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RESEARCH ARTICLE

Bilateral Panophthalmia as a Late Sequel of Leishmaniasis in Dogs

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ABSTRACT

Fifteen dogs were presented with complete blindness that progressed over 2-4 months. Diagnosis was confirmed that dogs had leishmaniasis through direct observation of the amastigotes within the blood cells, PCR testing and phylogenetic analysis. Gross pathologic and histopathologic examinations were performed for two dogs that were severely debilitated and humanely euthanized. Systemic involvement including decreased appetite (n=8), generalized weight loss (n=4), generalized lymphadenopathy (n=3), icterus (n=3), polyuria and polydepsia (n=2), lethargy (n=5) and four dogs were presented without any systemic involvement. All dogs had bilateral panophthalmia (n=30 eyes) manifested by cataract, anterior uveitis, posterior uveitis, retinal detachment, peri-ocular alopecia, conjunctivitis, blepharitis, keratoconjunctivitis and glaucoma. Detailed ultrasonographic ocular lesions were described; histopathological examination confirmed the ongoing changes within the eye. Leishmaniasis should be considered in the differential diagnosis of dogs with bilateral ocular involvement especially those not responding to symptomatic medicinal therapy.

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INTRODUCTION

Leishmaniasis is a zoonotic disease caused by the obligate intracellular protozoon of *Leishmania* spp. and transmitted by phlebotomine sand flies (Dantas-Torres, 2007; Jarallah, 2015; Aslan *et al.,* 2016). It is of great medical and veterinary significance with diverse epidemiological and clinical presentation. Based on World Health Organization (WHO) records, leishmaniasis is reported in approximately 12 million people worldwide with 0.9–1.3 million new cases and 20 to 30 thousand deaths annually (Kimutai *et al.,* 2009; Postigo, 2010). The geographical distribution of the disease is mostly in tropical and sub-tropical Africa, the Middle East, South and Central America, Southern Europe and Asia (Myler and Fasel, 2008; Bessat and El Shanat, 2013). Clinically, three major forms of the disease have been described including cutaneous (most common form), mucocutaneous and visceral leishmaniasis (most serious form) (Bessat and El Shanat, 2013).

Dogs can naturally be infected with *Leishmania*, also they may be a reservoir for disease transmission; they are more likely to be victims rather than reservoirs (Dantas-Torres, 2007; Jarallah, 2015; Pennisi and Persichetti, 2018). The disease seems to be under reported especially that infected dogs may remain asymptomatic for a long time (Moreno and Alvar, 2002; Karakuş *et al.,* 2015). Also, the diverse clinical manifestation of the disease along with the non-specific clinical signs including but not limited to change in appetite, generalized weight loss, facial alopecia, muscle wasting, painful joint, lymphadenopathy, splenomegaly, hepatomegaly, polyuria, polydipsia, polyphagia, epistaxis, melena, diarrhea, anterior uveitis, blepharedema, blepharitis, keratoconjunctivitis, or panopthalmitis makes the diagnosis difficult with a large differential list (Peña *et al.,* 2000, 2008; Baneth *et al.,* 2016; Abbehusen *et al.,* 2017). Canine leishmaniasis may be presented with ocular manifestation which may occur concurrently with other systemic signs or may be a sole clinical complaint (Peña *et al.,* 2000; Baneth *et al.,* 2016).

The present study aimed to document the clinical, ultrasonographic and histopathologic characteristics of confirmed ocular leishmaniasis in 15 dogs presented with bilateral panophthalmia.

MATERIALS AND METHODS

The present study was performed on 15 dogs admitted to the clinic of the Department of Surgery, Anesthesiology and Radiology- Faculty of Veterinary Medicine- Cairo University with bilateral panophthalmia. The dogs were referred by private veterinarians with complete blindness that was progressed over 2-4 months that remained non-responsive to treatment (topical antibiotics, anti-inflammatories and anti-glaucoma therapy). All study procedures were approved by Cairo University Institutional Animal Care and Use Committee (CU-IACUC. All dogs' owners were aware that their dogs will be included in research and signed a written consent form indicating their approval. Dogs included in the study were of both sexes (8 males and 7 females), of different breeds (9 Mongrel, 3 Labrador Retriever and 3 German Shepherd) and aged 3.5±1.1 years.

Historical data were recorded for each dog including the owner's complaint, onset and progression of clinical signs, previous medications and housing. Complete clinical examination including evaluation of the vital health parameters was performed for all dogs. Ophthalmic examination was done including inspection of the eyelids and globe, slit-lamp biomicroscopy and indirect ophthalmoscopy. Trans-eyelid ocular ultrasonography was performed using 8-10 MHz microconvex transducer. Hematological and biochemical examinations were also included for all dogs.

The diagnosis was made that all dogs had leishmaniasis through direct observation of the amastigotes of the parasite within the white blood cells using Giemsa Wright's stain.

Diagnosis was confirmed by PCR testing performed on peripheral blood sample. DNA extraction was performed from Ethylene Diamine Tetra Acetic acid (EDTA) blood by using a DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. PCR amplification was performed for the target region of the small subunit of ribosomal Ribonucleic acid (RNA) gene of *leishmania spp.* in dogs. The forward primer was 174 F (5'-GGTTCCTTTCCTGA TTTACG-3') and the reverse primer was 798 R (5'-GGCCGGTAAAGGCCGAATAG-3'). These primers generate amplicons of 560 bp and the amplification condition done (Osman *et al.*, 1997).

PCR product of positive samples was purified using Qiaquick purification kit (Qiagen, Germany) following the manufacturer's specifications. Sequencing was done using Big Dye Terminator V3.1 sequencing kit (Applied Biosystems, Waltham, USA) with the forward and reverse primer for 18S ribosomal RNA. The obtained nucleotide sequences were aligned with the sequences in GenBank using the NCBI BLAST server to confirm the identity with *Leishmania spp.*

The sequence (591 bp) of *Leishmania spp.* 18S rRNA was deposited in the GenBank database, under accession number MH916554. The submitted gene sequence was compared with the aligned sequences available in the NCBI GenBank database. Phylogenetic analysis revealed that the obtained nucleotide sequence was comparable to those available in public domains in GenBank using NCBI, BLAST server.

Publicly available 18S rRNA gene sequences of *Leishmania spp.* were downloaded from NCBI GenBank and imported into BioEdit version 7.0.1.4 for multiple alignments using the Clustral W program of the BioEdit. Phylogenetic analysis was done using MEGA version 7 with the Maximum likely hood. The bootstrap consensus tree was construed from 50 replicates. A similarity matrix was utilized using the DNASTAR program (Lasergene, version 8.0). The genetic distance values of species variations of *Leishmania spp.* were analyzed with MegAlign project of DNSTAR software.

Two dogs were severely debilitated and did not respond to medications, these dogs were humanely euthanized according to their owners' written request. Euthanasia was performed using an over dosage of pentobarbital (Beuthanasia-D®, Intervet/Schering-Plough Animal Health Corp, Kenilworth, NJ; 1mL/5kg) injected into the cephalic vein.

Gross pathologic examination and tissue samples were collected from both eyes, fixed in neutral formalin and processed routinely for histopathological examinations.

RESULTS

Clinical examination: The main clinical manifestation of the presented dogs was panopthalmia with subsequent bilateral disturbance in vision that was progressed to complete blindness over 2-4 months. All presented dogs were outdoor dogs and 4 of them were housed within the same animal shelter. All dogs did not receive any anti-parasitic medications during the last year.

The main systemic involvement of the dogs was decreased appetite (number; n=8), generalized weight loss (n=4), generalized lymphadenopathy (n=3), icterus (n=3), polyuria and polydepsia (n=2), lethargy (n=5) and four dogs were presented without any systemic involvement.

Ophthalmic examination: Upon examination, all dogs (n=15) had bilateral panophthalmia (n=30 eyes) manifested by cataract, anterior uveitis, posterior uveitis, retinal detachment, peri-ocular alopecia, conjunctivitis, blepharitis, keratoconjunctivitis and glaucoma (Fig. 1 and 2). Detailed signs of ocular leishmaniasis are demonstrated in Table 1.

Ocular Ultrasonography: The cornea lost its characteristic concave appearance in 12 eyes where it appeared as a straight hyperechoic line. In 18 eyes, the cornea maintained its thick echogenic curvilinear appearance. The anterior chamber was of mixed echogenicity (n=24 eyes) where multiple hypoechoic areas were seen within the normal anechoic pattern. The iris leaflets were visualized as thick echogenic bands attached to the thickened echogenic ciliary body (n=10 eyes). The anterior and posterior lens capsules were visualized as thick hyperechoic structures enclosing the hypoechoic nucleus (n=24 eyes). The lens dimensions were markedly increased in 21 eyes (6 immature and 15 mature cataract) and it was markedly decreased in 3 eyes (hyper-mature cataract).

Table 1: Detailed ocular lesions associated with leishmaniasis in the presented 15 dogs

Ocular sign	No. of eyes (30)	%	Findings
Cataract	21	70%	- Immature cataract (n=4) - Mature cataract (n=15) - Hypermature cataract (n=2)
Lens luxation	8	26.7%	- Posterior lens luxation (n=8)
Anterior uveitis	21	70%	- Corneal edema, miosis, intense vascular response (ciliary injection). - Cellular dots floating within the anterior chamber (aquas flares). - Corneal pigmentation (n=6).
Posterior uveitis	16	53.3%	- Multifocal grayish-white patches were detected with focal subretinal hemorrhage within the tapetal fundus. - Concurrent anterior uveitis was diagnosed in all dogs with posterior uvietis.
Glucoma	11	36.7%	- Increased intraocular pressure associated with anterior uveitis. - Buphthalamus and secondary corneal pigmentation.
Retinal detachment	13	43.3%	- Complete retinal detachment (n=10). - Incomplete retinal detachment (n=3).
Periocular alopecia	14	46.7%	- 3-5 mm wide areas of alopecia were seen adjacent to the eyelid margin containing wet seborrhiec secretions.
Blepharitis	8	26.7%	- Diffuse blepharitis (n=5) manifested by blepharal thickening and edema - Ulcerative blepharitis (n=3) manifested by excoriation of the skin along the eyelid margins.
Conjunctivitis	14	46.7%	- Conjunctival injection and chemosis with serous and/or purulent discharge within the conjunctiva
Chronic vascular/ pannus keratitis	7	23.3%	- Diffuse corneal edema with neovascularization, corneal epithelial dystrophy and reddish granulation tissue occupying either the periphery or the center of the cornea. - Corneal opacity was detected in 2 of these eyes.

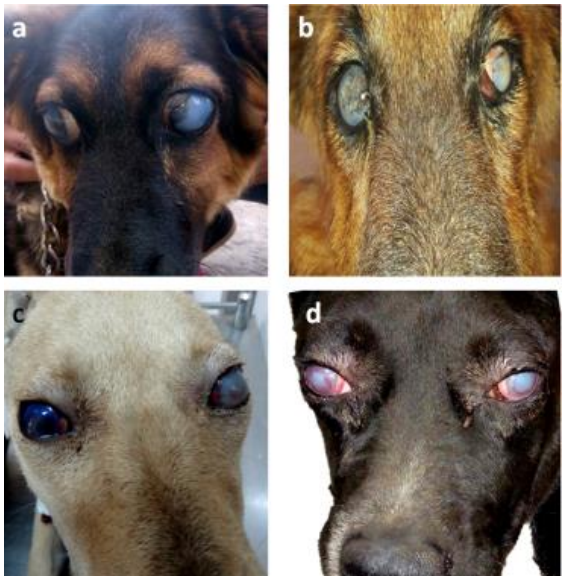

Fig. 1: Photograph demonstrating bilateral ocular involvement in dogs with leishmaniasis. a: Bilateral chronic glaucoma, buphthalmos, corneal edema and early pigmentation in both eyes in a 4-year-old male German Shepherd dog. b: Bilateral endophthalmitis with corneal edema (right eye) and chronic vascular (pannus) keratitis with early scarring in the left eye of 3-year-old male German Shepherd dog. c: Bilateral endophthalmitis with anterior lens luxation and corneal edema (right eye) and chronic keratoconjunctivitis with intense vascular response (left eye) of a 2-year-old male Mongrel dog. d: Bilateral endophthalmitis with chronic keratitis and corneal pigmentation in a 4-year-old female Mongrel dog.

Vitreous opacities were visualized in association with cataract (n=16 eyes). In 6 eyes, viteral hemorrhage was visualized as hyperechoic dots within the anechoic vitrous. Viteral membrane was visualized as a thick hyperechoic band in 3 eyes. The choroid was differentiated from the retina and sclera as a hypoechoic thickened structure (n=8 eyes). Complete retinal detachment was seen in 10 eyes where the retina was identified as a thick hyperechoic band between the ocular fundus and the ora cilliaris retinea forming the characteristic seagull wings. Incomplete retinal detachment (n=3) was visualized as thick hyperechoic band separated from the fundus at the level of the optic nerve. Ultrasonographic ocular changes of dogs presented with canine leishmaniasis are demonstrated in Fig. 3.

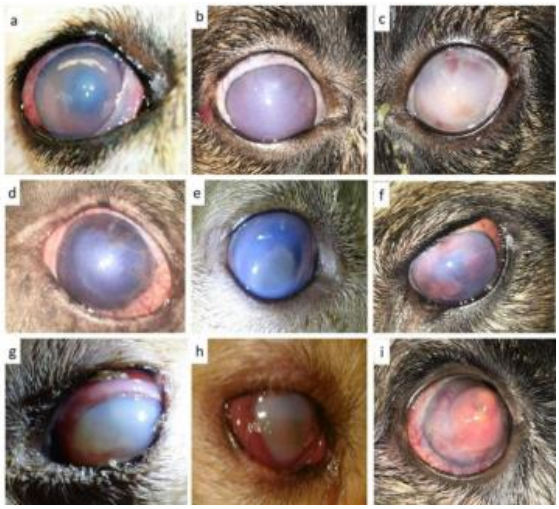

Fig. 2: Photograph demonstrating the ocular lesions of leishmaniasis in dogs. a: Uveitis with miosis, corneal edema, partial third eyelid prolapse and conjunctival injection. b: Endophthalmitis with corneal edema and superficial vascularization. c: Endophthalmitis with chronic vascular (pannus) keratitis. d: Endophthalmitis with chronic glaucoma and chronic vascular keratitis with early signs of granulation tissue formation. e: Endophthalmitis with secondary glaucoma, anterior lens luxation, corneal edema and intense vascular response (vascular fringe). f: Uveitis with corneal edema and corneal vascularization. g: Endophthalmitis, corneal opacity with inflammatory cell infiltrate within the corneal stroma and blepharitis with crusts formation and mucopurulent discharges. h: Endophthalmitis with keratoconjunctivitis and conjunctival chemosis. i: Endophthalmitis with corneal perforation and granulation tissue formation. Note the marked corneal vascularization and ciliary injection.

Hematologic examination: Leukocytosis with absolute lymphocytosis was recorded in all dogs. Direct observation of leishmania amastigotes was a consistent finding, the amastigotes appeared as a round to oval parasite with a round basophilic nucleus and a small rod-like kinetoplast within the macrophage or freed from ruptured cells (Fig 4.a). Marked increase in serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, blood urea nitrogen and creatinine was reported in 7 dogs compared to breed-specific normal reference range (Peavy *et al.*, 2003). The polymerase chain reaction (PCR) product of blood samples were positive and prominent bands on Agarose gel 2% electrophoresis at amplicon molecular weight 591 base pair (bp) (Fig. 4.b).

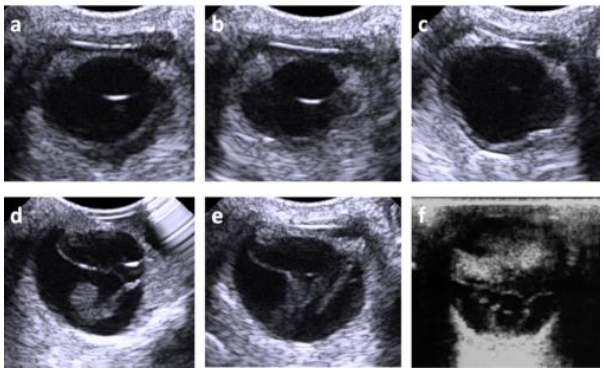

Fig. 3: Trans-eyelid ultrasonographic scan demonstrating multiple ultrasonographic changes within the same scan. a: The cornea lost its characteristic curvilinear appearance where it appeared as a thick straight hyperechoic line, thickened detached hypoechoic chorioretinal segment with presence of subretinal fluid. b: The lens became ovoid with thickened hypoechoic iridociliary junction and thickened chorioretinal membrane. c: Complete separation of chorioretinal membrane with incomplete retinal detachment. d: Complete retinal detachment with thickened hyperechoic floating retina within the anechoic vitreous. e: The eye lost its characteristic ultrasonographic appearance. f: The eye lost its characteristic ultrasonographic appearance with presence of mass of mixed echogenicity occupying the anterior chamber.

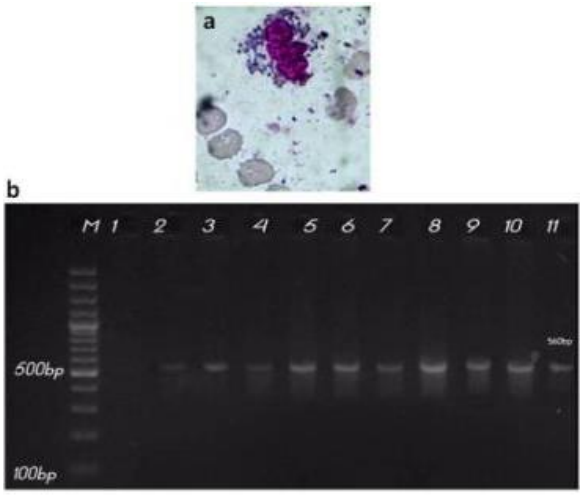

Fig. 4: The blood film stained with Giemsa stain demonstrating the presence of *Leishmania* amastigotes inside the phagocytes (a). Agarose gel electrophoresis demonestrating PCR product of blood samples. Lane 1 is representing DNA ladder 100bp, lane (2-11): positive canine blood samples of *leishmania spp.* with size marker 560bp.

Sequence analyses of purified PCR products were found 98% identical to *Leishmania* species (*L. spp.*) of GenBank database. The phylogenetic analysis revealed close relationship between detected *Leishmania spp.* in Egypt and *L. infantum, L. donovani, L. chagasi, L. major, L. tropica, Leishmania spp*. and *L. brasiliensis brasiliensis* in other countries (Fig. 5. A).

The comparison between inter- and intra-species analyses of genetic distance 16 isolates of *Leishmania spp.* available in public domains in GenBank with Egyptian *Leishmania* spp. were used in tree Maximum likely hood. The genetic identity of *Leishmania spp.* isolated from dogs in Egypt has a high sequence homology (97.9- 95.2 % similarity) with *Leishmania spp. Ghana* (EF524072) which was isolated from patients living in the Eastern Ghanaian community of Taviefe and *Leishmania spp.* (KU500888) which was isolated from

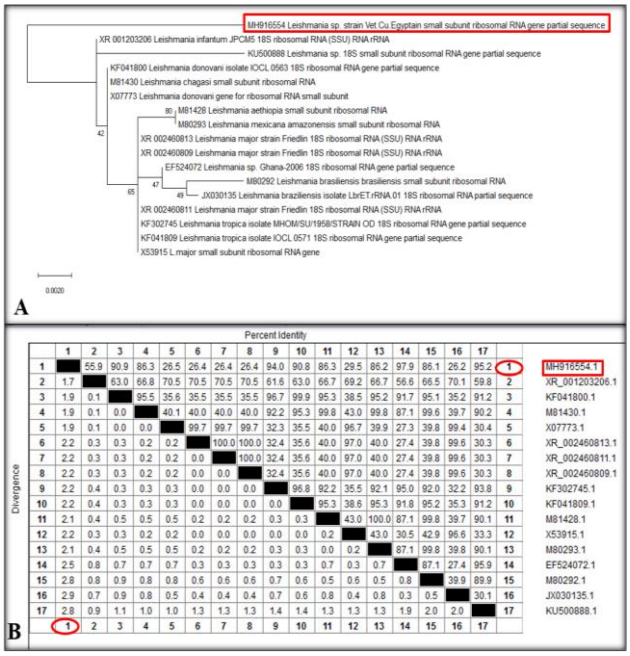

Fig. 5: Phylogenetic analysis of *leishmania spp.* in Egypt and in other countries. A: The evolutionary history was inferred using the Maximum likely hood. Evolutionary analyses were conducted in MEGA7. B: Similarity and genetic divergence of 18S ribosomal RNA sequences of *Leishmania species* from the dog in Egypt with the most similar references sequences from the GenBank database.

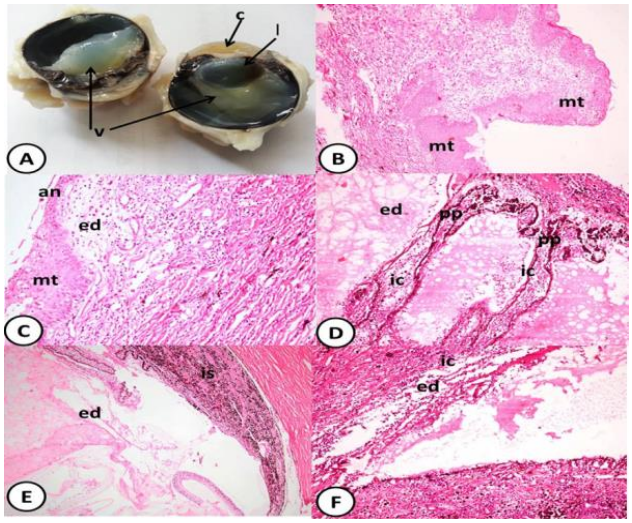

Fig. 6: Gross pathologic and histopathologic examination ocular lesion of canine leishmaniasis. A: Cross section in the eye demonstrating corneal opacity (c), cataract and hemorrhage in the eye lens (l) and turbid, opaque vitreous humour (v). B-F: Histopathological sections of the eye stained by H&E stain demonstrating B: Stratified squamous metaplasia (mt) in the cornea with edema in the corneal stroma (X 100). C: Metaplastic lesion (mt) compared with the apparently normal (an) pseudostratified epithelium of the cornea and marked stromal edema (ed) (X 200). D: Inflammatory cells aggregation (ic) in and around pars plicata (pp) of ciliary body (X 100). E: Inflammatory cells aggregation in iris stroma (is) with marked edema (ed) (X 40). F: Edema (ed) and inflammatory cells aggregation (ic) in the iris (X 100).

blood of female wolf in Brazil, respectively (Fig.5. b). Interspecies analysis based on the genetic distance values indicates 1.7 of genetic divergence (GD) within *L. infantum* JPCMS (XR001203206) isolated from human in USA. However, it was genetically more distant (GD 2.9) from *L. braziliensis* isolated from human in Brazil (JX030135) (Fig.5.b).

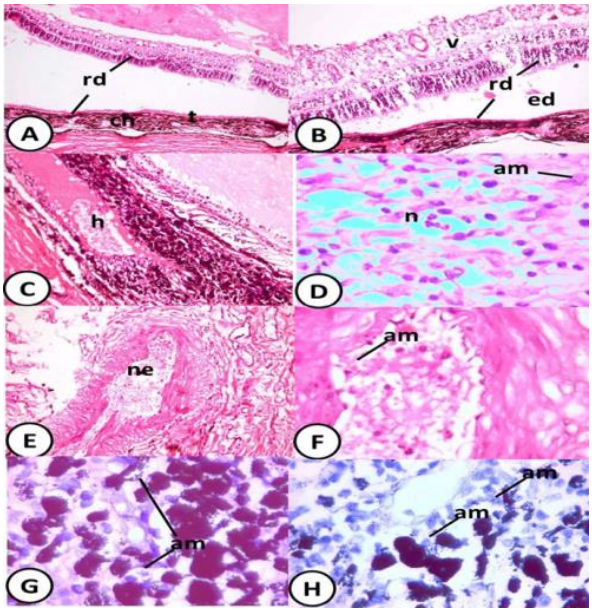

Fig. 7: Histopathological section of the eye with canine leishmaniasis. A-F stained by H & E stain, G stained by Giemsa stain and H stained by Toluidine blue stain. A: Retinal detachment (rd) showing separation of tapetum (t) and choroid (ch) from other layers of the retina (X100). B: Vacuolated retina epithelium (v) & retinal detachment (rd) with marked edema (ed) (X 200). C: Hemorrhage (h) in the pigmented epithelium of the retina (X 200). D: Inflammatory cells aggregation mainly neutrophils (n) and amastigotes (am) of *leishmania* in the macrophage cells (X 1000). E: Necrosis (ne) in the optic nerve (X 200). F: Amastigotes (am) of *leishmania* parasites in the necrotic tissue of the optic nerve (X 1000). G: Amastigotes stained by Giemsa surrounded with hallow zones (X 1000). H: Numerous amastigotes (am) of *leishmania* stained by toluidine blue (X 1000).

Gross pathologic examination: Gross pathologic examination of the two euthanized dogs revealed marked corneal opacity with thickened cornea and sclera. Focal hemorrhagic areas were seen within the lens close to the ciliary body. The vitreous appeared whitish; turbid with coagulated gelatinous mass adhered to the lens (Fig. 6).

Histopathologic examination: Numerous amastigotes of *Leishmania* were seen within the macrophages and histocytes which were confirmed by Giemsa and toluidine blue stains. The amastigotes appeared as multiple intracellular small parasites surrounded with hollow zone. Marked inflammatory cell infiltration and edema was seen all over the cornea, sclera, iris, vitreous, choroid and retina. Diffuse hyperplastic corneal epithelial proliferation was seen together with stromal edema and dispersion of collagen fibers within the stromal tissue. The iris and ciliary body showed marked inflammatory cell infiltration characterized by mononuclear cell infiltration mainly lymphocytes and macrophages together with edema at pars plicata of the ciliary body. Retinal detachment was manifested by retinal vasculitis (hemorrhage and multiple neutrophils) with separation between the retinal pigmented epithelium, tapetum and choroid. Marked necrosis was seen at the optic nerve. Histopathological changes of ocular lesions of leishmaniasis are represented in Figs. 6 and 7.

DISCUSSION

The current study presented 15 dogs with bilateral panopthalmia as a late sequel of canine leishmaniasis. The diagnosis was made through direct observation of *Leishmania* amastigotes within blood cells and confirmed by PCR amplification of *Leishmania* DNA obtained from peripheral blood samples. The genetic similarity between *Leishmania spp.* isolated from dogs in Egypt and high sequence homology (97.9%) of *Leishmania spp. Ghana* (EF524072) isolated from human supports that dogs are considered to be a reservoir for *Leismania spp.* (Osman *et al.,* 1997; Silva and Gontijo, 2005; Baneth *et al.,* 2008).

Ultrasonographic examination provided rapid, non-invasive diagnostic tool that allowed visualizing the ongoing pathologic changes within the globe; these changes were also confirmed by the gross pathologic and the histopathologic examinations.

The diverse clinical and pathological presentation of canine leishmaniasis reflects the difficulty in its diagnosis. Ocular leishmaniasis may be the only or the main clinical manifestation in 3.7 to 25% of *Leishmania* infected dogs (Ciaramella *et al.,* 1997; Peña *et al.,* 2008; Koutinas and Koutinas, 2014). Retrospective clinical studies have concluded that ocular leishmaniasis was reported in 16-25% of dogs naturally infected with *Leishmania* (Ciaramella *et al.,* 1997; Peña *et al.,* 2008). This variation could be attributed to the pathogenicity of the *Leishmania* involved, duration of illness or to the type of immune response developed by the patient (Koutinas and Koutinas, 2014).

Similar to previous reports, ocular lesions of dogs naturally infected by leishmaniasis were predominately bilateral reflecting the systemic involvement of the disease (Brito *et al.,* 2006). However, in earlier stages of the disease only one eye may be affected (Peña *et al.,* 2000; 2008).

Ocular involvement in dogs infected with leishmaniasis may be either a sequel of leukocytic infiltration secondary to the presence of *Leishmania* amastigotes or as a result of an immune mediated process with deposition of immune-complex at the blood aqueous barrier. This lymphoplasmacytic and granulomatous inflammatory infiltration involves (in order of frequency) the conjunctiva, limbus, ciliary body, iris, cornea, sclera, iridocorneal angle, choroid, and the optic nerve sheath (Peña *et al.,* 2000; Brito *et al.,* 2006) which could explain the presence of multiple ocular manifestations recorded in the present study and progression of these ocular lesions to panophthalmia and complete blindness over 2-4 months.

Similar to previous reports (Marcondes *et al.,* 2000; Brito *et al.,* 2006; Pietro *et al.,* 2016) anterior uveitis was the most common manifestation of ocular leishmaniasis in dogs included in this study. Uveitis may have an immunologic or allergic basis similar to post-kala-azar leishmaniosis of humans and may result in secondary glaucoma and panophthalmitis with permanent loss of vision (García-Alonso *et al.,* 1996; Ciaramella *et al.,* 1997). Uveitis regardless of its chronicity is characterized by uveal and corneal edema, miosis, fibrin formation in the anterior chamber, and multiple nodules within the iris stroma (García-Alonso *et al.,* 1996; Peña *et al.,* 2008; Pietro *et al.,* 2016). Posterior uveitis is less commonly reported and is usually accompanying anterior uveitis (Koutinas and Koutinas, 2014) explaining why all eyes in the present study with posterior uveitis were associated with anterior uveitis.

Keratoconjuctivitis may also appear as a sole manifestation in some dogs with *Leishmania*. It is characterized by purulent and sticky ocular discharge, corneal ulceration, and neovascularization. Neglected keratoconjunctivitis may progress to complete pigmentation of the cornea resulting in blindness (Bardagí *et al.*, 2010).

Retinal detachment reported in the present study may be due to systemic hypertension and inflammation of intraocular, extraocular, and adnexal smooth and striated muscles (Cortadellas *et al.*, 2006, Peña *et al.*, 2008).

Vitreous opacities seen within the eye of infected dogs could be explained by previous reports reporting significant increase of the level of total protein of the aqueous humor in dogs naturally infected with leishmaniasis compared to clinically healthy dogs (Brito *et al.*, 2004). This increase in total protein within the aqueous is due to the disruption of aqueous humor barrier due to iris and ciliary vessel dilatation (Brito *et al.*, 2006), or may due to presence of the parasite or deposition of immune complex in the iris stroma (Brito *et al.*, 2004, 2006).

In this study, ocular ultrasonography presented an easy and quick procedure for imaging the structures of the globe regardless of the opacities within the ocular structures (corneal opacities, uveitis and cataract). It was advantageous in identification, verification of the stage and location of cataract (immature, mature and hyper-mature cataract). Moreover, it was helpful for diagnosing retinal detachment and grading its severity (partial and complete).

Conclusions: In conclusion, leishmaniasis seems to be under-reported disease especially in outdoor dogs that lives in endemic areas that may subject to phlebotomine sand flies. Leishmaniasis should be considered in the differential diagnosis of dogs with bilateral ocular involvement that is not responding to symptomatic medicinal therapy.

Authors contribution: All authors conceived and designed the study. KMA, EAH and FAT performed the clinical, ophthalmic, ultrasonographic and gross pathologic examinations; MMA did the hematologic examination, PCR testing and phylogenetic analysis; MAM performed histopathologic examinations. All authors critically revised the manuscript for important intellectual contents and approved the final version.

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