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ORIGINAL ARTICLE

# The emergence of *Caryospora neofalconis* in falcons in Central Saudi Arabia

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## ABSTRACT

**Objective:** This study aimed to examine the occurrence of *Caryospora (C.) neofalconis* in falcons from the central region of the Kingdom of Saudi Arabia (KSA).
**Materials and methods:** Fecal samples (*n* = 149) from 149 healthy falcons including 56 saker falcons (*Falco cherrug*), 13 lanner falcons (*F. biarmicus*), 18 peregrine falcons (*F. peregrinus*), 40 Barbary falcons (*F. pelegrinoides*), and 22 gyrfalcons (*F. rusticolus*) were collected between October 2018 and May 2019. The fecal samples were examined for the presence of *C. neofalconis* by microscopic examination followed by confirmation by polymerase chain reaction targeting *18S rRNA* genes and their phylogenetic analyses.
**Results:** The overall prevalence of *C. neofalconis* in the falcons was recorded as 10.7% (16/149) by microscopic examination. The highest prevalence was found in *F. peregrinus* (6/18, 33.3%), followed by *F. rusticolus* (3/22, 13.6%), *F. cherrug* (5/56, 8.9 %) and *F. pelegrinoides* (2/40, 5.0%). There was no *C. neofalconis* infection observed in *F. biamicus*. The *18S rRNA* gene could be amplified in eight samples. The phylogenetic analysis of two *C. neofalconis* isolates exhibited a close relationship with the Mexican isolate (KT03081) with a 99.7% identity.
**Conclusions:** To our knowledge, based on the microscopic and molecular analysis, this is the first report of *C. neofalconis* in *F. cherrug*, *F. rusticolus*, *F. pelegrinoides*, and *F. peregrinus* from the central region of the KSA and it emphasize the value of adopting preventive measures to limit the spread of *C. neofalconis*.

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*Caryospora neofalconis*; falcon; Saudi Arabia; PCR



## Introduction

Enteroparasites are widespread in captive and wild raptors and can be critical pathogens if the birds are exposed to disease or stress. Coccidiosis is recognized by clinical signs such as vomiting and anorexia [1]. Genus *Caryospora (C.)* is the third most common of the family *Eimeriidae* and mainly affects reptiles and predatory birds [2,3].

There are 150 different *Caryospora* species worldwide, of which at least 25 have been recognized from raptorial birds [4,5]. Generally, seven species of *Caryospora* were identified from Europe, one from Russia, two from the Kingdom of Saudi Arabia (KSA), one from Western Australia, five from the USA, and six from the United Arab Emirates [4–8].

*Caryospora neofalconis* is one of the pathogens of captive raptors in the UK and it is the most common cause of death. Affected raptors have shown clinical signs of depression, anorexia, bloody feces, diarrhea, weight loss, and sometimes acute death [9,10]. Experimental infection in falcons with 1–3 × $10^3$ oocysts led to diarrhea and anorexia [11].

Molecular diagnosis and characterization of *Caryospora* targeting the *18S rRNA* gene have been increasingly and successfully identified in *C. neofalconis* from 60 *Falco* (*F.*) *peregrinus* in Central Mexico [12], *C. daceloe* from 30 laughing Kookaburras at a Wildlife Rehabilitation Centre in Kanyana, Western Australia [5], and *C. megafalconis* from 679 bustards in Dubai [13].

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More recently, in the KSA, infection with *C. biarmicusis* has been reported in the lanner falcon (*F. biarmicus*) [14], and *C. cherrughi* in the saker falcon (*F. cherrug*) [15] gathered from the markets of the city of Riyadh. To the best of our knowledge, there is no report of *C. neofalconis* from falcons in the KSA. Therefore, *C. neofalconis* in falcons from KSA was surveyed in this study and was identified morphologically and genetically.

## Materials and Methods

### *Sampling and microscopic examination*

Fresh fecal samples were collected from the cages of 149 healthy falcons between October 10, 2018, and May 5, 2019, from captive falcons in Riyadh and Qassim, KSA (Fig. 1). Meanwhile, the samples were collected from *F. cherrug* (*n* = 56), *F. biarmicus* (*n* = 13), peregrine falcons (*F. peregrinus*) (*n* = 18), Barbary falcon (*F. pelegrinoides*) (*n* = 40), and gyrfalcon (*F. rusticolus*) (*n* = 22). Fecal samples were gathered in individual plastic tubes before being recognized and stored in a cooler. They were directly transferred to the central Zoology laboratory department, College of Science, University of King Saud. Subsequently, the feces were mixed with 2.5% potassium dichromate solution ($K_2Cr_2O_7$). Feces with coccidian oocysts (unsporulated) were laid in thin layers of potassium dichromate, incubated at room temperature, and observed every day till 75% of the oocysts were sporulated. The oocysts were collected using the method of Sheather's flotation with a solution of sucrose and examined with a microscope [16]. The image was then captured using a digital camera coupled with a light microscope (Olympus, CX41, Tokyo, Japan). Oocyst measurements were undertaken to identify parasites species. Positive *Caryospora* samples were stored for further molecular analysis.

### *DNA extraction and polymerase chain reaction (PCR) analysis*

For the total DNA extraction, oocyst samples were washed five times in distilled water by centrifugation. The pellets of the oocysts were resuspended and sonicated. The DNA from the oocysts was extracted and purified using QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany, cat. no. 51604), according to the instructions of the manufacturer. Briefly, InhibitEX Buffer (1 ml) was mixed with each specimen, vortexing was done, and centrifuged for 1 min. Subsequently, 200 µl of supernatant was gently mixed with proteinase K (25 µl) and mixed gently. An amount of 600 µl Buffer AL was added and then incubated at 70°C for 10 min. 200 µl ethanol (96%–100%) was

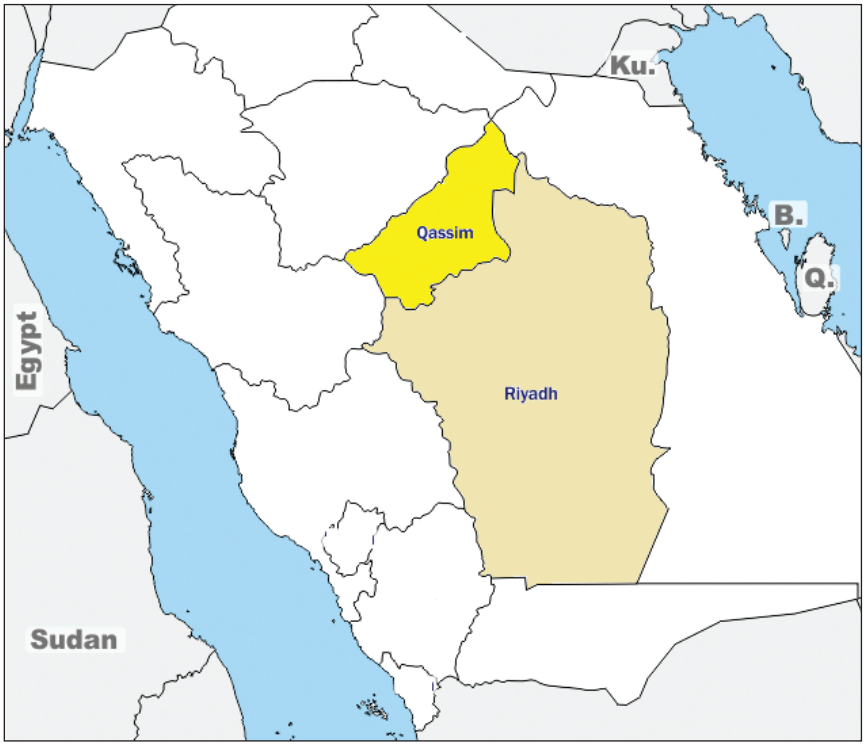


**Figure 1.** The location of the central region of the KSA that was sampled.

added and vortexed. The DNA (200 µl) was obtained after loading of the spin-column supplied in the kit. Aliquots of DNA were kept at −20°C until used for PCR. The PCR mixture (25 µl) contained 15 µl GoTaq® Green Master Mix 2X, 1 µl (20 pmol) of each primer, 100 ng extracted DNA and nuclease-free water to 25 µl final volume. The nested PCR was done by using the following primers: the first amplification contained forward primer, EIF1 5′-GCT TGT CTC AAA GAT TAA GCC-3′ and reverse primer, EIR3 5′-ATG CAT ACT CAA AAG ATT ACC-3′. Then, the first PCR product was used as a template for the second PCR amplification: forward primer EIF3 5′-CTA TGG CTA ATA CAT GCG CAA TC-3′ and the reverse primer EIR3 to get a 1,399–1,407 bp fragment [5]. The amplification of PCR was performed as follows: initial cycle at 94°C for 5 min and then 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec, and finally a cycle at 72°C for 10 min. The PCR product was monitored by electrophoresis by 1.5% agarose gel containing ethidium bromide (0.5 µg/ml), and the amplified DNA image was taken by a gel documentation system (Upland, CA).

### *DNA sequencing and phylogenetic analysis*

The PCR targeting the *18S rRNA* gene was purified and sequenced using an automated DNA sequencer (ABI 3730XL, Solgent Co. Ltd., *Seoul*, South Korea). The nucleotides were read by using the DNA BaserV3 software, and the blastN search was done using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST) to identify the *18S rRNA* gene entries in the GenBank database, with the highest identities of nucleotide sequences. Evolutionary analysis by the method of Maximum Likelihood was inferred by using the method of Maximum Likelihood and the Tamura-Nei model. The study involved 24 nucleotide sequences of the *18S rRNA* gene. There were 2,846 positions in the final dataset. Evolutionary analysis was conducted in MEGA X [17]. The obtained sequences ($n$ = 2) identified in this study were deposited in the GenBank with the accession numbers MN629229 and MN629230.

## Results

### *Detection of C. neofalconis in fecal samples by microscopy*

Testing of fecal specimens collected from 149 falcons from the KSA using microscopy revealed that 16 falcons were infected with *Caryospora* sp., giving a prevalence rate of 10.7%. The prevalence of *C. neofalconis* was higher in *F. peregrinus* (6/18, 33.3%), followed by *F. rusticolus* (3/22, 13.6%), *F. cherrug* (5/56, 8.9%) and *F. pelegrinoides* (2/40, 5%). There was no infection with *C. neofalconis* but was detected in *F. biarmicus* (Fig. 2).

Oocyst of *C. neofalconis* was subspherical in shape, having a smooth bilayered wall. The length and width measurements of oocysts were studied; their length ranged from 25.8 to 28.4 µm with an average of 26.9 µm, and the width ranged between 21.0 and 24.7 µm with an average of 23.2 µm. The mean shape index was 1.1. There was an absence of oocyst residuum, micropyle, and polar granules of this parasite (Fig. 3, Image A). Each sporulated oocyst contained only one sporocyst, and the sporocyst was subspherical to ovoid (Fig. 3, Images B-D), and was surrounded by a smooth, uni-layered wall. The length ranged between 15.8 and 19.5 µm, with an average of 18.3 µm and its width

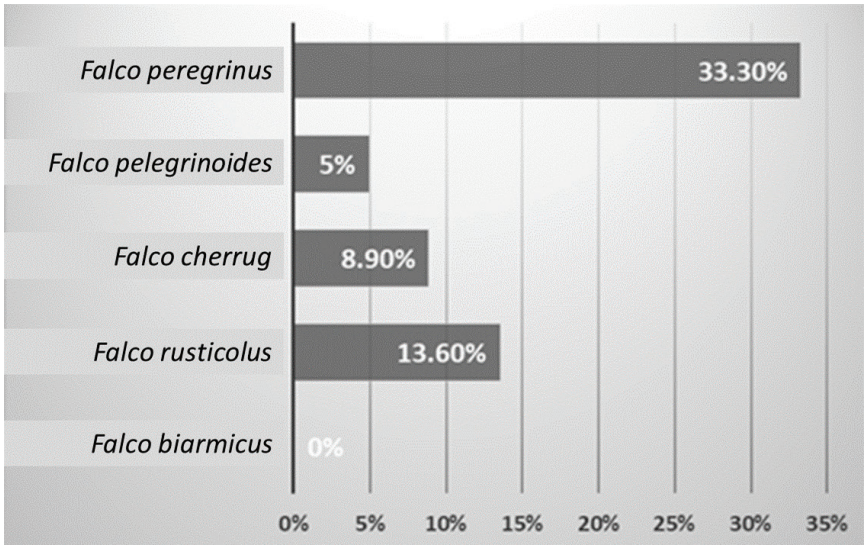


**Figure 2.** Prevalence of *C. neofalconis* in central Saudi Arabia.

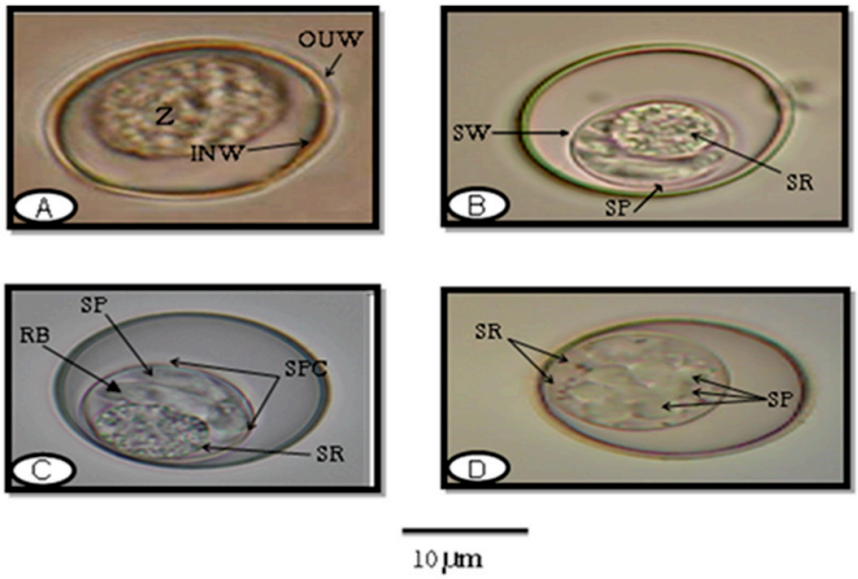


**Figure 3.** [Image A] Unsporulated oocyst of *C. neofalconis* collected from *F. peregrinus* (100x), INW = Inner wall; OUW = Outer wall; Z = Zygote. [Image B] Sporulated oocyst of *C. neofalconis* collected from *Falcon pelegrinoides*, SR = Sporocyst residuum; SP = Sporozoite; SW = Sporocyst wall. [Image C] Sporulated oocyst of *C. neofalconis* collected from *F. cherrug*, SR = Sporocyst residuum; SP = Sporozoite; RB = Refractile body; SPC = Sporocysts. [Image D] Sporulated oocyst of *C. neofalconis* collected from *Falcon rusticolus*, SR = Sporocyst residuum; SP = Sporozoite.

ranged from 13.8 to 16.0 µm with an average of 14.5 µm. There was an absence of the body of Stieda and sub-Stieda of the sporulated oocyst.

Furthermore, the sporocyst residuum existed and was either combined as an average ball measuring 10 µm (Images B and C) or diffuse (Image C). When the growth of the sporocyst was shown, the sporozoite was found to be elongated and curved little with an average length of 11.2 µm and an average width of 2.7 µm and contained a refractile body (Image C). Tracing the time of growth for this parasite turned out to be from 60 to 72 h.

### *PCR analysis and partial 18S rRNA gene sequencing*

PCR was performed, targeting the *18S rRNA* gene. The *18S rRNA* was successfully amplified in eight samples. Two samples, one from Riyadh and the other from Qassim, with confirmation of *C. neofalconis* occurrence using both microscopy and PCR, were used for sequencing analyses. The partial nucleotide sequence of *C. neofalconis* (GenBank Accession MN629229, MN629230) revealed two identical sequences of 99.15%. Whereas, they showed a 99.7% identity to a *C. neofalconis* sequence from Mexico (KT037081), over the 1,413 nucleotides of the *18S rRNA* gene characterized. Among the nucleotide sequences of *C. neofalconis* isolates in this study, heterogeneity appeared over 9 bp substitutions. In contrast, when compared with the reference sequence (KT037081), heterogeneity appeared over 1–10 bp substitutions (Fig. 4), as well as over 4 amino acids. Also, BLAST search of the *C. neofalconis* sequences revealed over 91%, 97.8%, 98% identity to *Besnoitia besnoiti*, *Cystoisospora* sp., *Hammondia* sp. sequences, respectively, as well as a 97.8% to *Isospora belli* and ≥ 98.4% to *Toxoplasma gondii* and *Neospora caninum* sequences published in GenBank.

### *Phylogenetic analysis*

The phylogenetic tree dependent on *18S rRNA* gene sequence within two sequences of the present study and reference Eimeriorina isolates (1 *C. neofalconis*, 7 *Besnoitia besnoiti*, 3 *Cystoisospora* sp., 5 *Hammondia* sp., 1 *Isospora belli*, 2 *Toxoplasma gondii*, and 3 *Neospora caninum*) available in GenBank revealed that there is a genetic identity and all 24 sequences were grouped into two clusters (cluster 1: *Besnoitia besnoiti*, *Cystoisospora* sp., *Isospora belli*, and *C. neofalconis* and cluster 2: *Hammondia* sp., *Toxoplasma gondii*, and *Neospora caninum*). The tree also showed that *C. neofalconis* isolates (MN629229, MN629230, this study) are closely related to *C. neofalconis* (KT037081, Mexico) and are grouped in the same subclade

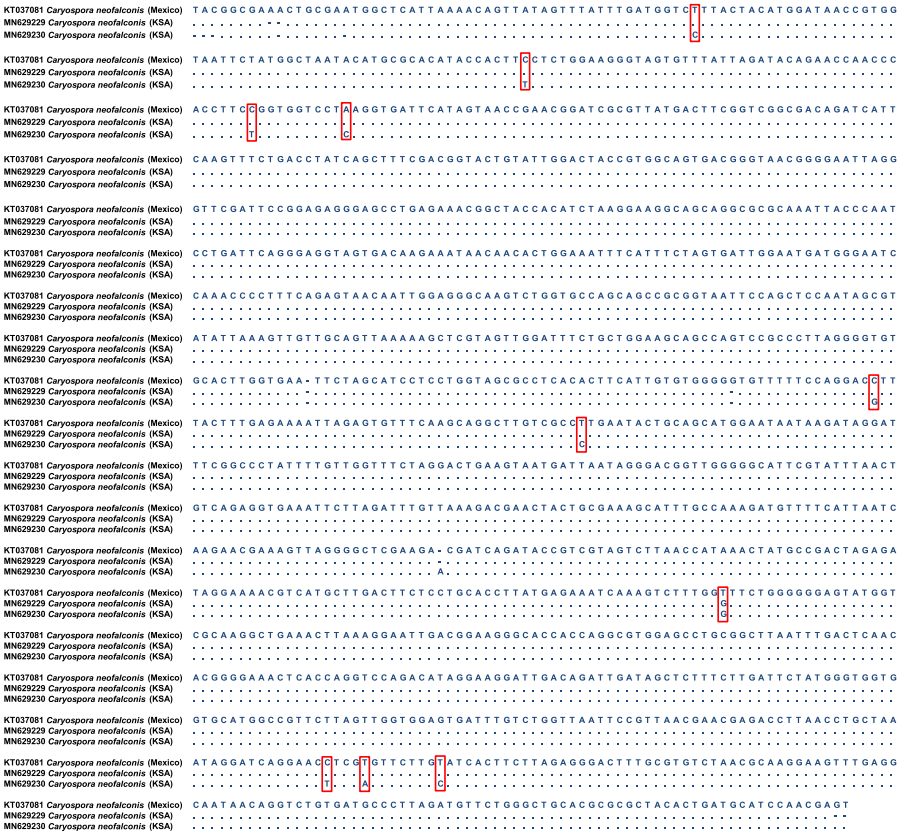


**Figure 4.** Nucleotide sequence alignment of *C. neofalconis 18S rRNA* gene variants from KSA (this study, GenBank Accession MN629229, MN629230) with a reference strain of *C. neofalconis* from Mexico (GenBank Accession KT037081). Identical bases are shown as dots, and sites of variation are shown inboxes. Gaps generated during alignment are shown as dash, and the difference in nucleotide sequence is represented by a single-letter nucleotide code.

with a 91% nodal support, except two sequences of the study were placed in a separate clade (Fig. 5).

## Discussion

This study revealed that 16 out of the 149 falcons examined from the KSA belonged to the *Caryospora* species. The prevalence rate was 10.7%. Previous studies indicated that the prevalence rate of *Caryospora* species in falcons were 12% in Germany [11], 24% in the UK [9], 27% in the UAE [8], 31% in the US [18], and 40% in the KSA [19].

Previously in the KSA, *Caryospora* infection other than *C. neofalconis* was reported, as was *C. cherrughi* sp. from *F. cherrug* [6], *C. biarmicusis* sp. from *F. biarmicus* [14], and *C. maxima* from viperid snake [20]. As per our preliminary literature review, this is the first report of *C. neofalconis* between falcons native to Riyadh and Qassim, KSA. The highest prevalence was observed in *F. peregrinus* (33.3%), as compared to the other examined falcons; furthermore, there was no infection detected in *F. biarmicus* (Fig. 2). The results of the study proved that the *F. cherrug*, *F. pelegrinoides*, *F. peregrinus*, and *F. rusticolus* were infected with *C. neofalconis*, and are considered as new hosts of this parasite in the central region of KSA. It was first described in the following five falcons in Europe; these are *F. Mexicanus*, *F. Biarmicus*, *F. Peregrinus*, *F. Tinnunculus*, and *F. Subbuteo* [11], redescribed by Klüh [21].

In this study, sporulated oocysts were observed to be oval to sub-spherical (Fig. 3, Image D). Böer [11] reported that it could only assume the oval shape. This can be attributed to the loading process. In Central Mexico, Santana-Sánchez et al. [12] reported the presence of *C. neofalconis* in falconiformes and the shape of sporulated oocysts was spherical to subspherical.

Böer [11] mentioned that the estimated time of sporulation was between 60 and 80 h at 21°C, whereas it ranged

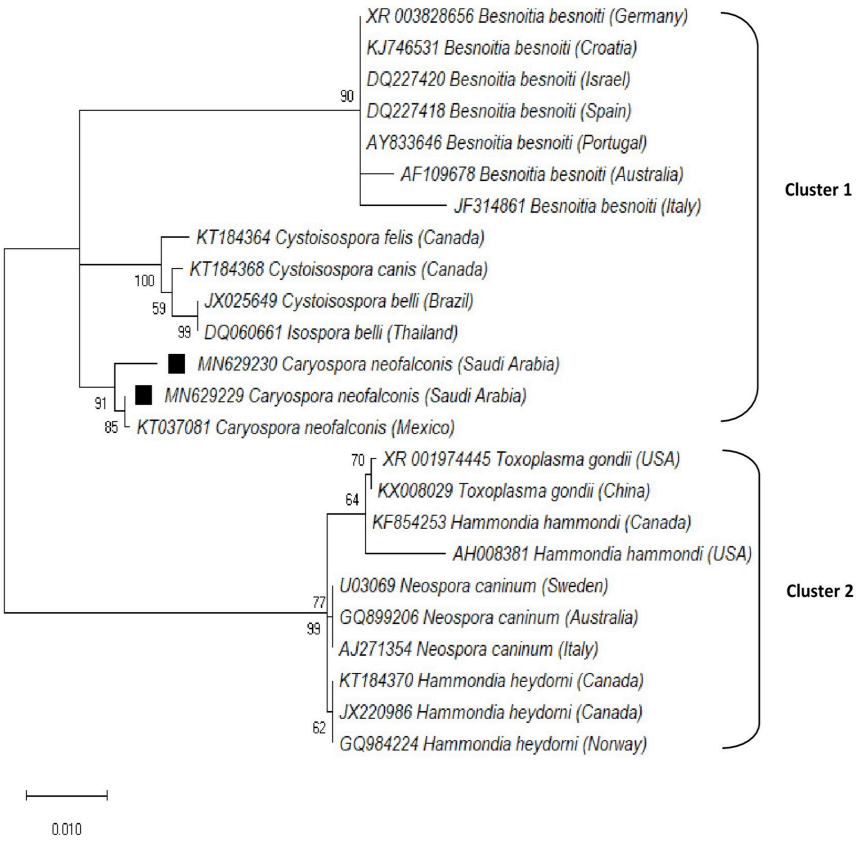


**Figure 5.** Phylogenetic tree of *C. neofalconis* using evolutionary analysis by Maximum Likelihood method of *18S rRNA* gene sequences. The numbers at nodes indicate 1,000 bootstrap values. The scale bar shows sequence variation.

between 60 and 72 h in this study. This difference in sporulation time could be due to the difference in the temperature wherein it was 24°C ± 2°C in the current study.

According to Upton et al. [4] and Pavlík et al. [22], *Caryospora* is one of the pathogens that causes problems in falcons, especially *C. neofalconis*, which are characterized by diarrhea and dullness. Microscopic fecal examination revealed that 10.7% of the falcons were infected with *C. neofalconis*, as confirmed by PCR in 8 falcons. A previous study detected *C. neofalconis* oocysts from 68 fecal samples collected from 30 birds [22]. In another study, *C. neofalconis* was detected in 7 fecal samples collected from 60 raptors by microscopy and PCR from Central Mexico [12]. Sequence comparison of the *18S rRNA* gene in the identified *C. neofalconis* strains in the KSA showed a higher level of nucleotide homology (> 99%), indicating that the circulating coccidian parasites do not subject to quick genetic changes. For genetic characterization of circulating *C. neofalconis*, the nucleotide sequences were aligned with other coccidian sequences available in GenBank and a phylogenetic tree was constructed. The phylogenetic tree showed that circulating *C. neofalconis* isolates were clustered together with *Isospora*, *Cystoisospora*, and *Besnoitia.* Furthermore, it is also showed a close relationship between *C. neofalconis* and *Isospora*, *Cystoisospora*, and *Besnoitia* than *Hammondia*, *Toxoplasma*, and *Neospora*. Besides, it is closely (99.7% identity) related to *C. neofalconis* in Mexico 2015 strain.

Interestingly, two sequences of *C. neofalconis* of this study had over 99% similarity, and there were 9-bp substitutions. This suggests that two distinct populations of *C. neofalconis* circulating in falcons of the KSA and which were made the source of these parasites is controversial. Furthermore, our sequences and the reference strain (KT037081) showed four amino acid substitutions. The substitutions in a number of amino acids indicate the circulation of many subtypes, and the possibility of identifying novel strains in the same host and/or in the various host species [23,24]. Furthermore, nucleotide sequences of *C. neofalconis* isolate targeting multiple genes are needed, which will provide further in-depth analysis of the evolutionary relationships of *C. neofalconis*.

## Conclusion

To our knowledge, this is the first report of *C. neofalconis* in *F. cherrug*, *F. pelegrinoides*, *F. peregrinus*, and *F. rusticolus* from the KSA based on microscopic and molecular techniques. These falcons are new hosts within the central Saudi territory for *C. neofalconis.*


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## Conflict of interest

The authors declare that they have no conflict of interest.

## Authors' contribution

FA: conceptualization, methodology, investigation, data curation, writing – original draft, writing – review, and editing. MA: conceptualization, data curation, supervision, writing – review, and editing. ME: sequencing and phylogenetic analyses, writing – original draft, writing – review, and editing. All the authors read the final version and approved it for publication.

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