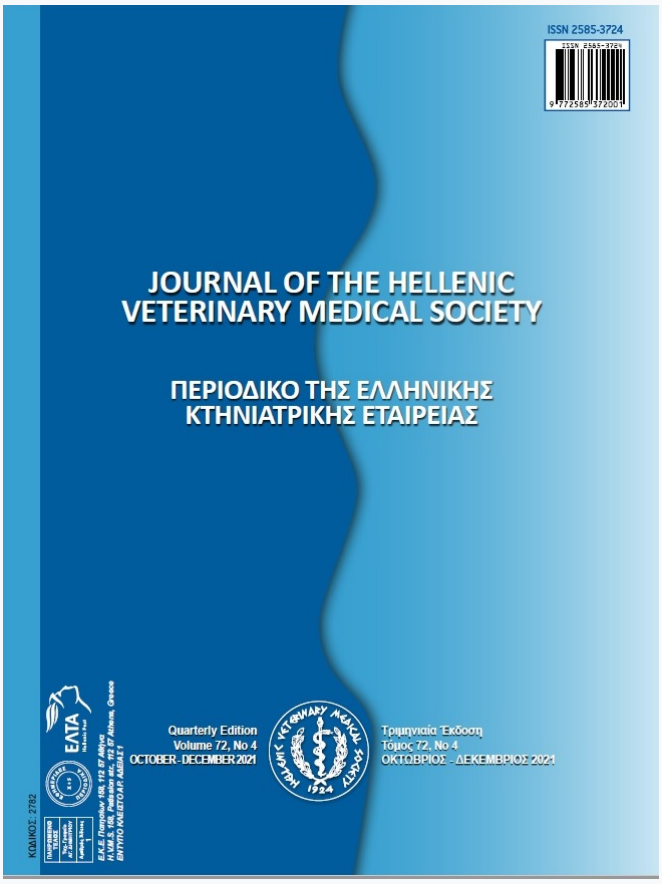
Journal of the Hellenic Veterinary Medical Society

Vol 72, No 4 (2021)



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M MESHKAT, B SHEMSHADI, K AMINI

doi: 10.12681/jhvms.29429

To cite this article:

MESHKAT, M., SHEMSHADI, B., & AMINI, K. (2022). Investigation of bovine interleukin-6 gene polymorphism and its association with Cryptosporidium infection in calves. *Journal of the Hellenic Veterinary Medical Society*, *72*(4), 3371–3376. https://doi.org/10.12681/jhvms.29429

J HELLENIC VET MED SOC 2021, 72(4): 3371-3376
ΠΕΚΕ 2021, 72(4): 3371-3376

Research article
Ερευνητικό άρθρο

Investigation of bovine interleukin-6 gene polymorphism and its association with *Cryptosporidium* infection in calves

M. Meshkat[1], B. Shemshadi[1]*, K. Amini[2]

[1]*Department of Pathobiology, Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran*

[2]*Department of Microbiology, Faculty of Basic Sciences, Saveh Branch, Islamic Azad University, Saveh, Iran*

ABSTRACT: Interleukin-6 (IL-6) is associated with inflammatory diseases, but its connection with *Cryptosporidium* in Holstein calves remains unknown. This study aimed to investigate the effect of single nucleotide polymorphisms (SNPs) of IL-6 on the resistance and susceptibility to *Cryptosporidium* in calves and to prepare a phylogenetictree in order to show the relation between *Cryptosporidium* species. Seventy-two samples were studied from healthy and infected with *Cryptosporidium* calves and genotyped using the tetra amplification refractory mutation system (ARMS). The phylogenetic tree was constructed by the neighbor-joining method using the MEGA 7.0 software. The results showed a frequency of 76.40% for T allele and 23.60% for C allele in the healthy calves, while the results showed a frequency of 73.60% for T allele and 26.40% for C allele in calves infected with *Cryptosporidium.* The results did not reveal a significant difference between healthy and infectious animals according to the allele frequency (P=0.637). The phylogenetic tree demonstrated that *C. parvum* (HQ259589.1) with an 81% bootstrap were clustered with *C. hominis* (KM012041.1). The results also indicated that *C. parvum* (HQ259589.1) and *C. hominis* (KM012041.1) had a common ancestor with *C. cuniculus.* Additionally, *C. andersoni*(HQ259590.1) with an 88% bootstrap of support was placed in the same clade of *C. muris* (L19069.1), and both of them had a common ancestor with *C. serpentis*(KF240618.1). Further studies are required to investigate the relation between SNPs of IL-6 in other regions and the resistance or susceptibility to *Cryptosporidium* in calves.

Keywords: SNP, interleukin-6, phylogenetic tree, calves

Corresponding Author:
Bahar Shemshadi, Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran
E-mail address: bshemshadi@yahoo.com

Date of initial submission: 22-09-2020
Date of acceptance: 11-06-2021

INTRODUCTION

Intestinal parasites are one of the most common challenges for health in countries with poor economy (Saki and Asadpouri, 2017). The species infect various animals and humans (Firoozi et al., 2019). *Cryptosporidium* species are observed in humans and animals and cause high water-borne prevalence all over the world (Alsmark et al., 2018). *Cryptosporidium* species are the most important species in inducing infection in calves and are a potential reservoir for zoonotic infection (Saki and Asadpouri, 2017). Intestinal epithelial cells are the initial cellular sites for *Cryptosporidium* infection in animals (Thomson et al., 2017). The induction of infection by *Cryptosporidium* species increases the expression of TLR2 and TLR4 that activate NFκB and some interleukins (Yang et al., 2015). Interleukins are commonly produced by macrophages, dendritic cells, and lymphocytes during response to infections (Diez-Fraile et al., 2003). Interleukin-6 (IL-6) increases the production of antibodies in response to inflammation, regulates leukocyte populations, and promotes the T cell production (Dienz and Rincon, 2009). The interleukins, particularly IL-6, are commonly considered a marker for early inflammation and prognosis in calves (Rincon, 2012). Lacroix et al. (2001) reported the key role of IL-6 in *C. parvum* infection, showing the increased expression level in adult knockout mice. The changes in some sequences and/or single nucleotide polymorphisms (SNPs) are related to *Cryptosporidium* infection (Widmer et al., 2012). SNPs could change the expression and activity of the genes and the related gene products. SNPs as the genetic markers can be used in the dairy industry, and farmers can raise genetic lines with lower susceptibility to *Cryptosporidium* infection. The association between IL-6 and inflammatory diseases is well known, but its connection with infected calves from *Cryptosporidium* is still unknown. Therefore, this study aimed to investigate the genetic polymorphism of the bovine IL-6 gene. The study also investigated the effect of the identified SNP of IL-6 on the resistance and/or susceptibility to *Cryptosporidium* in infected calves.

Furthermore, the use of phylogenies based on larger datasets of sequences from multiple genes provides greater resolution of phylogenetic relationships between organisms (Naushad et al., 2015a, 2015b). We also prepared genome sequencing for *Cryptosporidium* isolates and utilized the data to construct broad and well-resolved phylogenetic trees based on all genes in the core genome of *Cryptosporidium*. We aimed to prepare a highly reliable base to understand interspecies relatedness among *Cryptosporidium* species.

MATERIALS AND METHODS

Animals

The present study was conducted on 72 calves of the Holstein-Friesian breed, comprising 36 healthy calves and 36 calves infected with *Cryptosporidium* diarrhea. The samples were collected from preweaned calves (≤2 months) in the town of Shahrood (Semnan-Iran). The calves were randomly selected from different dairy farms. It is essential to mention that dairy farms used artificial insemination and did not use conventional methods for mating, resulting in a lower rate for inbreeding.

DNA extraction and Tetra ARMS

Blood samples (5 mL per animal) were collected from the jugular vein into EDTA containing vacutainer tubes. The samples were stored at -91°C until future use. DNA was extracted by the commercial kits of CinnaGen Company. The quality and quantity of the isolated DNA were investigated by agarose gel electrophoresis (2%) and NanoDrop spectrophotometer (GE Healthcare) prior to use in PCR and DNA sequencing. To investigate the DNA quality, purified DNA was run in agarose gel, and the OD 260/280 ratio for all the samples was between 1.8 and 2.

The PCR conditions were as follows: denaturation at 95°C for 5 minutes, denaturation at 95°C for 45 seconds, annealing at 58°C for 30 seconds, extension at 72ºC for 30 seconds and final extension at 72°C for 10 minutes. To amplify the IL-6 gene, specific primers were designed using the Primer3 software (http://frodo.wi.mit.edu/; NW_001494874.2). The specified forward and reverse sequence primers used were compared to other bovine DNA sequences by the basic alignment search tool (BLAST) option from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) by the input of a DNA reference sequence. One SNP was observed at amplicon E +519, and primers were genotyped at individual cow DNA at this locus (c.+472-152C>T). Table 1 shows the used primers.

The samples were genotyped and conducted by Tetra ARMS primers generated using a publicly available software program (Ye et al., 2001).

Table 1: The primers used for the Tetra ARMS PCR

Tetra ARMS Primer Sequence	Temperature	bp size
Forward inner primer C allele: 5 GGGCTCAGAGCAGAGGACCTCCCACC-3	67.80	225
Reverse inner primer T allele: 5-GCCACTGGCCTTGACTGCCCAGCTA-3	68.20	255
Forward outer ARMS primer: 5-AGGCCCCCGAAGAACCCATTAAAATGCCT-3	65.30	428
Reverse outer ARMS primer: 5- TCCAGCAGGTCAGTGTTTGTGGAG-3	65.60	428

Phylogenic tree

The sequence for *Cryptosporidium* species was downloaded from the NCBI'sGene Bank database. The alignments were used for phylogenetic analysis. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site. The analysis involved 17 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 516 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Tamura et al., 2013).

Statistical methods

The results for genotypic and allelic frequencies were analyzed by the FREQ procedure of the SAS software (SAS Institute Inc., v. 9.2). The Hardy-Weinberg equilibrium of the mutation was investigated by the chi-square test.

RESULTS

Figure 1 presents the results for genotype frequency in healthy calves and those with *Cryptosporidium*. The results indicated that TT genotypic frequency was 52.80% (n=19), and the CT genotype frequency was 47.20% (n=17) in healthy calves. The genotypic frequencies for the CT genotype (n=19) and the TT genotype (n=17) in infected calves were 52.80% and 47.20%, respectively. The CC genotype was not observed in the calves. The frequencies were 23.60% and 76.40% of C and T alleles, in healthy calves,respectively, while, the frequencies were 26.40% and 73.60% of C and T alleles, in infected calves, respectively (Figure 2). The results did not show a significant difference between the allelic frequencies in healthy and infected calves (X^2=0.222, P=0.637). This result indicates a lack of association between polymorphism of the IL-6 gene and the occurrence of *Cryptosporidium*.Figure 3 depicts the amplicons +472-152C>T SNP of genotype determination by gel electrophoresis.

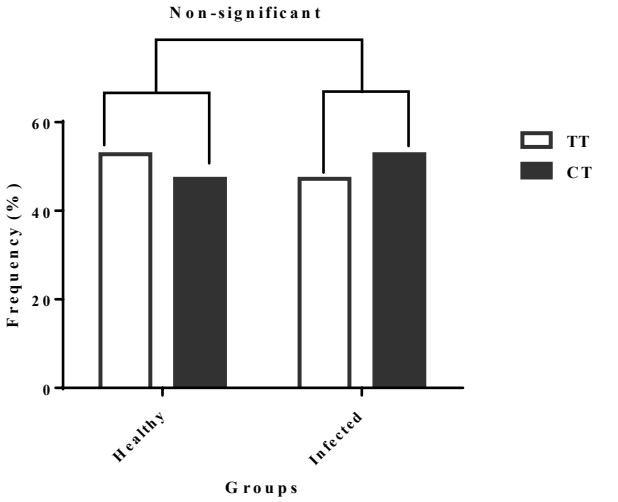

Figure 1: The frequency of genotypes in healthy and infected calves. The results did not show a significant difference between healthy and infected calves

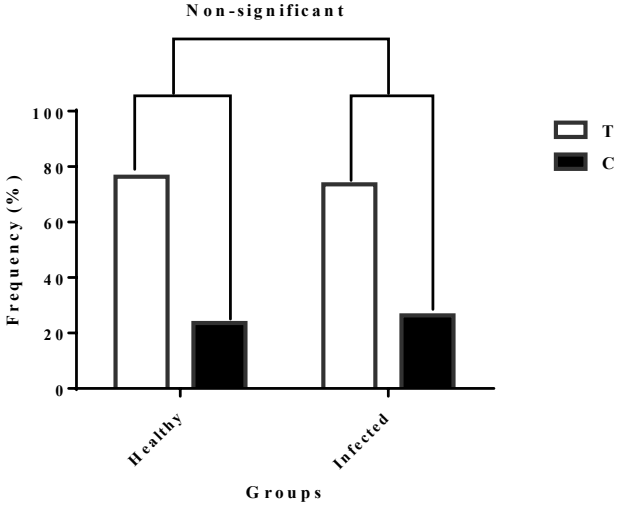

Figure 2: The frequency of alleles in healthy and infected calves. The results did not show a significant difference between healthy and infected calves

Figure 4 displays the phylogenetic relationships of *Cryptosporidium* parasites. The phylogenetic analysis showed that our samples were grouped with identity values ranging from 80.2 to 100%. *C. parvum*, *C. bovis*, *C. andersoni* and *C. muris* were distinguished.

The results showed that *C. parvum* (HQ259589.1) with an 81% bootstrap was clustered with *C. hominis* (KM012041.1). The results also revealed that *C. parvum* (HQ259589.1) and *C. hominis* (KM012041.1) had a common ancestor with *C. cuniculus.* In addition, *C. andersoni*(HQ259590.1) with an 88% bootstrap of support was placed in the same clade of *C. muris* (L19069.1), and both had a common ancestor with *C. serpentis*(KF240618.1).

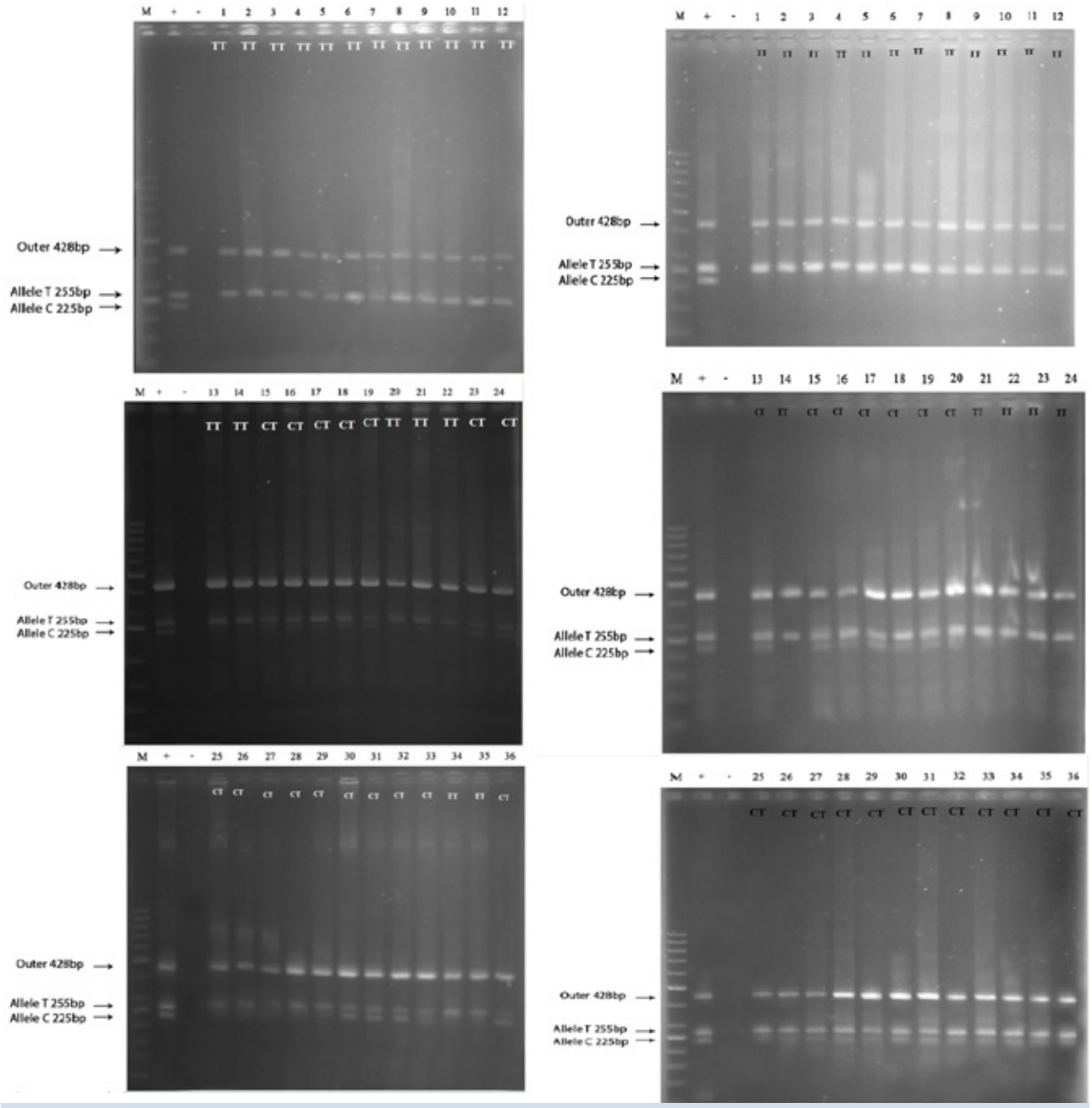

Figure 3: Gel electrophoresis of IL-6 SNP genotyping by specific Tetra-ARMS primers. M shows molecular weight markers, where CC and CT show the calves genotype at the IL-6 c.+472-152 C>T SNP locus. At the left column gels are shown the PCR amplicons of healthy and at the right column gels the PCR amplicons of infected calves

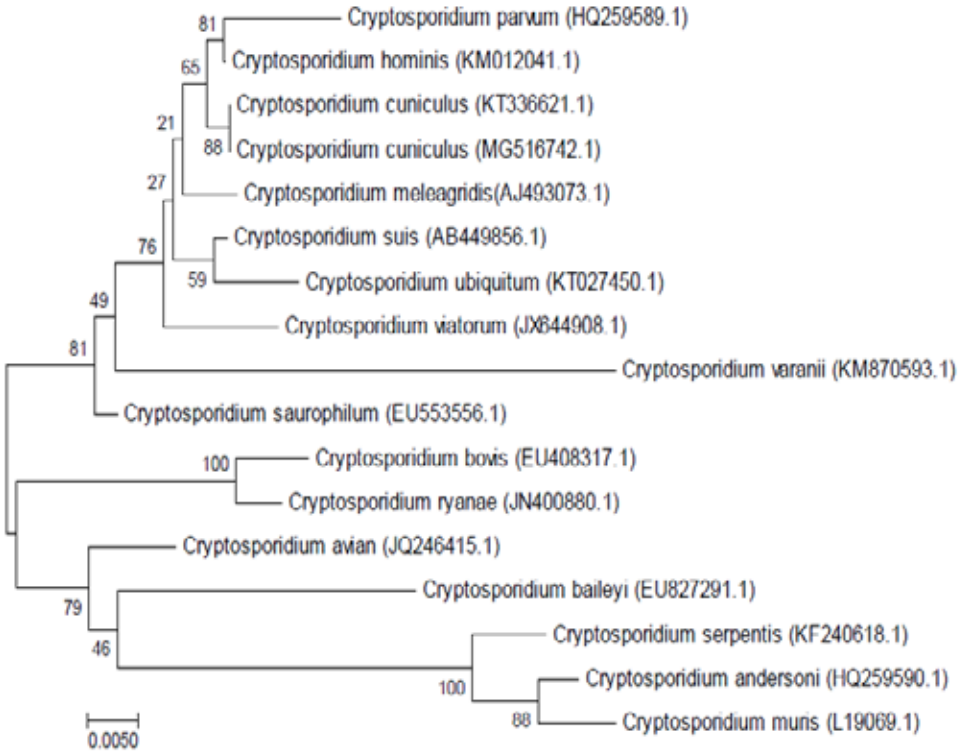

Figure 4: Phylogenetic relationships of Cryptosporidium parasites

DISCUSSION

The current study investigated polymorphism in the bovine interleukin-6 gene and its association with *Cryptosporidium* in calves in Iran. Until now, studies have not investigated SNPs in the bovine interleukin-6 gene and their association with *Cryptosporidium* infection. However, some studies have shown the relation between other interleukins and *Cryptosporidium* (Yang et al., 2015). The relation between IL-6 and *Cryptosporidium* in mice has been reported (Lacroix et al., 2001). Some studies have reported IL-6 as a prognostic marker associated with a specific pathogen in people like children with hemolytic uremic syndrome(Karpman et al., 1995), shigellosis (de Silva et al., 1993) and viral diarrhea (Jiang et al., 2003; Azevedo et al., 2006). The diseases have been related to intronic polymorphisms or nucleotide repeats (Stangl et al., 2000; Sanghera et al., 2004; Kumar and Ghosh, 2008). However, our results did not show any association between the polymorphism of bovine IL-6 and *Cryptosporidium* in calves. It might be attributed to the location of the SNPs. SNPs are potential diagnostic and therapeutic biomarkers for some diseases. The location of SNPs influences resistance and sensitivity to diseases (Deng et al., 2017). Ghavimi et al. (2016) showed that polymorphisms within the promoter or other regulatory regions of cytokine genes only influence the transcriptional activity.Donath &Shoelson(2011) reported that sensitivity and resistance to inflammatory diseases depend on the location of SNPs of IL-6. It could be said that studied region has not any relation with *Cryptosporidium*but the relationship might be in different regions of IL-6. Genetic diversity could be increased by selecting calves with unrelated pedigrees to the maternal and paternal grandsire for *Cryptosporidium*. Our results demonstrated that the T allele frequency was significantly higher than the C allele frequency. It is considered that SNP may have recently emerged in Holstein calves, and the C allele is probably new for the population or that the T allele is more appropriate. The results did not show any frequency for the CC genotype. The absence of CC might indicate that this genotype is genetically inappropriate.The results obtained in the current study do not follow Mendalian genetics. Blake et al. (2009) reported similar results by indicating the lack of a relationship between polymorphisms of IL-6 and mastitis. In sum, our results also suggest the lack of a relationship between polymorphisms of IL-6 and *Cryptosporidium* in Holstein calves.

Several studies have conducted phylogenetic analyses of *Cryptosporidium* parasites. Contrary to our findings, an initial SSUrRNA sequence analysis showed more than 99% identity between *C. parvum* and *C. muris*(Cai et al., 1992), while other studies failed to

separate *C. parvum*, *C. muris*, and *C. baileyi*(Tzipori and Griffiths, 1998). The differences among species suggest the biological differences between *Cryptosporidium* parasites. The two *Cryptosporidium* groups are differentiated from each other, since *C. parvum* and *C. baileyi*initially infect the small intestine and the respiratory tract, while *C. muris*and *C. serpentis*commonly infect the stomach (Cai et al., 1992).

CONCLUSION

In conclusion, the results suggest the lack of an association between polymorphisms of bovine IL-6 and *Cryptosporidium* infection. These findings suggest that alleles in IL-6 in the studied region cannot play a role in protecting against *Cryptosporidium* infection in calves. The phylogenetic analysis showed that the sequence analysis showed more than 99% identity between *C. bovis, C. parvum* and *C. muris*. The major limitation of the current study is that it was limited to calves; therefore, the results cannot be used for other animals. We recommend that more studies be conducted on other animals to investigate the relation between polymorphisms of bovine IL-6 and *Cryptosporidium* infection.

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