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Presence of *Neospora caninum* DNA of Wild Birds from Turkey

Türkiye'deki Vahşi Kuşlarda Neospora caninum DNA'sının Varlığı

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ABSTRACT

Objective: *Neospora caninum* is a protozoon parasite that has a worldwide distribution and mainly causes abortion in cattle and current serological evidence shows that the disease may be also zoonotic. Wild birds play a role as a reservoir of the disease in nature. The study aimed to determine the prensence of N. caninum in wild birds.
Methods: In this study, the presence of neosporosis in wild birds (n=55) including 22 different species found in the western side of Turkey, was investigated by polymerase chain reaction (PCR). In addition, PCR positive samples were confirmed by sequencing, BLAST, and phylogenetic analysis using MEGA7.
Results: Obtained results showed that the presence of *N. caninum* DNA was 5.45% (3/55) in brain-heart homogenates wild birds. The bird species which were found positive for *N. caninum* were little owl (*Athene noctua*), common buzzard (*Buteo buteo*), and little tern (*Sternula albifrons*). According to phylogenetic analysis and BLAST, all samples were compatible with reference *N. caninum* isolates.
Conclusion: To the best of authors' knowledge, this is the first study detecting *N. caninum* in little tern. In future studies, it may be interesting to investigate the prevalence of *N. caninum* in other wild animals to elucidate the transmission properties.
Keywords: Little tern, *Neospora caninum*, PCR, Turkey, wild birds

ÖZ

Amaç: *Neospora caninum*, dünya çapında dağılım gösteren ve esas olarak sığırlarda düşüklere neden olan protozoon bir parazittir ve güncel serolojik kanıtlar hastalığın zoonotik olabileceğini göstermektedir. Yabani kuşlar, doğada hastalığın rezervuarı olarak rol oynamaktadır. Çalışma, yabani kuşlarda N. caninum varlığının belirlenmesini amaçladı.
Yöntemler: Bu çalışmada, Türkiye'nin batısındaki 22 farklı yabani kuşta (n=55) neosporosis varlığı polimeraz zincir reaksiyon (PZR) ile araştırılmıştır. Ek olarak, PZR pozitif örnekler sekanslanarak MEGA7 kullanılarak BLAST ve filogenetik analiz ile doğrulanmıştır.
Bulgular: Elde edilen sonuçlara göre, yabani kuşların beyin-kalp homojenatlarının %5,45'inde (3/55) *N. caninum* DNA'sı saptanmıştır. Kukumav (*Athene noctua*), bayağı şahin (*Buteo buteo*) ve küçük sumru (*Sternula albifrons*) *N. caninum* pozitif bulunan kuş türleridir. Filogenetik analiz ve BLAST sonuçlarına göre, tüm örnekler referans *N. caninum* izolatları ile uyumlu olarak tespit edilmiştir.
Sonuç: Yazarların bildiği kadarıyla bu çalışma, küçük sumruda *N. caninum* tespit eden ilk çalışmadır. Gelecekteki çalışmalarda *N. caninum* bulaşma özelliklerini aydınlatmak amacıyla diğer vahşi hayvanlarda prevalansın araştırılması faydalı olabilir.
Anahtar Kelimeler: Küçük sumru, *Neospora caninum*, PZR, Türkiye, vahşi kuşlar

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Türkiye PARAZİTOLOJİ Dergisi

INTRODUCTION

Neospora caninum (N. caninum) is a protozoon parasite that can cause neurological disorders, paralysis, and skeletal muscle problems in dogs. Cattle are by far the most affected species by this parasite because of abortions (1-3).

Up to date, viable parasites have been isolated only from dogs, gray wolves, cattle, sheep, water buffalo, europian bison, and white-tailed deer. However, many studies using molecular techniques showed that *N. caninum* can be detected in other mammals and birds (4-11). In humans, although antibodies against this parasite have been described, the zoonotic potential is unclear because neither parasite nor its DNA has not been detected in human tissues (4,12).

N. caninum was first described by Bjerkas and Presthus (13) from dogs in Norway in 1988 and later in a wide range of other animals (14). In wild bird species, *N. caninum* infection is transmitted by hunting and eating small animals containing tissue cyst or their carcasses, as well as through consuming food and water contaminated by oocyst. Thus, predator birds are more frequently infected by *N. caninum*. Different researches have highlighted the role of birds in the transmission of *N. caninum* (1,15,16). In experimental animal models using chickens, partridges, and quails, *N. caninum* caused death in all of the partridges and neurological disorders as well as dose-dependent variable mortality rates in chickens and quails (17-19).

Wild birds require different areas for hunting, feeding, and breeding and these properties are very important for the transmission of *N. caninum* in nature (20-22). Turkey has important geographical locations on the migration routes of wild birds and also hosts them. İzmir and Manisa provinces are located in the western side of Turkey next to the migration routes of wild birds. Moreover, İzmir is the third biggest city in Turkey and has a huge wildlife park and bird sanctuary. The present study aimed to investigate the presence of *N. caninum* DNA in deceased wild birds found in the western part of Turkey by polymerase chain reaction (PCR).

METHODS

Characteristics of the Study Area

İzmir and Manisa provinces (latitude: 37-38 °N, longitude: 26-29 °E) which have a mild Mediterranean climate and are located on the western part of Turkey which have a neighborhood to the Gediz Delta Plain. İzmir Bird Paradise sheltering 289 species of birds has a size of 8.000 hectares. Overall, 50 thousand birds come to this region during migrating. In addition, İzmir bird paradise is the popular resting and hunting area of wild birds coming to Gediz Delta Plain.

Birds and Sample Collection

Dead wild birds were found in the İzmir Bird Paradise or brought to İzmir Natural Life Park Clinics from eight different districts of İzmir province (Bornova, Konak, Bergama, Kemalpaşa, Balçova, Karabağlar and Seferihisar) and four districts from Manisa province (Saruhanlı, Turgutlu, Salihli and Centrum) between 2015 and 2017. İzmir Bird Paradise and İzmir Natural Life Park are located in Çiğli district next to Gediz Delta Plain. Organs (brain and hearth) were collected from 55 dead birds. The species of wild birds are listed in Table 1.

Organ Homogenization and DNA Extraction

The organs homogenates were prepared both the brain and heart of wild birds as previously described (23-25). Firstly, the brain and heart of the dead birds were weighed together. 125 mL (125 mL NaCl/10 gr organs) 0.9% NaCl was added and homogenized using a blender (Waring, USA). Thereafter, trypsin was added to the homogenate (0.5 gr trypsin/10 gr organs) and incubated with 120 rpm at 37 °C for 60 min in an incubator shaker (New Brunswick, USA). After incubation, the homogenate was filtered through sterile two-layered gauzes and centrifuged at 910×g. Next, the supernatant was discarded and the pellet was washed two times with 0.9% NaCl. After the last centrifugation, the pellet was homogenized with 5 mL 0.9% NaCl and, 500 µL aliquot was kept for DNA extraction. DNA isolation from bird organ samples was performed using QIAamp DNA Mini Kit (Qiagen, USA) according to the manufacturer's protocol.

Molecular Detection

PCR targeting a ~340 bp product of the *N. caninum Nc5* gene was performed as described using the Np6 (Forward primer, 5'-CTCGCCAGTCAACCTACGTCTTCT-3') and Np21 (Reverse primer, 5'-CCCAGTGCGTCCAATCCTGTAAC-3') primers (1,26). The 20 µL amplification reactions included 2 µL template DNA, the primers (0.5 µM each), 1.25 U Taq DNA Polymerase (Thermo Scientific, USA), 200 µM dNTPs, 3.25 mM $MgCl_2$ and 1xTaq reaction buffer. The PCR amplification reaction was performed using the following protocol: 10 min initial denaturation step at 95 °C, followed by 40 cycles of 60 s at 95 °C, 60 s at 56 °C, and 60 s at 72 °C, and a final extension of 10 min at 72 °C. All PCR products were separated by 1.5% agarose gel electrophoresis, stained by ethidium bromide and, visualized under DNR bio-imaging systems (Israel). A DNA sample confirmed to be belonging to *N. caninum* was used as the positive control (1) and, distilled water was used as the negative control.

Sequencing

PCR products of 340 bp fragments belonging to positive samples were sequenced by ABI3730XL. Generated sequences were edited and aligned by MEGA 7.0 software. Also, BLAST analysis was performed to compare with reference *N. caninum* samples in National Center for Biotechnology Information (NCBI).

The phylogenetic analysis was performed by MEGA 7.0 software. The phylogenetic tree based on Nc5 gene sequences belonging to *Neospora* isolates was constructed by MEGA7.0 software according to the Neighbour Joining/Maximum Likelihood method using Kimura 2 Gamma distribution (K2+G) model with 500 Bootstrap replications.

RESULTS

According to the PCR results, *N. caninum* Nc5 gene was detected in organ samples of three birds (3/55, 5.45%) (Figure 1) among all wild birds. The presence of *N. caninum* was 2.5% (2/40) among predator wild birds. In the remaining birds, presence of *N. caninum* was 16.6% (1/15). This birds are not predators but eat fish, insects, and reptiles. *N. caninum* positive bird species were a little owl, common buzzard, and little tern.

Among the *N. caninum* positive birds, the location of common buzzard was unknown, others were detected in the Çiğli (İzmir Bird Sanctuary) district (Figure 2).

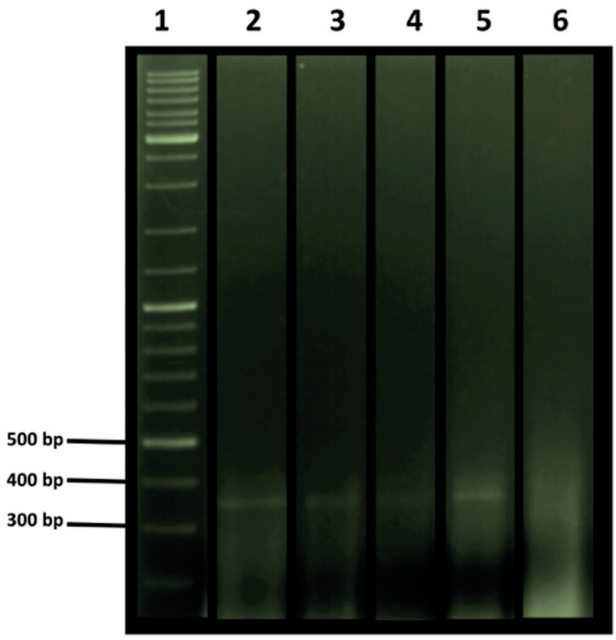

Figure 1. Agarose gel image result of PCR positive samples. Lane 1: DNA ladder; Lane 2: Little owl (sample #7); Lane 3: Common buzzard (sample #46); Lane 4: Little tern (sample#48); Lane 5: Positive control; Lane 6: Negative control

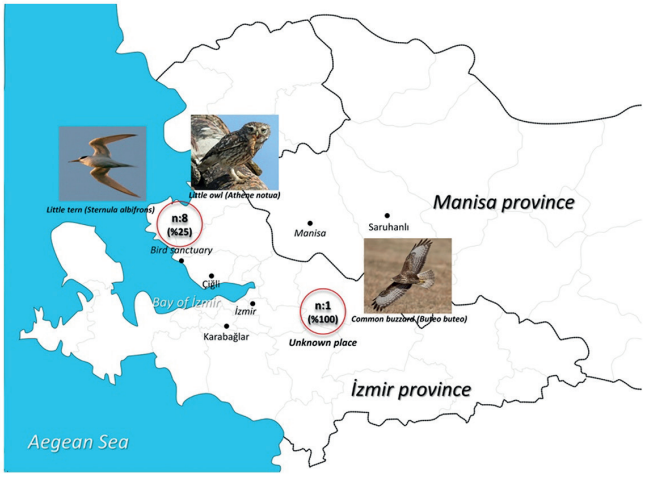

Figure 2. Geographic distribution of *N. caninum* positive deceased wild birds

Nc5 amplicons from positive samples were sequenced using the Np5-Np21 primer pair, and *N. caninum* positivity was confirmed. The Nc5 genomic DNA region displayed homology at levels of 96%, 97%, and 98%, respectively, with a *N. caninum* isolated from naturally infected cattle from Italy (KP715563.1).

Phylogenetic analysis results were compatible with BLAST and *N. caninum* isolates were more close to KP715563.1 (Cattle 2) than other *N. caninum* isolates (Figure 3). Similarity rates changed from 96% to 98.07%.

DISCUSSION

N. caninum leads to major economic losses in livestock worldwide. For example, *N. caninum* causes 2.38 billion dollars' losses per

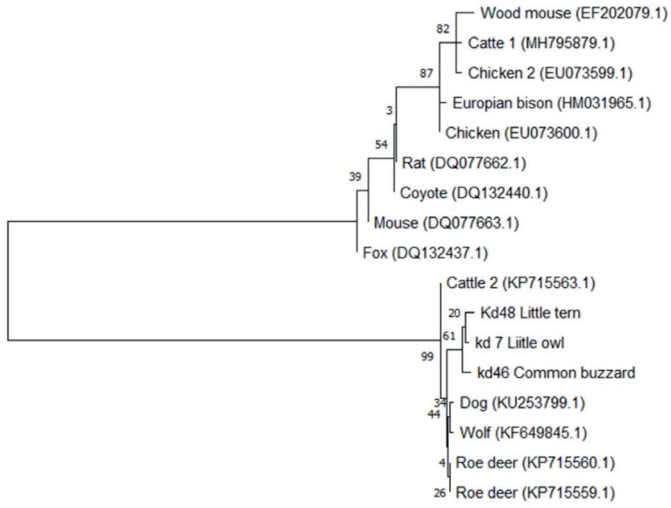

Figure 3. Phylogenetic tree showing the association of *N. caninum* isolates with *N. caninum* isolates

year in the USA due to abortions in cattle (15). In addition, it was reported in Spain that abortions rates in sheep and goats caused by *N. caninum* were 6.75% and 12.5% (27).

Globally, the prevalence of *N. caninum* varies between 1.5-83.6% in wild birds using PCR or serological assays in distinctive regions of the world (4). In Brazil, *N. caninum* DNA was detected in six out of 100 free-range chickens (28). In a study conducted in Iran, the prevalence of *N. caninum* using PCR was 8% in sparrows (29). In Spain, *N. caninum* DNA was detected in three birds (1.5%; n=200) which were two magpies and a common buzzard (16).

There is little information describing the prevalence of *N. caninum* in birds found in Turkey. In a study conducted in Hatay province located in southern Anatolia and Van province located in eastern Anatolia, *N. caninum Nc5* gene was detected in six wild avian species among 103 wild birds (16.5%) belonging to 20 different species (1).

In our study, *Neospora* positivity detected by PCR in wild birds was 5.45%. To our knowledge, this is the first report showing the presence of *N. caninum* in little tern in Turkey and the world. This result indicates that the wild birds can be important as intermediate hosts for *N. caninum* transmission according to the phylogenetic analysis results, the isolates obtained from birds were found to be close to each other. Also, according to Genbank data, it was found to be closely related to isolates obtained from Italy. The reason for this is that migratory birds can carry *N. caninum* strains to countries in the near geographic region.

This research was carried out in İzmir and Manisa provinces of Turkey which are one of the most important resting, hunting, feeding, and breeding areas on the migration routes of wild birds. These results indicate that natural water sources can be contaminated with *N. caninum* oocysts as well as small animals such as rodent or wild type mouse strains containing tissue cysts that may play an important role in transmission of *N. caninum* to wild birds. Moreover, since wild birds that are resistant to many pathogens can transmit neosporosis as well as other important zoonotic diseases such as avian influenza, toxoplasmosis, and *Acanthamoeba* keratitis to humans and animals, further epidemiological studies are required on this area (1,25,30-32).

Table 1. Bird species included in the study, external examination findings, and *Neospora caninum* positivity

No	Name of the wild bird	Predator bird	Cause of death	Local/migratory bird	Location of the bird	Result
1	Barn owl (*Tyto alba*)	Pr	Fracture of wing	L	Çiğli (INLP)	NEGATIVE
2	Caspian gull (*Larus cachinnans*)	Pr	Euthanasia	PM (wintering in North Africa, European coast, South-West Asia)	Konak	NEGATIVE
3	Eurasian eagle-owl (*Bubo bubo*)	Pr	Paralysis	L	Kemalpaşa	NEGATIVE
4	Thrush nightingale (*Luscinia luscinia*)	-	Diarrhea	M (wintering in Africa)	Konak	NEGATIVE
5	Wood pigeon (*Columba palumbus*)	-	Diarrhea	M (wintering in Europe)	Konak	NEGATIVE
6	Caspian gull (*Larus cachinnans*)	Pr	Car crash	PM (wintering in North Africa, European coast, South-West Asia)	Balçova	NEGATIVE
7	Little owl (*Athene noctua*)	Pr	Paralysis	L	Çiğli (IBS)	POSITIVE
8	Common buzzard (*Buteo buteo*)	Pr	Paralysis	PM (wintering in Africa and Southern Asia)	Çiğli (INLP)	NEGATIVE
9	Eurasian stone curlew (*Burhinus oedicnemus*)	-	Paralysis	M (wintering in Southern Europe, the Middle East and Africa)	Çiğli (Kaklıç)	NEGATIVE
10	White stork (*Ciconia ciconia*)	-	Fracture of wing and beak	M (wintering in Africa)	Konak	NEGATIVE
11	Common buzzard (*Buteo buteo*)	Pr	Fracture of wing	PM (wintering in Africa and Southern Asia)	Seferihisar	NEGATIVE
12	Common buzzard (*Buteo buteo*)	Pr	Fracture of wing	PM (wintering in Africa and Southern Asia)	Manisa (Muradiye)	NEGATIVE
13	Great cormorant (*Phalacrocorax carbo*)	Pr (fish)	Poaching	PM (wintering in Africa, southern Asia, Australia)	Çiğli (INLP)	NEGATIVE
14	Common buzzard (*Buteo buteo*)	Pr	Fracture of wing	PM (wintering in Africa and Southern Asia)	Manisa	NEGATIVE
15	Common buzzard (*Buteo buteo*)	Pr	Fracture of leg	PM (wintering in Africa and Southern Asia)	Manisa	NEGATIVE
16	Barn owl (*Tyto alba*)	Pr	Fracture of wing	L	Konak	NEGATIVE
17	Common buzzard (*Buteo buteo*)	Pr	Fracture of wing	PM (wintering in Africa and Southern Asia)	Kemalpaşa	NEGATIVE
18	Common buzzard (*Buteo buteo*)	Pr	Keratitis	PM (wintering in Africa and Southern Asia)	Çiğli	NEGATIVE
19	Eurasian sparrowhawk (*Accipiter nisus*)	Pr	Fracture of wing	PM (wintering in southern Europe, southern Asia, Africa)	Salihli	NEGATIVE
20	Common buzzard (*Buteo buteo*)	Pr	Fracture of wing	PM (wintering in Africa and Southern Asia)	Manisa	NEGATIVE
21	Common buzzard (*Buteo buteo*)	Pr	Fracture of wing	PM (wintering in Africa and Southern Asia)	Salihli	NEGATIVE
22	Eurasian sparrowhawk (*Accipiter nisus*)	Pr	Fracture of wing	PM (wintering in southern Europe, southern Asia, Africa)	Çiğli	NEGATIVE
23	Dalmatian pelican (*Pelecanus crispus*)	Pr (fish)	Fracture of leg	PM (wintering in Eastern Europe, Asia, Nile river)	Çiğli (IBS)	NEGATIVE
24	Common buzzard (*Buteo buteo*)	Pr	Car crash	PM (wintering in Africa and Southern Asia)	Turgutlu	NEGATIVE
25	Common buzzard (*Buteo buteo*)	Pr	Euthanasia	PM (wintering in Africa and Southern Asia)	Bergama	NEGATIVE
26	Common buzzard (*Buteo buteo*)	Pr	Systemic infection	PM (wintering in Africa and Southern Asia)	Çiğli (INLP)	NEGATIVE
27	Common buzzard (*Buteo buteo*)	Pr	Keratitis	PM (wintering in Africa and Southern Asia)	Salihli	NEGATIVE

28	Common buzzard (*Buteo buteo*)	Pr	Unilateral Keratitis	PM (wintering in Africa and Southern Asia)	Bornova	NEGATIVE
29	Common buzzard (*Buteo buteo*)	Pr	Keratitis	PM (wintering in Africa and Southern Asia)	-	NEGATIVE
30	Common buzzard (*Buteo buteo*)	Pr	Respiratory infection	PM (wintering in Africa and Southern Asia)	-	NEGATIVE
31	Eurasian eagle-owl (*Bubo bubo*)	Pr	Respiratory infection	L	Salihli	NEGATIVE
32	Common buzzard (*Buteo buteo*)	Pr	Keratitis	PM (wintering in Africa and Southern Asia)	Salihli	NEGATIVE
33	Common buzzard (*Buteo buteo*)	Pr	Fracture of wing	PM (wintering in Africa and Southern Asia)	Salihli	NEGATIVE
34	Common buzzard (*Buteo buteo*)	Pr	Keratitis	PM (wintering in Africa and Southern Asia)	Bergama	NEGATIVE
35	Eurasian sparrowhawk (*Accipiter nisus*)	Pr	Keratoconjuntctivitis	PM (wintering in southern Europe, southern Asia, Africa)	Bornova	NEGATIVE
36	Common buzzard (*Buteo buteo*)	Pr	Hyphema	PM (wintering in Africa and Southern Asia)	Turgutlu	NEGATIVE
37	Common buzzard (*Buteo buteo*)	Pr	Paralysis	PM (wintering in Africa and Southern Asia)	Konak	NEGATIVE
38	Greater flamingo (*Phoenicopterus roseus*)	-	Electric shock	PM (wintering in all Mediterranean basin and Africa)	Konak	NEGATIVE
39	Common buzzard (*Buteo buteo*)	Pr	Diarrhea	PM (wintering in Africa and Southern Asia)	Saruhanlı	NEGATIVE
40	Eurasian sparrowhawk (*Accipiter nisus*)	Pr	Respiratory infection	PM (wintering in southern Europe, southern Asia, Africa)	Karabağlar	NEGATIVE
41	Common buzzard (*Buteo buteo*)	Pr	Paralysis	PM (wintering in Africa and Southern Asia)	Konak	NEGATIVE
42	Eurasian sparrowhawk (*Accipiter nisus*)	Pr	Respiratory infection	PM (wintering in southern Europe, southern Asia, Africa)	Saruhanlı	NEGATIVE
43	Peregrine falcon (*Falco peregrinus*)	Pr	Fracture of wing	M (wintering in Africa)	Çiğli	NEGATIVE
44	Common kestrel (*Falco tinnunculus*)	Pr	Fracture of wing	PM (wintering in Africa)	Konak	NEGATIVE
45	Black stork (*Ciconia nigra*)	Pr (fish)	Fracture of wing	M (wintering in Africa)	-	NEGATIVE
46	Common buzzard (*Buteo buteo*)	Pr	Poaching	PM (wintering in Africa and Southern Asia)	-	POSITIVE
47	Common buzzard (*Buteo buteo*)	Pr	Diarrhea	PM (wintering in Africa and Southern Asia)	-	NEGATIVE
48	Little tern (*Sternula albifrons*)	Pr (fish)	Incoordination	M (wintering in Africa Arabian Peninsula, southeast Asia, and Australia)	Çiğli (IBS)	POSITIVE
49	Northern pintail (*Anas acuta*)	-	Shot by hunters	M (wintering in Europe)	Çiğli (IBS)	NEGATIVE
50	Mallard (*Anas platyrhynchos*)	-	Shot by hunters	M (wintering in Europe)	Çiğli (IBS)	NEGATIVE
51	Mallard (*Anas platyrhynchos*)	-	Shot by hunters	M (wintering in Europe)	Çiğli (IBS)	NEGATIVE
52	Mallard (*Anas platyrhynchos*)	-	Shot by hunters	M (wintering in Europe)	Çiğli (IBS)	NEGATIVE
53	Eurasian coot (*Fulica atra*)	-	Shot by hunters	M (wintering in Europe)	Çiğli (IBS)	NEGATIVE
54	Eurasian sparrowhawk (*Accipiter nisus*)	Pr	Respiratory infection	PM (wintering in southern Europe, southern Asia, Africa)	Çiğli (INLP)	NEGATIVE
55	Eurasian woodcock (*Scolopax rusticola*)	-	Systemic infection	M (wintering in Europe)	Bornova	NEGATIVE

Samples are numbered according to the date they have arrived to the laboratory. Pr: Predator bird that hunts other animals, L: Local bird, M: Migrating bird, INLP: İzmir Natural Life Park, IBS: İzmir Bird Sanctuary

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The pictures on the map are taken from the International Union for Conservation of Nature's Red List of Threatened Species (www. https://www.iucnredlist.org/).

* Ethics

Ethics Committee Approval: All experiments were performed under the instructions and approval of the Institutional Animal Care and Use Committee (IACUC) of Ege University for animal ethical norms (Permit number: 2014-016).

Informed Consent: There is no need for patient consent information as the dead bird is being studied.

Peer-review: Internally peer-reviewed.

* Authorship Contributions

Surgical and Medical Practices: H.C., D.A., Ö.D., Concept: M.K., H.C., T.K., H.G.Ö., M.N.M., A.Y.G., M.D., Design: M.K., H.C., H.G.Ö., M.N.M., A.Y.G., M.D., Data Collection or Processing: M.K., D.A., Ö.D., T.K., E.K., M.D., Analysis or Interpretation: M.K., D.A., Ö.D., T.K., E.K., M.D., Literature Search: M.K., T.K., E.K., Writing: M.K., H.C., M.D.

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