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Original Article

Vitamin B_{12} deficiency in newly weaned goat kids associated with clinical infection with *Eimeria arloingi*

Deficiência de vitamina B_{12} em cabritos recém-desmamados associada à infecção clínica por *Eimeria arloingi*

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Abstract

A severe outbreak of diarrhea associated with poor growth was reported in ten newly weaned goat kids that originated from a research farm (Group A). Two of these kids underwent necropsy examination. Five goat kids of the same age maintained in the same pen showed no clinical signs (Group B). The clinical, gross pathological and histopathological features of the clinically sick animals were consistent with severe coccidiosis. Group A animals had significantly lower levels of serum vitamin B_{12} (<200 pg/ml) compared with group B animals (2000 pg/ml). In addition, kids belonging to group A had significantly higher *Eimeria arloingi* oocysts per gram (OPG) of faeces (101,400/g) compared with kids of group B (9,154/g). Microscopy and molecular tools (18S rRNA and COI genes) confirmed that the goat kids were infected with the caprine protozoan parasite *E. arloingi*. This study provides a definitive association between low levels of serum vitamin B_{12} and clinical *E. arloingi* infection, and also provides support to our previous studies that demonstrated how low levels of serum vitamin B_{12} leads to an impairment of neutrophil function and thereby potential lowered immunity to pathogens.

Keywords: Cobalt, vitamin B_{12}, *Eimeria arloingi*, 18S rRNA, COI, goats.

Resumo

Um surto grave de diarreia, associado à baixo crescimento, foi relatado em dez cabritos recém-desmamados, originários de uma fazenda de pesquisa (Grupo A). Dois animais foram submetidos a exame necroscópico. Cinco cabritos da mesma idade e mantidos na mesma instalação não apresentaram sinais clínicos (Grupo B). As características clínicas e as lesões macroscópicas e microscópicas dos animais clinicamente doentes eram consistentes com coccidiose grave. Os animais do grupo A apresentaram níveis significativamente mais baixos de vitamina B12 sérica (<200 pg / ml) em comparação com os animais do grupo B (2000 pg/ml). Além disso, os animais pertencentes ao grupo A apresentaram um número de oocistos de *Eimeria arloingi* por grama (OPG) de fezes (101,400/g) significativamente mais alto do que os animais do grupo B (9,154/g). As análises microscópica e molecular (genes 18S rRNA e COI) confirmaram que os cabritos estavam infectados com o protozoário *E. arloingi*. Este estudo fornece uma associação definitiva entre baixos níveis de vitamina B12 no soro e infecção clínica por *E. arloingi*. Também fornece suporte aos estudos anteriores, que demonstraram como baixos níveis de vitamina B12 no soro comprometem a função dos neutrófilos e, consequentemente, a imunidade a patógenos.

Palavras-chave: Cobalto, vitamina B_{12}, *Eimeria arloingi*, 18S rRNA, COI, cabras.

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Introduction

In previous studies, we demonstrated that newly weaned goat kids having low levels of serum vitamin B_{12} (< 200 pg/ml) exhibited poor growth rates and bone lengths (Kadim et al., 2006), decreased nutrient digestibility (Kadim et al., 2003) and poor meat quality (Kadim et al., 2004). In addition, we also observed hepatic lipidosis in goat kids fed diets with low levels of cobalt resulting in deficiency of serum vitamin B_{12} (Johnson et al., 1999, 2004). Deficiency of vitamin B_{12} in serum significantly impairs antibody response, and also leads to lower oxidative respiratory burst of neutrophils during phagocytosis (Johnson et al., 2010, 2016). Other authors have observed that sheep deficient in vitamin B_{12} are more susceptible to parasites such as *Ostertagia ostertagi* (Paterson & MacPherson, 1990).

Eimeria infections in goats are commonly asymptomatic. However, some species such as *E. ninakohlyakimovae* and *E. arloingi* are considered pathogenic and have been associated with poor body growth (Chartier & Paraud, 2012; Ruiz et al., 2012). A positive correlation has been seen between undetermined *Eimeria* species oocyst counts and low levels of serum vitamin B_{12} levels in six month old goats (Al-Zadjali et al., 2004).

In the present study, we describe an outbreak of diarrhea and poor growth in a group of newly weaned goat kids that were found infected with *E. arloingi*. Based on previous experience with this farm it was also suspected that some of the animals might also have low levels of serum vitamin B_{12} and decreased red blood cell counts despite all of the animals having access to mineral blocks containing cobalt. We measured serum vitamin B_{12} levels in these clinically affected animals, as well as in asymptomatic kids from the same pen, and also evaluated their stool samples not only quantitatively for the presence of *Eimeria* oocysts but also employed morphological and molecular tools to confirm the species of *Eimeria* causing pathogenicity.

Material and Methods

Study animals

Faecal and blood samples were collected at the Agricultural Experiment Station (AES), Sultan Qaboos University from two groups (A and B) of goat kids. These kids were separated from their dams at weaning, yet kept in the same pen at densities of 20-30 heads per pen. Group A was comprised of kids (*n*=10) that presented severe diarrhea and a history of rapid weight loss. Group B was comprised of apparently healthy kids (*n*=5), with no obvious clinical signs of diarrhea or weight loss. Animals at the AES were free of internal parasites, as they were routinely subjected to anthelmintics. Both groups were fed 150 g/day per head of pelleted concentrate (containing 0.2 ppm Co/kg DM), Rhodes grass hay (*Chioris gayanna*) *ad libitum* and provided with access to mineral blocks (containing 40 mg/kg of Co).

Microscopic procedure

Sporulation of *Eimeria* oocysts was done at room temperature (~32 °C) by adding 2% $K_2Cr_2O_7$ to 2g of faeces. Sporulated oocysts of *Eimeria* were isolated 72-97 hr later by flotation technique using saturated salt or 50% sucrose solution, as previously performed by Al-Habsi et al. (2017a). Oocysts per gram (OPG) of faeces were determined by a modified McMaster test, as described by Zajac & Conboy (2012). A 3-axis hydraulic micromanipulator (MO-102, Nirashige, Japan) was used as previously performed by Al-Habsi et al. (2017a) to isolate selected morphotypes and to transfer *Eimeria* oocysts to separate slides for morphometric measurements. Length and width measurements (*n*=50 from total samples) were done and species were confirmed from works of Levine et al. (1962) and Al-Habsi et al. (2017a).

A week after the collection of faecal and blood samples, two kids from group A died and were necropsied. Tissue samples from the liver, mesenteric lymph nodes, and small and large intestine were fixed in 10% buffered formalin. Later, tissues were embedded in paraffin, sectioned at 5µm, stained with hematoxylin and eosin, and examined microscopically. The remaining eight kids were treated for three consecutive days with oral coccidiostat (55 mg/kg body weight sulfadimethoxine, Albon®, Zoetis Inc USA), and a vitamin B_{12} supplement (Lovit Amino Plus Liquid, Kaesler Nutrition®, Germany).

Chemical procedure for vitamin B_{12}

Serum vitamin B_{12} levels were determined for animals from both group A and group B goat kids, as previously performed by Johnson et al. (2004) using a Microparticle Enzyme Immunoassay (MEIA) (IMX B_{12}; Abbott Laboratories) according to the manufacturer's recommendations. The assay was based on the competitive binding of serum vitamin B_{12} and a standard B_{12} where the alkaline phosphatase conjugate attaches to an intrinsic factor bound on a matrix.

Molecular procedure

DNA was extracted from 0.25 g of faecal material using repeated bead beating plus column (RBB+C) method as performed by Yu & Morrison (2004). Briefly, cell lysis was achieved by bead beating in the presence of 4% (w/v) sodium dodecyl sulfate (SDS), 500 mM NaCl, and 50 mM EDTA. The impurities and the SDS were removed by precipitation with ammonium acetate, and then the nucleic acids were recovered by precipitation with isopropanol. Genomic DNA was purified via sequential digestions with RNase and proteinase K, followed by the use of QIAamp columns. Subsequently, DNA was also extracted from single *Eimeria* oocysts using micromanipulator (as delineated under the microscopic procedure) and transferred into a PCR tube for extraction. At annealing temperature of 58 °C, DNA was amplified at 18S rRNA and COI loci, using two-step PCR, with primers as shown in Table 1. PCR products of ~1510 bp and 723 bp were expected from 18S rRNA and COI amplifications respectively. Horizontal electrophoresis was performed using 1.0% agarose gels (Promega, Madison, USA).

Sequence searches of positive isolates for *Eimeria* were conducted using BLAST (NCBI, 2020) and nucleotide sequences were analyzed using Chromas Lite version 2.0 (Technelysium, 2020) and aligned with reference genotypes from GenBank using ClustalW (2020). Maximum likelihood (ML) and neighbor-joining (NJ) analyses were conducted using Tamura-Nei model and pairwise deletion for gaps based on the most appropriate model selection using Model Test in MEGA 7: Molecular Evolutionary Genetics Analysis version 7.0 (Tamura et al., 2013). Bootstrap analyses were conducted using 1000 replicates to assess the reliability of inferred tree topologies.

Results

Eimeria morphometry and density

Eimeria arloingi was identified by microscopy in faecal samples of all animals in both groups. All examined oocysts (*n*=50) were elliptical with bi-layered walls with an average length and width of 26.8 µm (range; 24.4-30.4 µm) and 19.6 µm (range; 17.1-21.6 µm). Sporocysts were ovoid with an average length size of 12.1 µm (10.4-14.1). No polar granules were noted, while sporocyst residuum was observed in most of the examined oocysts (Figure 1A, B).

Table 1. Target genes and primers used for amplification and sequencing of *Eimeria* isolates in Omani goats.

Target gene/ Primer	Nucleotide sequence (5'-3')	PCR	Reference
18S rRNA			
EiGTF1	TTCACTGGTCCCTCCGATC	Primary	Yang et al. (2016)
EIGTR1	AACCATGGTAATTCTATGG		
EiGTF2	TTACGCCTACTAGGCATTCC	Secondary	Yang et al. (2015)
EiGTR2	TGACCTATCAGCTTTCGACG		
cytochrome c oxidase subunit I (COI)			
COIF1	GGTTCAGGTGTTGGTTGGAC	Primary	Ogedengbe et al. (2011)
COXR1	CCAAGAGATAATACRAARTGGAA		Dolnik et al. (2009)
COIF2	TAAGTACATCCCTAATGTC	Secondary	Dolnik et al. (2009)
COXR2	ATAGTATGTATCATGTARWGCAA		

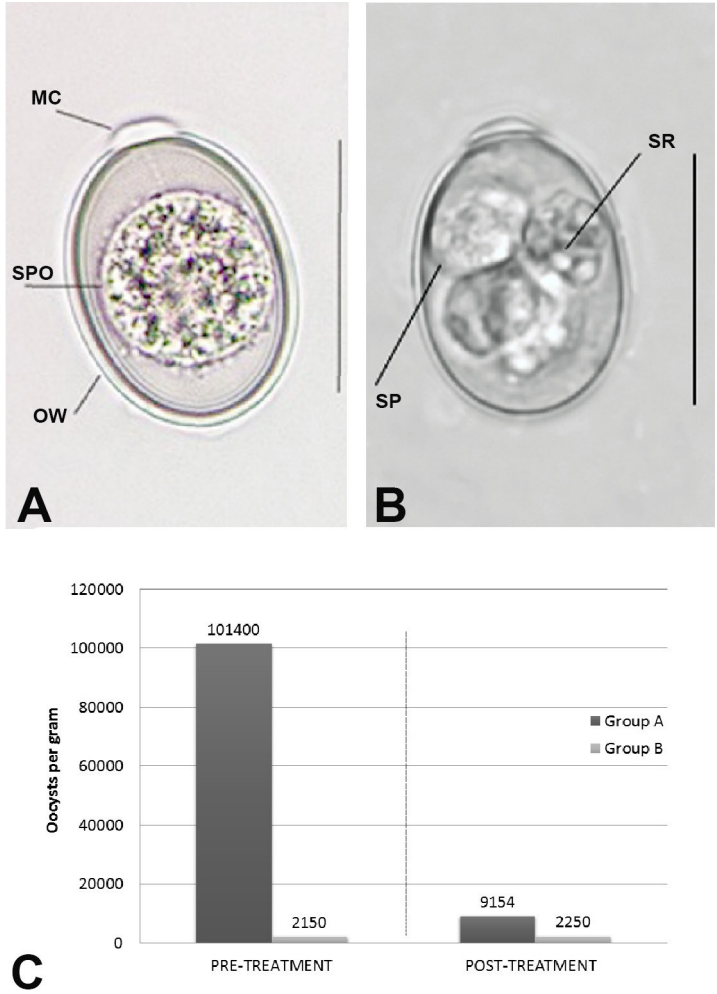

Figure 1. Nomarski interference-contrast photomicrographs of the *Eimeria* oocysts from weaned Omani goats; (A) unsporulated *E. arloingi,* and (B) sporulated *E. arloingi* showing oocyst wall (OW), micropolar cap (MC), sporont (SPO), sporozoites (SP), and sporocyst residuum (SR), Scale bar = 20 µm; (C) Density of *Eimeria* spp. using modified McMaster in Omani goat kids before and after treatment with a coccidiostat.

An average of 101,400 (range; 86,190-116,610) OPG of faeces was found in kids from group A. In group B kids, this value was considerably lower, i.e., 2,150 (range; 1,825-2,472) OPG. After treatment of group A kids with a coccidiostat, the OPG got reduced to 9,154 (range; 7,781-10,527) (Figure 1C).

Histopathology and vitamin B_{12} levels

Whitish yellowish foci were noted through the jejunal serosa with severe enlargement of the mesenteric lymph nodes (Figure 2A). Fatty livers with rounded borders and yellowish brown colour were observed (Figure 2B). Examined sections from hepatic tissues revealed periportal diffuse macrovesicular steatosis (fatty liver) (Figure 2C). Examined jejunal tissue sections showed replacement of the intestinal villi and the crypts with lymphocytes and different sexual developmental stages of *Eimeria* spp. Mature microgamonts containing and shedding microgametes, mature macrogamont and, young oocysts were seen. The majority of the crypts of Lieberkühn revealed various asexual developmental stages of *Eimeria* spp. Moreover, myriads of trophozoites and multiple first generation schizonts were detected. The jejunum exhibited complete loss of the intestinal villi and the presence of macroschizonts in the intestinal lumen. Severe necrotic enteritis with lymphocytic infiltrations and multiple dilated blood vessels were observed in the terminal part of the jejunum (Figure 2D-I).

The average serum vitamin B_{12} measured in group A kids (with diarrhea and reduced weight gains) was 188.4 pg/ml (range; 109-294 pg/ml). This result was significantly lower (P<0.05) than the mean values of the group B goat kids which was >2000 pg/ml.

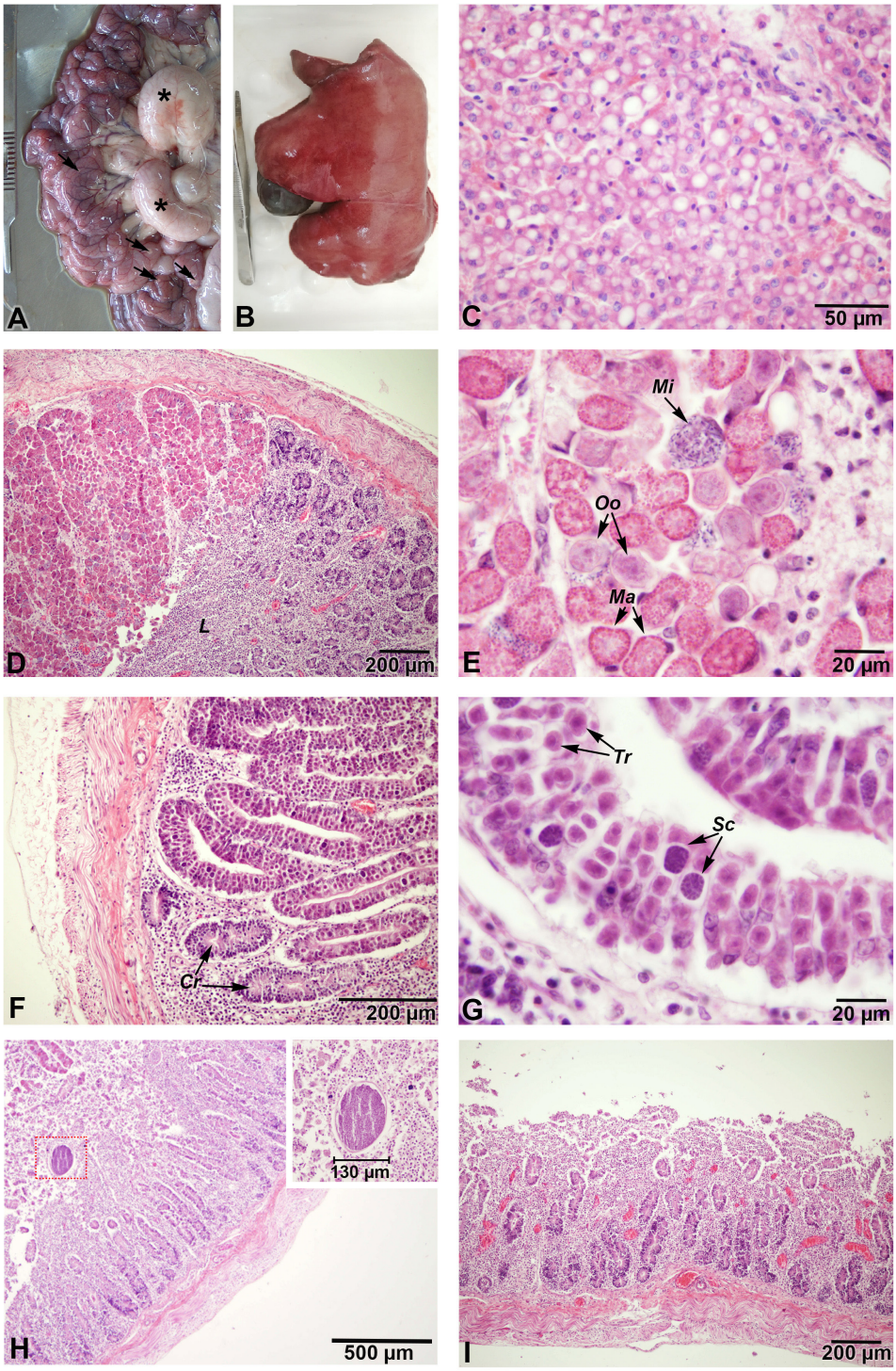

Figure 2. Pathological findings in naturally infected goat kid due to *E. arloingi* (Stain: H&E): (A) severely enlarged mesenteric lymph nodes (*) with presence of whitish yellowish patches (arrows) in the jejunum that can be seen through the serosa; (B) fatty liver with rounded borders and yellowish brown colour; (C) micrograph of (B) revealing periportal diffuse macrovesicular steatosis; (D) jejunum with complete replacement of the intestinal villi and the majority of the crypts with lymphocytes (L) and different sexual developmental stages of *E. arloingi*; (E) higher magnification of (D) showing mature microgamonts (Mi) containing and shedding microgametes, mature macrogamont (Ma) and young oocysts (Oo); (F) few normal crypts of Lieberkühn (Cr) with the majority of the crypts invaded with the asexual developmental stages of *E. arloingi*; (G) higher magnification of (F) showing myriads of trophozoites (Tr) and multiple first generation schizonts (Sc); (H) jejunum with complete loss of the intestinal villi and the presence of a macroschizonts in the intestinal lumen (inset: higher magnification of the macroschizonts); (I) terminal part of the jejunum showing severe necrotic enteritis with complete loss of the intestinal villi, lymphocytic infiltrations and multiple dilated blood vessels.

Amplification of *Eimeria* at the 18S rRNA and COI genes

PCR amplification of the *Eimeria* microscopically positive samples resulted in successfully generating 1280 bp identical products which were confirmed by sequencing in both directions. Distance, Parsimony and ML analyses were used to construct the phylogeny. Two Iranian and Australian goat-derived *E. arloingi* 18S rRNA sequences were retrieved from GenBank (KC507792) (Khodakaram-Tafti et al., 2013) and (KX845686) (Al-Habsi et al., 2017a) with a length of 637 and 1270 bp respectively. *Eimeria arloingi* from Omani goats (MK830054) grouped in one clade with *E. arloingi* (KX845686) which was derived from captured rangeland goats (*Capra hircus*) in Western Australia (Al-Habsi et al., 2017a) with 99.4% homology (Figure 3A).

At the cytochrome c oxidase I (COI) locus, 677 bp PCR products were successfully amplified from all the microscopy positive isolates. Single ovine *E. ahsata* (KT184373) and caprine *E. arloingi* (KX857470) (Al-Habsi et al., 2017a) cytochrome c oxidase I (COI) sequences were available in the GenBank database. Phylogenetic analysis of the 677 bp COI sequence grouped *E. arloingi* from Omani goats (MK861046) of the present study in one clade with *E. arloingi* isolate from rangeland goats of western Australia (Al-Habsi et al., 2017a), with 99.8% similarity (Figure 3B).

Discussion

The present study revealed that Omani goat kids with low levels of serum vitamin B_{12} (<200 pg/ml) had severe clinical manifestations as a result of infection with *E. arloingi*. Ten of the animals had severe diarrhea and showed poor growth compared to the five healthy ones. Two of the ten goats that succumbed to the infection had histopathological inflammatory alterations in the jejunum consistent with *Eimeria* infection with different developmental stages of the parasite seen. In addition these animals had enlarged pale livers with macroscopic and microscopic evidence of hepatic lipidosis; pathological lesions that we have also previously experimentally reproduced by inducing low levels of serum vitamin B_{12} in newly weaned goats by feeding them a diet with low levels of cobalt (<0.1 ppm Co per kg DM) (Johnson et al., 2004). In contrast to these clinically sick group A goat kids, the group B goat kids had normal levels of vitamin B_{12} and showed no clinical signs, despite being affected with *E. arloingi*.

Previous studies have suggested that there might be a causal relationship between low levels of serum vitamin B_{12} and susceptibility to infection. Al-Zadjali et al. (2004) reported that goats with low levels of serum vitamin B_{12} at the field level under a variety of management conditions had significantly higher coccidia counts. Paterson & MacPherson (1990) reported that cobalt-deficient calves had significantly higher *Ostertagia* egg counts than cobalt-supplemented calves. Our study provides evidence of clinical severity due to *E. arloingi* infection associated with vitamin B_{12} deficiency.

Morphological characterization of sporulated oocysts and host specificity were conventionally used as the basis for *Eimeria* species identification. Based on microscopic examination, a total of 17 *Eimeria* species have been described worldwide in goats (Al-Habsi, 2017b). However, microscopy has limitations of time consumption and possible human error (Kawahara et al., 2010; Carvalho et al., 2011; Khodakaram-Tafti et al., 2013; Nahavandi et al., 2016).

More recently, to address these limitations, molecular tools have been employed to accurately identify *Eimeria* spp. from goats (Al-Habsi et al., 2017a; Mohamaden et al., 2018). The 18S rRNA locus has been used extensively as a molecular marker in phylogenetic analysis. However, as it is a conservative gene, the 18S rRNA locus may not be the ultimate compatible gene for differentiating closely related *Eimeria* species and therefore it is best utilized simultaneously with other gene loci (e.g., COI locus) which are acceptable for discriminating morphologically similar *Eimeria* species in small ruminants (Ogedengbe et al., 2011; Al-Habsi et al., 2017a).

In the present study, the morphological characteristics of oocysts and sporocysts of the identified *E. arloingi* were consistent with those of previous reports (Levine et al., 1962; Al-Habsi et al., 2017a) and these findings were confirmed by molecular tools and sequencing analysis retrieved from GenBank database. Employing both of these tools increases the accuracy of differentiation of those species sharing nearly identical morphological characteristics (Kawahara et al., 2010; Nahavandi et al., 2016). The phylogenetic analysis at the 18S rRNA and COI loci revealed that *E. arloingi* from the newly weaned clinically positive and control goats were almost identical to pathogenic *E. arloingi* (KX845686 and KX857470) from captured rangeland goats in Australia (Al-Habsi et al., 2017a).

Our lab in previous studies provided evidence that neutrophils from goats with low levels of serum vitamin B_{12} exhibit impaired oxygen-dependent respiratory bursts during the phagocytic process (Johnson et al., 2010, 2016) when compared to neutrophils from goats with normal levels of serum vitamin $B_{12.}$ Neutrophils are the first

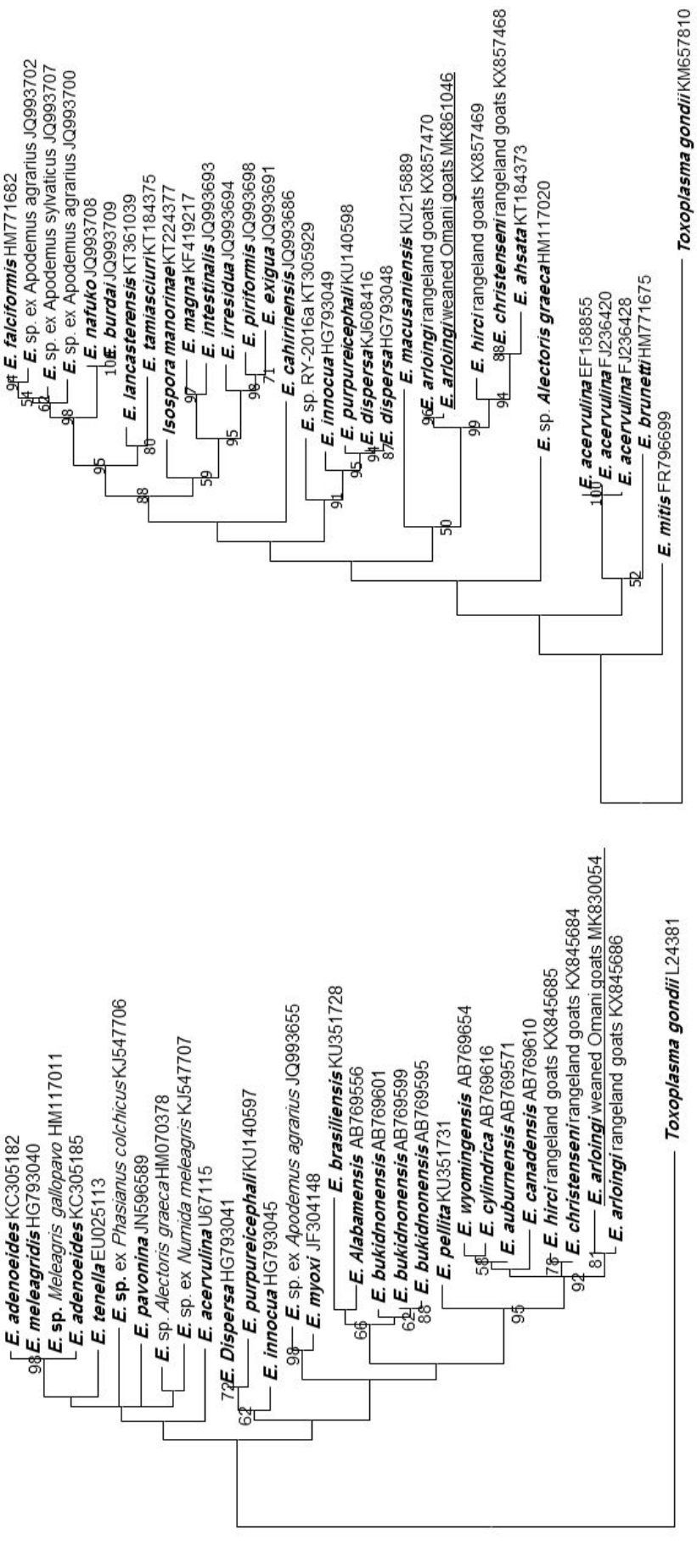

Figure 3. Evolutionary relationships of *Eimeria* spp. inferred by distance analysis of 18S rRNA gene (A) and cytochrome c oxidase subunit I (COI) gene (B). Accession numbers of samples follow the species name. Bootstrap values (> 50%) based on 1000 replicates are indicated at the left of each supported node. The scale bar is the proportion of base substitutions per site.

line of immunological defense utilized by the host to fight infections and have potentially a broad repertoire of mechanisms that they can employ. They produce reactive oxygen (superoxide anion and hydrogen peroxide) during the respiratory burst and release antimicrobial peptides. Recently, a study demonstrated the role of NETosis, a mechanism employed by neutrophils to immobilize pathogens by releasing neutrophil extracellular traps (NETs) which are protein-labeled DNA matrices capable of extracellular trapping and killing of invasive sporozoites and oocysts of *E. arloingi* (Silva et al., 2014). Bovine neutrophils have also been recognized to cast NETs against *Neospora caninum* (Villagra-Blanco et al., 2017).

Conclusions

This study provides evidence that low levels of serum vitamin B_{12} in goat kids increases susceptibility to clinical infection with pathogenic *E. arloingi*. Goat kids with normal levels of vitamin B_{12} shed *E. arloingi* oocysts, however, they did not present clinical signs of weakness and diarrhea. This study also provides further support to our *in vitro* studies which demonstrated that low levels of serum vitamin B_{12} leads to an impairment of neutrophil function and thereby has the potential to lower immunity to pathogens. As there are only a few goat-derived sequences available in the GenBank for both 18S rRNA and COI loci, molecularly characterized *E. arloingi* from the present study will serve as an invaluable addition to the database to be used in future caprine studies.

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