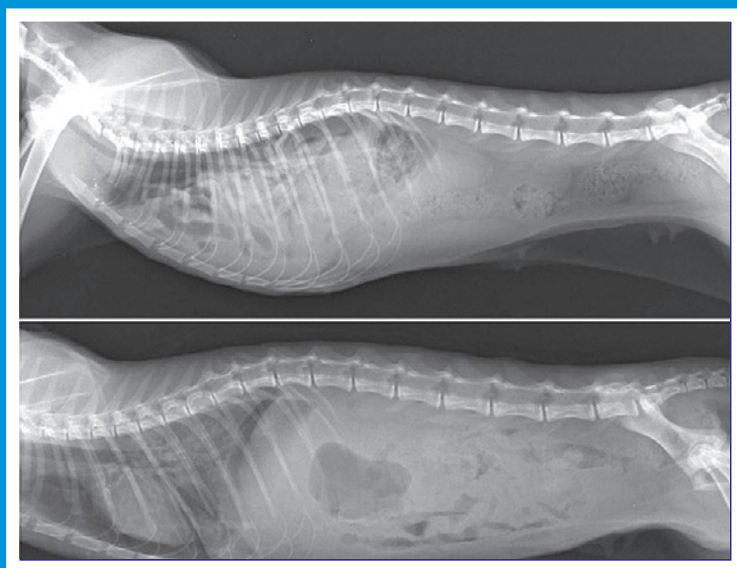
ISSN 1580-4003

THE SCIENTIFIC JOURNAL OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA

SLOVENIAN VETERINARY RESEARCH

SLOVENSKI VETERINARSKI ZBORNIK



Volume
59 1

Slov Vet Res • Ljubljana • 2022 • Volume 59 • Number 1 • 1–70

ISSN 1580-4003

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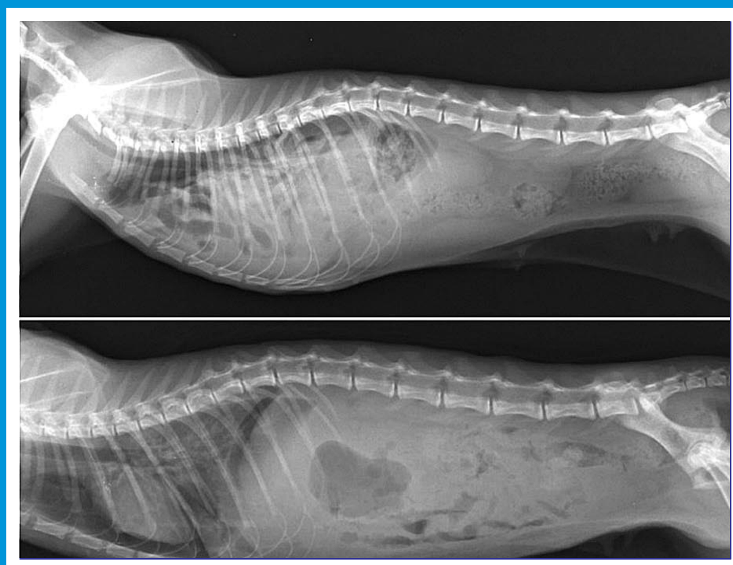

Slov Vet Res 2022; 59 (1): 1–70

Volume 59 1

Slov Vet Res • Ljubljana • 2022 • Volume 59 • Number 1 • 1–70

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The Scientific Journal of the Veterinary Faculty University of Ljubljana

SLOVENIAN VETERINARY RESEARCH
SLOVENSKI VETERINARSKI ZBORNIK

Previously: RESEARCH REPORTS OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA
Prej: ZBORNIK VETERINARSKE FAKULTETE UNIVERZA V LJUBLJANI

4 issues per year / izhaja štirikrat letno
Volume 59, Number 1 / Letnik 59, Številka 1

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Published by / Založila: University of Ljubljana Press / Založba Univerze v Ljubljani
For the Publisher / Za založbo: Gregor Majdič, the Rector of the University of Ljubljana / rektor Univerze v Ljubljani

Issued by / Izdala: Veterinary Faculty University of Ljubljana / Veterinarska fakulteta Univerze v Ljubljani
For the Issuer / Za izdajatelja: Breda Jakovac Strajn, the Dean of the Veterinary Faculty / dekanja Veterinarske fakultete

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Sponsored by the Slovenian Research Agency
Sofinancira: Javna agencija za raziskovalno dejavnost Republike Slovenije

ISSN 1580-4003
Printed by/tisk: DZS, d.d., Ljubljana, March 2022
Number of copies printed / Naklada: 220
Indexed in/indeksirano v: Agris, Biomedicina Slovenica, CAB Abstracts, IVSI
Urlich's International Periodicals Directory, Science Citation Index Expanded,
Journal Citation Reports – Science Edition
https://www.slovetres.si/

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SLOVENIAN VETERINARY RESEARCH
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DOI 10.26873/SVR-1354-2022
UDC 616.993.19-078:616-039.71:598.2

Review Article

AVIAN CRYPTOSPORIDIOSIS: A SIGNIFICANT PARASITIC DISEASE OF PUBLIC HEALTH HAZARD

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Abstract: Cryptosporidiosis is one of the most important zoonotic parasitic diseases affecting a wide range of host species. The disease is widely distributed all over the world. *Cryptosporidium* species can affect different avian hosts, causing severe economic losses. The severity of avian cryptosporidiosis symp-toms vary from asymptomatic disease to severe enteric and/or respiratory manifestations with high mortality. Diagnosis of *Cryptosporidium* infection is mainly based on microscopic detection of oocysts, serological methods, or molecular techniques to identify different *Cryptosporidium* species. Humans and animals are highly susceptible to infection by different *Cryptosporidium* species as a result of the ingestion of contaminated food and water by oocysts or direct contact with infected hosts. Different prevention and control strategies have been applied either in the surrounding environment or for the infected animals, birds, and humans. Therefore, this review article was designed to shed light on avian cryptosporidiosis species and its distribution, susceptibility and infection, clinical pictures, laboratory diagnosis, zoonotic importance in humans, and prevention and control strategies.

Key words: avian; control; *Cryptosporidium*; diagnosis; human

Introduction

Cryptosporidiosis is a zoonotic enteric parasitic disease affecting humans, mammals, birds, and fish (1, 2). Globally, cryptosporidiosis is ranked fifth among the 24 most important foodborne parasites (3-5). Avian species could be infected with *Cryptosporidium* parasite with variable mortality and morbidity rates, causing great economic losses (6-10). The most important pathogenic species of *Cryptosporidium* that affect birds are *Cryptosporidium* meleagridis (*C. meleagridis), C. baileyi*, *C. galli*, and *C. avium* (11). In chickens and/or turkeys, infection is usually caused by C. baileyi and *C. meleagridis* (12), and rarely *C. parvum* (13) and C. galli (14). There are great differences in the prevalence and distribution rates of *Cryptosporidium* species among various avian species. The clinical pictures of *Cryptosporidium* infections vary from intestinal to respiratory diseases according to the oocysts of each species, susceptibility and age of the birds, and presence of concurrent infections (15). Conventional microscopical staining techniques (16), serological methods, and recent molecular techniques are used for the diagnosis of cryptosporidiosis (17, 18). Several studies around the world demonstrated the zoonotic potential of *Cryptosporidium* species like *C. parvum*, *C. hominis*, and *C. meleagridis* in different hosts like dogs, cattle, mice, and cats. Humans, especially children and patients with immunodeficient diseases, could be infected through direct contact with infected animals (19) or birds (20). Application of hygienic measures, prevention of direct contact with infected animals or birds, and treatment of infected

Received: 3 July 2021
Accepted for publication: 3 January 2022

animals, especially diarrheic animals or humans, are the most important strategies for the prevention and control of cryptosporidiosis.

Accordingly, this review article was designed to shed light on avian cryptosporidiosis distribution, susceptibility and infection, clinical pictures, laboratory diagnosis, zoonotic importance in humans, and prevention and control strategies.

History and distribution

Table 1 show the incidence and distribution of Cryptosporidium species in different avian hosts all over the world. The first detection of avian Cryptosporidium oocysts was in the cecum of apparently healthy chickens (21) as these oocysts were similar to C. parvum and C. hominis in humans. Later, for the first time, a unique Cryptosporidium species was described as C. meleagridis by Slavin (22), and this species was molecularly differentiated from C. parvum as a different avian species with zoonotic nature (23). In an Algerian study by Baroudi et al. (24), the prevalence rates of C. meleagridis were 57.9%, 43.9%, and 5.5% in turkey poults, adult turkeys, and broiler chickens, respectively. However, the infection rate of C. meleagridis in chickens of the previous study was 28.9%.

Furthermore, C. meleagridis was previously found in 5% (25), 3% (26), and 3.2% (27) of broiler chickens and 10% of layer chickens in China (28) and in 9% of broilers in Algeria (29).

Another species of Cryptosporidium called C. baileyi was detected in the intestinal tracts, bursae of Fabricius, and cloacae of birds (30), in the upper and lower respiratory tracts of broilers (31, 32, 26) and geese (33), and in the urinary tract as kidneys (34, 35). Recently, C. baileyi was molecularly detected in broiler and layer chickens, ducks, and pigeons as a zoonotic species (36, 27, 37). It has been documented that C. baileyi is the most prevalent avian Cryptosporidium species worldwide and has a wide host range (38-41, 28, 11, 42).

Although C. parvum has zoonotic importance in humans, it is sporadically found in poultry species (11). In 2017, in Germany, the prevalence rates of Cryptosporidium species were 5.7% in broilers and 8.3% in layers, and C. parvum was the most predominant isolate of chickens and turkeys (20, 40). In Brazil, C. parvum was detected in chicks (15), while in the Unites States it was detected in turkeys (43).

Other species of Cryptosporidium have also been identified earlier. Levine (44) found C. tyzzeri in chickens, while Proctor and Kemp (45) found C. anserinum in geese. In 1990, C. blagburni was identified in finches (46, 39), but this species was further described as C. galli with a zoonotic nature. The latter was previously identified in chickens' proventriculi (47, 48, 14).

Different avian species of Cryptosporidium have been reported among Egyptian poultry flocks. The first morphological detection of C. meleagridis, C. baileyi, and C. galli was in quails in 2011. The study showed that the prevalence rates of Cryptosporidium oocysts were 30.8% in bobwhite quails and 33.3% in brown quails with a total percentage of 31.9% (49). From a wide range of avian species in different seasons, Cryptosporidium in all the examined bird species was prevalent in winter (15.4% for fowl, 3.6% for pigeons, 44.2% for ducks, 15.7% for turkeys, and 30% for geese), while the lowest prevalence rate of Cryptosporidium was in spring (8.3% for fowl and 2.6% for pigeons). Cryptosporidium showed less incidence in ducks in autumn (2.4%) and summer (3.2%) and in turkeys (4.4%) (50). In the study of Kalifa et al. (51), the incidence of Cryptosporidium species in ducks was 39.9 %, with the highest rate in winter (74.6%) and the lowest rate in autumn (7.1%). Approximately 55% of 100 ducks had antibodies against cryptosporidiosis. The molecular analysis revealed positive amplification at 435 bp, and sequencing confirmed the presence of C. meleagridis.

Genetically, five genotypes of Cryptosporidium (I, II, III, IV, and V) were identified in birds (9, 52). In Canada, genotypes I to V were identified in wild geese and the black duck (53, 54). Genotype I was detected in canaries and Indian peafowl (41, 55). Genotype II was found in ostriches (56, 57) and other several species of Psittaciformes and Passeriformes (41, 58-62). Genotype IV was identified in Japanese white-eye woodcocks, in addition to the Eurasian woodcock genotype that was detected in Eurasian woodcocks (41). Genotype V was found in cockatiels (52), Psittaciformes (59, 62), and reptiles (63). Phylogenetically, C. meleagridis, C. baileyi, goose genotypes I and II, and the duck genotype belong to the Cryptosporidium intestinal clade, while C. galli, C. andersoni, C. muris, C. serpentis, and genotypes III and IV belong to the gastric clade (64, 41).

Table 1: Incidence and distribution of *Cryptosporidium* species in different avian hosts all over the world

Country	Incidence and distribution	Reference
Brazil	*C. baileyi, C. galli,* and *C. meleagridis* have been characterized in ostriches using morphological, biological, and molecular techniques.	[9]
	C. baileyi was found in ducks and quail, while *C. melagridis* was detected in chicken.	[36]
	Positive amplification for *Cryptosporidium* species was observed in 12.6% (24/190) of the samples, including *C. baileyi* (9.8%; 18/190), *C. meleagridis* (0.5%, 1/190), *C. parvum* (2.1%; 4/190), and *Cryptosporidium* species (0.5%; 1/190). Sub-genotyping of *C. meleagridis* revealed the presence of the subtype IIIgA23G3R1.	[42]
	Sequencing of *Cryptosporidium* species revealed presence of *C. baileyi* in a black vulture, a domestic chickens, and a saffron finch; *C. galli* in canaries, a cockatiel, and lesser seed-finches; *C. meleagridis* in a domestic chicken; *C. parvum* in a cockatiel; *Cryptosporidium* avian genotype I in a canary and an Indian peafowl; *Cryptosporidium* avian genotype II in ostriches, and *Cryptosporidium* avian genotype III in a cockatiel and a peach-faced lovebird.	[55]
	Molecular analysis of nucleotide sequences grouped the ostrich isolate of *Cryptosporidium* species as *C. baileyi* which was genetically distinct from all other species.	[56]
	Among the 242 fecal samples from wild birds, 16 (6.6%) were positive for the presence of *Cryptosporidium*. Molecular characterization of 16 samples showed *C. meleagridis*. *C. galli* was identified in rufous-bellied thrush, green-winged saltators, slate-coloured seedeater, goldfinch, and saffron finches. Isolates in Goldfinch isolate, buffy-fronted seedeater, red-cowled cardinal, and saffron finch isilates were identified as *C. baileyi*. Avian genotype II was found in an isolate from a white-eyed parakeet.	[60]
	Out of 103 fecal samples of exotic birds, 7 (6.8%) were positive for *Cryptosporidium*. Sequencing analyses showed *C. parvum* in Bengalese finch and avian genotype III in Java sparrow and cockatiel. The sequences of the *Cryptosporidium* species isolated from canaries presented a higher genetic similarity with *C. parvum*.	[61]
	A total of 1027 fecal samples were collected from Psittaciformes and Passeriformes. Molecular analysis showed positive results in 580 (56.47%) and 21 (2.04%) samples, respectively, for *C. galli* and *Cryptosporidium* avian genotype II, and in 28 (2.73%) and 3 (0.29%) samples, respectively, for *C. galli* and *Cryptosporidium* avian genotype III. *C. baileyi* and *Cryptosporidium* avian genotype V were also identified.	[62]
	Microscopic examination of fecal smears of carrier pigeons revealed presence of 4% (4/100) positive *Cryptosporidium*. While, 7% (7/100) were molecularly positive. *C. parvum* was genetically identified.	[75]
	C. baileyi was identified in broiler chickens. This species was able to infect Japanese quails.	[79]
	Cryptosporidium was observed in 44.4% of the examined 77 ostriches. However, 100% of the ostriches shed oocysts in their feces.	[86]
	C. galli infection was microscopically, histologically, and molecularly characterized in canaries, a cockatiel, and in lesser seed-finches with clinical complaints of apathy and sporadic mortality.	[134]
	Cryptosporidium species were detected in 24.5% samples of adult and 13% of young species of birds including great-billed seed-finch, lesser seed-finch, ultramarine grosbeak, and rusty-collared seedeater. The sequencing analyses showed identification of *C. galli*. The protozoon infection was associated with concomitant infection with *Escherichia coli* and *Isospora* species.	[135]

Table 1: continnuation

	Cryptosporidium species were microscopically demonstrated in young quails experienced high mortality, diarrhea, and clear fluid content in the intestine.	[6]
	Cryptosporidium species and reovirus were identified in bobwhite quails with white-watery diarrhea, dehydration, and death, 30%-45% mortality rate, and mucoid enteritis.	[7]
	The overall 23 of 56 (41%) broiler chickens had *C. baileyi* tracheitis. The infection rates among *C. baileyi*-infected flocks ranged from 10%-60%.	[32]
	Cryptosporidium species were microscopically detected in the urinary tract of adult laying hens.	[35]
	C. parvum was found in turkeys, especially in 4-9-week-old birds.	[43]
	Genotypes I to V of *Cryptosporidium* were identified in wild geese.	[53]
	Cryptosporidium goose genotype I, *Cryptosporidium* goose genotype II, *Cryptosporidium* duck genotype, *C. parvum*, and *C. hominis* were identified.	[54]
	Oocysts of *Cryptosporidium* species were observed in the feces, and the developmental stages of the parasite were observed in tissue sections of turkeys and Muscovy ducks but not bobwhite quail.	[66]
	Oral inoculation of ducks with *C. baileyi* induced no clinical signs, while intratracheal inoculation produced mild respiratory disease, no deaths, and airsacculitis.	[69]
	Proventriculus and intestinal samples from 70 North American red-winged blackbirds were examined. Twelve birds (17.1%) were genetically positive for the *Cryptosporidium*. Sequence analysis of the gastric species revealed presence of *C. galli* and *Cryptosporidium* avian genotype VI.	[90]
	Microscopic examination of the intestinal contents of turkey poults suffering from diarrhea and mucoid enteritis and typhlitis showed presence of *Cryptosporidium* species in the enterocytes, villi atrophy, and infiltration with inflammatory cells.	[95]
The United states	*Cryptosporidium* oocysts were detected in the droppings of 16/20 (80%) 17-day-old and of 38/100 (38%) 24-day-old turkeys without signs. The protozoon was frequently found in the ceca, colon, and cloaca of inoculated turkeys and chickens.	[98]
	Outbreaks of sinusitis due to *Cryptosporidium* were documented in 7-and 3-week-old turkeys.	[100]
	Concurrent infection with *Mycoplasma sturni* and *Cryptosporidium* species was detected in cliff swallows manifested clinical, gross, and microscopic lesions.	[106]
	Mixed infection with *C. baileyi* and infectious bursal disease virus resulted in more severe bursal lesions, more infected birds, and greater numbers of the parasite in infected tissues.	[114]
	Double infection with *C. baileyi* and either reovirus promoted shedding of both. Reovirus infection did not modify lesions caused by *C. baileyi* infection.	[115]
	Cryptosporidium infection promoted systemic spread of reovirus, and reovirus intensified *Cryptosporidium* infection, but no significant synergistic effect on mortality or weight gain was observed.	[126]
	Cryptosporidium species were detected during histologic examination of small intestine from a budgerigar with chronic weight loss and from a cockatiel that died acutely.	[128]
	C. baileyi was microscopically detected in the small intestine of cockatiels.	[129]
	Cryptosporidium avian genotype III was demonstrated in lovebird (*Agapornis* species) manifested gastrointestinal signs ad lesions.	[136]
	Mixed infection with adenovirus and *Cryptosporidium* species was demonstrated during examination of tracheal mucosa of 7-week-old broiler chickens that had excessive exudate in the tracheas and congestion of the nasal turbinates.	[139]

Table 1: continnuation

Australia	The phylogenetic analysis supported the existence of *C. baileyi* and C. *meleagridis* in finches, a black duck, and brown quail.	[39]
	Of 430 avian-derived fecal specimens, 27 *Cryptosporidium*-positive isolates were detected and characterized. Genotypes I to IV were molecularly identified. *C. galli*, *C muris*, and *C. andersoni* were also identified in a tawny frogmouth and a quail-crested wood partridge.	[41]
	Cryptosporidium infection was found in 4-week-old quails with respiratory lesions and shrunken bursa of Fabricius. Deciliation, hyperplasia, and inflammatory cell infiltration in the respiratory cells and in the oesophageal and salivary glands were detected. Besides, follicular bursal atrophy and presence of *Cryptosporidium* species were observed.	[76]
	Cryptosporidium associated conjunctivitis was detected in 8-week-old ducks with conjunctivitis.	[101]
	Respiratory cryptosporidiosis was diagnosed in a 2-week-old peacock chicks. Microscopic examination of conjunctiva, nasal-sinus, and trachea showed the different developmental stages of *Cryptosporidium*.	[104]
	C. meleagridis was detected in an Indian ring-necked parrot using morphological and molecular methods.	[130]
Spain	Histological examination and antigenic diagnosis by enzyme-linked immunosorbent analysis revealed the presence of *Cryptosporidium* species in respiratory and intestinal tracts of red-legged partridges. Morbidity (diarrhoea and cough) was 60%-70% and mortality was 50%. *C. meleagridis* was molecularly identified in faecal samples.	[10]
	Intestinal cryptosporidiosis was identified in young pigeons manifested diarrhea and body weight loss. Hyperplasia of the intestinal crypts with inflammatory infiltration were seen.	[71]
	An ocular and respiratory disease were associated with *C. baileyi* infection in wild scops owl. Molecular analysis confirmed the presence of *C. baileyi* in the conjunctival cells and the nasal respiratory epithelium.	[89]
Canada	Developmental stages of *Cryptosporidium* were observed in tracheal epithelium of turkey poults. Lesions associated with the parasite included excess mucus, epithelial hyperplasia, metaplasia, and necrosis, and infiltration with macrophage and heterophil in thickened lamina propria.	[65]
	Cryptosporidium species were found in the feces of 14 out of 165 (8.5%) ostriches. The oocysts failed to infect chickens, turkeys, or quail. *Cryptosporidium* species from ostriches was different from *C. meleagridis*, *C. baileyi*, and *Cryptosporidium* species of bobwhite quail.	[82]
Scotland	Ziehl-Neelsen staining of the fecal smears, bursae of Fabricius, or respiratory organs of broilers revealed infection rates of 18.7% of *Cryptosporidium* species.	[16]
	C. baileyi was molecularly identified in wild red grouse with sinusitis, conjunctivitis, and swollen head.	[105]
The Czech Republic	*C. galli* was isolated from the stomach of hens and it was transmitted from hens to chickens.	[48]
	C. baileyi was identified in 15 out of the 22 avian-derived isolates, while *C. meleagridis* was identified in 5 avian-derived isolates. One isolate (B1-30), from a rose-ringed parakeet, exhibited a mixed infection of both *C. meleagridis* and *C. baileyi.*	[138]
Germany	*C. baileyi* was microscopically and molecularly identified from raptors and from a German falcon breeder with a history of respiratory distress.	[88]
England	*C. baileyi* was detected in red grouse moors with bulgy eye signs.	[108]
France	*C. baileyi* infection didn't prevent the induction of immunity against Marek's disease virus serotype 1 vaccine (CVI988/Rispens) in chickens.	[112]
Hungary	Chicken anemia virus infection may increase the reproductive potential of *C. baileyi* in chickens and both pathogens have synergistic effect on each other.	[113]
The Nether-lands	*Cryptosporidium* infection was associated with colitis and cystitis in green iguanas. The disease was characterized by cloacal prolapses and cystitis. Based on molecular gene identification, *Cryptosporidium* species were c belonging to the intestinal *Cryptosporidium* lineage, but not to *C. saurophilum* or C. *serpentis*.	[63]
Denmark	In 73 of 128 ducklings, and in 44 of 74 goslings, *Cryptosporidium* species were detected. Tissues from the bursa of Fabricius were positive in both species of birds. The presence or absence of the parasite could not be correlated with clinical signs or lesions and/or poor performance of the birds.	[68]

Table 1: continnuation

Poland	A total of 499 fecal dropping from 308 free-ranging, 90 captive, and 101 domestic birds were tested by conventional, immunological, and molecular techniques for *Cryptosporidium*. *C. parvum* was found in 19 (3.8%).	[93]
Greece	*C. baileyi* was molecularly identified in 7-week-old Saker falcon died with a history of severe respiratory signs and lesions and otitis media.	[107]
	Ziehl-Neelsen staining of the fecal smears, bursae of Fabricius, or respiratory organs of broilers showed infection rates (24.3%) of *Cryptosporidium* species.	[142]
South Africa	Heavy infection of the cloacal and bursal tissues with *Cryptosporidium* species was observed in ostriches showed phallus prolapse as well as in normal birds. Loss of the microvilli borders, epithelial hyperplasia, swelling of organelles, and nuclear changes were detected microscopically.	[84]
	The histological findings in emaciated 4-month-old ostriches revealed presence of *Cryptosporidium* species in the necrotic and inflamed pancreatic epithelium.	[127]
Nigeria	The total prevalence of *Cryptosporidium* oocysts was 7.4% in different avian species. Local birds had the highest prevalence rate (9.5%), followed by exotic birds (6.6%) and the wild ones (5.3%).	[4]
China	The infection rate of *Cryptosporidium* among broiler chicken was 10% (38/385). *C. baileyi, C. meleagridis*, and *Cryptosporidium* avian genotype II were molecularly identified.	[25]
	The overall infection rate of *Cryptosporidium* was 10.6% (163/1542) in layer chickens, 3.4% (16/473) in broilers, and 16.3% (92/564) in Pekin ducks. C. *baileyi* (184/187) on chicken and duck farms, and *C. meleagridis* (3/187) on layer chicken farms were detected.	[28]
	C. baileyi (33/471; 7.0%) and *C. meleagridis* (15/471; 3.2%) were identified in broiler chickens. Two subtypes of *C. meleagridis* including IIIbA26G1R1b and IIIbA22G1R1c were characterized.	[27]
	The overall prevalence of *Cryptopsoridium* in psittacine birds was 8.1% (35/434). Three *Cryptosporidium* species and two genotypes were identified, including *C. baileyi* (18/35 or 51.4%) in red-billed leiothrixes, white Java sparrows, common mynas, zebra finches, a crested Lark, a Gouldian finch, and a black-billed magpie; *C. meleagridis* (3/35 or 8.6%) in a Bohemian waxwing, a Rufous turtle dove, and a fan-tailed pigeon; *C. galli* (5/35 or 14.3%) in Bohemian waxwings and a silver-eared Mesia; *Cryptosporidium* avian genotype III (3/35 or 8.6%) in cockatiels and a red-billed blue magpie; and *Cryptosporidium* avian genotype V (6/35 or 17.1%) in cockatiels.	[59]
	The overall prevalence of *Cryptosporidium* infection in pigeons was 0.82% (2/244). *C. baileyi* and *C. meleagridis* were identified.	[74]
	The overall prevalence of *Cryptosporidium* in quail was 13.1% (29 of 47 farms). The highest prevalence was observed in autumn and the lowest in winter. *C. baileyi* and *C. meleagridis* were molecularly detected.	[78]
	C. baileyi infection in baby chicks may induce bursal atrophy, immunosuppressive effects against avian influenza (H5N1), and increase the susceptibility to the virus.	[121]
	A total of 303 fecal samples were collected from ostriches and 31 samples (10.2%) were *Cryptosporidium*-positive upon microscopic analysis. The infection rate was 27.6% in ostriches aged 16-60 days, 1.2% in those aged 61-180 days, and 20.4% in those aged >10 years. Genetic analysis of the isolated parasite revealed presence of *C. muris* and *C. baileyi*.	[137]
Japan	Eleven *Cryptosporidium* isolates were identified molecularly in cockatiels. Three new genotypes in *C. meleagridis*, avian genotype III, and a new avian genotype V were characterized.	[52]
	Cryptosporidium avian genotype III was molecularly identified in peach-faced lovebirds.	[58]
	Mixed infection with *Mycoplasma gallisepticum* and *Cryptosporidium* species or other bacteria was detected in Japanese quail. Birds showed swelling of the head, nasal discharge, increased lacrimation, decreased egg production, mortality rate of 5.7% per day, caseous exudate in the sinuses, egg peritonitis, and airsacculitis. Microscopically, non-purulent or purulent inflammation accompanied by lymphoid hyperplastic tissue with germinal centers were observed in the oculofacial respiratory mucosa.	[77]
Korea	Infection with *C. baileyi* induced an immunosuppressive effect on Newcastle disease virus vaccine in 2-day-old chicks and the infection with the parasite may increase the susceptibility to this virus infection.	[118]
	C. baileyi infection could suppress the immune response against infectious bronchitis virus vaccine and perhaps increase the susceptibility to this virus infection in chickens.	[120]

Table 1: continnuation

Malaysia	A total of 90 samples were screened for *Cryptosporidium* from different avian species. Phylogenetic trees identified all the isolates as *C. parvum*.	[94]
Bangla-desh	Microscopic examination revealed presence of *Cryptosporidium* species in 19.8% (39/197) of avian specimens. Molecular characterization showed that 15.7% (31/197) of the samples were *Cryptosporidium* positive. Of these 31 samples, 17 were *C. baileyi* (8.7%), 12 were *C. meleagridis* (6.0%), and 2 were *C. parvum* (1.0%). *C. meleagridis* had two subtypes (IIIbA21G1R1 and IIIbA23G1R1), which were found in broiler, native and sonali chickens, and a pigeon. Two novel subtypes (IIIbA21G2R1 and IIIbA20G2R1) were identified in sonali chickens, a broiler chicken, and a layer chicken.	[37]
Vietnam	*C. baileyi* genotype II was detected in 2-3-month-old ostriches with a prevalence rate of 23.7% (110 out of 464 samples).	[57]
Thailand	*C. meleagridis* was identified in pigeons, however, *Cryptosporidium* avian genotype III was detected in seagulls.	[73]
Egypt	The prevalence rates of *C. meleagridis, C. baileyi*, and *C. galli* were 30.8% in bobwhite quails and 33.3% in brown quails with a total percentage of 31.9%.	[49]
	Cryptosporidium showed less incidence in ducks in autumn (2.4%) and summer (3.2%) and in turkeys (4.4%).	[50]
	The incidence of *Cryptosporidium* species in ducks was 39.9%, with the highest rate in winter (74.6%) and the lowest rate in autumn (7.1%). Approximately 55% of 100 ducks had antibodies against the parasite.	[51]
	C. baileyi could be one cause of vaccination failure against Newcastle disease and/or avian influenza viruses in chicken's farms.	[119]
Iran	*Cryptosporidium* species were identified in pigeons with overall prevalence rate of 2.94%.	[72]
	C. meleagridis was histologically detected in the intestinal tract of turkey poults suffering from diarrhea and unthriftness. The oocyst shedding was detected only in 29% of positive birds.	[96]
	Microscopic examination of the intestine and trachea demonstrated that 23.75% of 240 broiler samples were infected	[148]
Algeria	*Cryptosporidium* infection rates were 34% and 44% in chickens and turkeys, respectively, with all positive turkeys (25) and most positive chickens (26/31) having *C. meleagridis*. All *C. meleagridis* isolates belonged to a new subtype family.	[24]
	A total of 345 faecal samples were collected from domestic, captive, and wild birds. *Cryptosporidium* species were detected in 31 samples. Sequence analysis revealed the presence of *C. baileyi* in domestic chicken broilers, captive ostriches, and a wild mallard, and *C. meleagridis* in a graylag goose, chickens, and turkeys. The overall prevalence of *Cryptosporidium* in chickens and turkeys was 2% and 6%, respectively. Both *C. meleagridis* and *C. baileyi* were detected in chicken broilers, with a prevalence ranging from 9% to 69%. Turkeys were positive only for *C. meleagridis*, with a 13% prevalence at the animal level. Subtyping of *C. meleagridis* isolates showed subtype IIIgA22G3R1 in graylag goose and chicken broilers and IIIgA23G2R1 in chicken and turkey broilers.	[29]
Morocco	Examination of intestine, bursa of Fabricius, and trachea revealed existence of *Cryptosporidium* species in 37% of 225 broilers flocks. The prevalence of infection within flocks varied from 14%-100%. High incidence of *Cryptosporidium* infection occurred in 36-45-day-old broilers (52%). *Cryptosporidium* species were detected in bursa (24%), intestine (15%), and trachea (2%). In the bursa of Fabricius, *Cryptosporidium*-induced epithelial lesions, lymphoid atrophy, and depletion.	[143]
Tunisia	*Cryptosporidium* was detected in 9 out of 200 broiler chicken (4.5%). Molecular characterization showed presence of *C. meleagridis* in one broiler chicken.	[144]
Turkey	Clinical signs of cryptosporidiosis in 10-day-old pigeon were depression, ruffled feathers, and diarrhea. Gross lesions were mild hyperaemic segments of small intestine distended with typical green watery ingesta. *Cryptosporidium* species were found in the villi of lower portions of atrophic and misshapen small intestine.	[70]
Dubai	*C. parvum* was molecularly detected from an outbreak of catarrhal enteritis and mortality among stone curlews.	[132]

Susceptibility and infection

Cryptosporidiosis has been recorded worldwide in more than 30 species of domestic, wild, and captive birds (23). The disease was demonstrated in chickens, turkeys, ducks, geese, quails, pheasants, ostriches, partridges, and peacocks (65-67, 1).

In water fowl (ducks and geese), Richter et al. (68) detected the presence of *Cryptosporidium* species in the intestinal and respiratory tracts of ducklings and goslings at incidence rates of 57% and 59%, respectively, using indirect immunofluorescence assay. Experimentally, *C. baileyi* was identified in ducks in Brazil (36). However, *C. baileyi* was detected using the in situ hybridization technique in the conjunctival and bursal tissues of geese experimentally infected with Usutu virus (33).

Moreover, *C. baileyi* and *C. meleagridis* could infect the intestine, bursa, and cloaca of ducks after the incubation period up to 9-10 days (69).

In pigeons, cryptosporidiosis has been identified by some researchers (70, 71). Qi et al. (59) identified *C. meleagridis* in one pigeon using molecular techniques, while Radfar et al. (72) found that the prevalence rate of *Cryptosporidium* species was 3.4% in adult pigeons and 2.3% in squabs. Moreover, cryptosporidiosis infection has been microscopically and molecularly identified in different countries like Thailand (73), China (74), and Brazil (75).

In Australia, quails gain natural infection with *Cryptosporidium* species like *C. baileyi* and *C. meleagridis* (76, 39). The natural infection of quails with *C. baileyi* has been recorded in Japan using molecular techniques (77). Both *C. baileyi* and *C. meleagridis* were molecularly characterized in China (78). The experimental infection of quails with *C. baileyi* isolates of chicken was first unsuccessful (6, 7), while Cardozo et al. (79) succeeded in the induction of infection in quails using isolates of broiler chickens.

Infection with *C. meleagridis* has also been reported in red-legged partridge chicks (10).

Ratite bird species also showed susceptibility to cryptosporidiosis. The first detection of *Cryptosporidium* infections in ostriches was in the 1990s (80, 8, 81-84). Gajadhar (81) identified *Cryptosporidium* in the feces of 14/165 (8.5%) African ostriches. However, in 1994, Gajadhar (82) investigated these oocysts and the host specificity. The identified oocysts were identical to *C. meleagridis*, but they were non-infectious to chickens, turkeys, quails, and mice (82). Later on, Ponce Gordo et al. (85) identified *Cryptosporidium* oocysts in 2-month-old to 5-year-old ostriches, but with a low prevalence rate (2/336, 0.6 %). Microscopically, the prevalence rate of *C. baileyi* in Europe was 60% in adult ostriches and rheas (85), and that of the Brazilian isolate was 44% in adult ostriches (86). *Cryptosporidium* avian genotype II species was molecularly identified in ostriches (56, 41), which was similar to the Brazilian isolate of *C. baileyi* (9). In Vietnam, Nguyen et al. (57) identified *Cryptosporidium* avian genotype II oocysts of *C. baileyi* in 2-3-month-old ostriches with a prevalence rate of 23.7% (110 out of 464 samples).

Pet birds could be infected with *C. galli*, *C. meleagridis*, and *C. baileyi*. These species have been described as important zoonotic parasites to humans, especially kids (87). In addition, *C. baileyi* has been isolated from the upper respiratory tracts of three mixed-bred falcons (88). In owls, Molina-Lopez et al. (89) reported an infection with *C. baileyi* associated with an outbreak of ocular and respiratory diseases. *C. galli* and novel *Cryptosporidium* avian genotype VI have been found in North American red-winged blackbirds (90).

The age group susceptibility to cryptosporidiosis is variable according to the *Cryptosporidium* species. It has been proposed that young birds are the most important risk group since their immune system is not yet fully developed (43). The infection rate of *Cryptosporidium* was 4.9% in 1-20-day-old broiler chickens, 24.6% in 31-60-day-old layer chickens, and 40.3% in 11-30-day-old Peking ducks (28). Moreover, it has been shown that chickens of all ages are susceptible to *C. baileyi*, while 31-120-day-old layer chickens are more susceptible to *C. meleagridis* infection (28). In addition, C. *baileyi* and *C. meleagridis* were detected in chicken aged above four months (27). In the United States, the infection is predominant in 4-9-week-old turkeys (43). In the study of Helmy et al. (20), infections with *Cryptosporidium* were noted in 13.8% of 13-20-week-old turkeys, 5.7% of 1-6-week-old broilers, and 8.3% of layer chickens above 20 weeks of age.

Perhaps broiler chickens act as a source of cryptosporidiosis disease infection and transmission of oocysts (91). Birds are infected with *Cryptosporidium* by ingestion or inhalation of sporulated oocysts in contaminated litter, feces,

water, and dust. The main route of *Cryptosporidium* transmission is the fecal-oral route as oocysts are shed in the droppings, contaminate the soil and water, and thus provide many paths into the food chain (92). Mechanical transmission through migratory birds has also been reported. Although these birds have a low level of *Cryptosporidium* infections, they shed oocysts and contaminate the environment (93). Moreover, asymptomatic birds act as a mechanical source of transmission of infective oocysts to other birds, animals, and humans (94). Poor hygienic measures are associated with increased incidence of the infection in poultry flocks (15).

Clinical picture

The severity of *Cryptosporidium* clinical disease in poultry depends on birds' hygienic conditions, stocking density, crowding, and mixing of different ages and species of birds (15). Intestinal *Cryptosporidium* infection is characterized by diarrhea, lowered weight gain, and distention of the intestinal lumen with mucus and gases, in addition to the presence of different stages of *Cryptosporidium* in different parts of the small intestine (95, 96).

Infection of turkeys with *C. meleagridis* presents either subclinically (97, 98) or clinically in the form of enteritis (22, 95, 99). *C. meleagridis* may infect chickens (69) with intestinal involvement.

It has been shown that *C. baileyi* mainly affects the respiratory and intestinal tracts of poultry (38, 40). Reports of *C. baileyi* respiratory tract infections have been detected in chickens (31), turkeys (100), ducks (101, 102), geese (33), and pheasants (103). In addition, some wild birds, such as falcons, owls, swallows, and the red grouse, showed infection of the upper respiratory system, middle ear, and eyes with *C. baileyi* (104, 88, 89;105-108). Co-infection of *C. baileyi* with Escherichia coli and infectious bronchitis (IB) virus was reported, and the affected birds showed mortality associated with lower respiratory tract infections, such as bronchitis, pneumonia, and air sacculitis (109-111). Moreover, *C. baileyi* is associated with other causative agents with high mortality and lowered body weight gain. Among these infectious agents are the virus vaccine of Marek's disease (MD) (Rispens) (112), chicken infectious anemia virus (113), infectious bursal disease (IBD) virus (114), and reoviruses (115).

In addition, it has been reported that *C. baileyi* induces humoral immuno-suppression effect through the infection of the bursa of Fabricius (116). This effect is controversial and shows different results. For example, the infection of chickens with *C. baileyi* suppresses the immune response to the virus vaccines of MD (117, 112), IBD (114), reoviruses (115), Newcastle disease (118, 119), IB disease (120), and avian influenza disease (121, 119). Although the experimental challenges of chickens with *C. baileyi* induced purulent bursitis and hyperplasia as well as slight lymphoid atrophy (122, 123), no effect on the humoral immune response has been detected (110, 123).

Severe sinusitis with a morbidity rate of 5%-10% was observed in young turkey poults infected with Mycoplasma species (100). Respiratory signs were observed in thirteen turkeys that were histologically positive for *Cryptosporidium* species (124). However, a history of self-limiting diarrhea was identified in a flock of turkey poults suffering from invasive *Cryptosporidium* (95). In Iran, C. *meleagridis* oocysts were detected in diarrheic unthrifty turkey poults (96). Sneezing, frothy eyes, swollen sinuses, coughing, and rattling were reported in a case of *C. baileyi* and C. meleagridis infections in turkeys (125).

Cryptosporidium species were detected in five-day-old quails with high mortality (6). Ritter et al. (7) reported a mortality rate up to 45% that was associated with acute fatal diarrhea in 1-17-day-old quails as the infection was associated with *Cryptosporidium* and reovirus. A similar study was carried out by Guy et al. (126) who observed severe diarrhea and mortality as well as high oocyst shedding after the experimental coinfection of quails with *Cryptosporidium* and reovirus. Respiratory signs and lesions, drop in egg production with peritonitis, and a daily mortality rate of 5.7% were reported in Japanese quails due to mixed infection with Mycoplasma gallisepticum and *Cryptosporidium* species (77). However, in a study of Wang et al. (78) in China, no signs were detected in a *Cryptosporidium*-positive quail flock.

In pigeons, invasive stages of *Cryptosporidium* were identified in the intestine of a pigeon with depression, and diarrhea soiled the feather around the vent (70). Moreover, yellow watery diarrhea, weight loss, and dehydration were associated with 40% morbidity and 5% mortality rates (71).

Diarrhea, cough, morbidity rates of 60-70%, and a mortality rate more than 50% were reported

in red-legged partridge chicks having mixed infection with *C. meleagridis* and *C. baileyi* (10).

In ostriches, prolapsed cloacae, recta, and bursae were observed in dead 4-week-old chicks heavily infected with *Cryptosporidium* (8, 83, 84). Moreover, it has been proposed that *Cryptosporidium* infection is associated with pancreatic necrosis (80, 127). Infected 7-30-day-old ostrich chicks with *Cryptosporidium* genotype II showed sudden death with cloacal prolapse, and the oocysts were identified in the rectum, coprodeum, urodeum, and bursa (9).

Pet birds like budgerigars (128), cockatiels (128, 129), parrots (130), and lovebirds (131) exhibited intestinal cryptosporidiosis with high mortality.

When *C. parvum* or *C. galli* infects chickens or turkeys, no clinical signs could be detected (14, 43). However, in Dubai, an outbreak of catarrhal enteritis and mortality were reported among stone curlews (132). Birds infected with gastric *C. galli* showed diarrhea, weight loss, and sometimes mortality (46, 133, 39). Chronic proventriculitis is associated with *C. galli* with secondary infections (134, 135). In psittacine birds, strains of *C. galli* genotype III induced chronic vomiting, weight loss, and proventriculitis (58, 136).

Other species, *C. muris* and *C. andersoni*, were found in birds' droppings perhaps due to mechanical transmission from mammals (41). In China, adult ostriches showed infection with *C. muris* (137). In the Czech Republic, a novel genotype of *Cryptosporidium* (Eurasian woodcock) was molecularly identified, and it caused proventriculus and death of Eurasian woodcock species of birds (138).

The life cycle of *Cryptosporidium*

The shed oocysts are usually present in high numbers, resist the environmental conditions, and do not require special conditions for maturation. Infection with infective oocysts through ingestion is followed by excitation in the small intestine with the release of sporozoites. Released sporozoites proliferate in the intestinal epithelial cells where the asexual multiplication phase begins. As a result of asexual multiplication, invasion of merozoites to the neighboring cells is followed by the sexual multiplication phase. After this stage, production of macrogamonts and microgamonts occurs, followed by fertilization of macrogamonts and production of oocysts that sporulate in the host before shedding. The sporulated oocysts containing four sporozoites are shed in feces and respiratory secretions (especially in birds and children) in case of respiratory cryptosporidiosis (139, 140).

Laboratory diagnosis

Various methods including microscopic, immunological, and molecular techniques are used for the detection of *Cryptosporidium* infection (Figure 1). Microscopic examination includes concentration flotation and sedimentation techniques. Fecal samples that were concentrated using Sheather's sugar flotation technique followed by bright-field microscopy showed overall infections rates of *Cryptosporidium* of 3.4% in broilers and 10.6% in layer chickens in China (28). Iodine-saline wet mount method is another method for microscopic examination. However, routine fecal examination methods have some disadvantages like difficulties in distinguishing *Cryptosporidium* oocysts from other small debris particles, molds, algae, or yeasts (141).

Ziehl-Neelsen staining of the fecal smears, bursae of Fabricius, or respiratory organs of broilers revealed infection rates of *Cryptosporidium* of 18.7% in Scotland (16), 24.3% in Greece (142), 37% in Morocco (143), and 4.5% in Tunisia (144). In acid-fast stained smears, *Cryptosporidium* oocysts appear as pink to red and spherical to ovoid bodies with a blue or purple background. This technique is useful in smears with a high number of oocysts or even a low number until one oocyst and can be stored permanently for a long time (145). For the detection of oocysts in smears, the sensitivity was 67.5% for modified acid-fast staining and 53.75% for Giemsa staining (146). In Japan, the histological examination of tissues detected 36.8% of *Cryptosporidium* in broilers and 33.3% in layers (147). However, in Iran, this rate was 23.8% in broilers (148).

Flow cytometry is also used for the demonstration of *Cryptosporidium* after staining with fluorescent markers (149).

Cryptosporidium antigens can be detected through many immuno-chromatographic dipstick tests, enzyme immunoassays, reverse passive hemagglutination tests, and immunofluorescence

techniques (150-152). Some Egyptian studies used enzyme linked immunosorbent assay (ELISA) for the detection of antibodies against *Cryptosporidium* species in humans. For example, Gabr et al. (146) noted that the sensitivity of ELISA was 90% for the detection of cryptosporidiosis. In addition, Hassanein et al. (153) used this assay for the demonstration of *C. parvum* immunoglobulin G in the serum of Egyptian children with persistent diarrhea and acute lymphoblastic leukemia. Nanogold-beads-based ELISA was used for the detection of *Cryptosporidium* in the stool samples of diarrheic patients in Benha Province, Egypt. However, in an animal study, Fereig et al. (154) reported marked sero-prevalence of *C. parvum* in cattle in South Egypt using ELISA. A similar study was recently conducted in Japan, where the indirect ELISA showed total sero-positivity of 96.3% for *C. parvum* in cattle (155).

Unfortunately, the most common diagnostic traditional tools such as microscopy and immunology cannot discriminate *Cryptosporidium* species or subtypes to understand the transmission pathways and dynamics in humans (156). In addition, these tools have a much lower sensitivity than polymerase chain reaction (PCR) (17, 18). Accordingly, genotyping and subtyping of *Cryptosporidium* were done using restriction fragment length polymorphism (RFLP) analysis of the 18S rRNA gene (157, 158) and *Cryptosporidium* oocyst wall protein (COWP) gene (159) and sequence analysis of the 60-kDa glycoprotein gene (160). There are differences in the prevalence and infection rates according to the method used to detect *Cryptosporidium* (e.g., microscopic examination vs. PCR) (161, 156). The first molecular report regarding the genotyping and subtyping of *Cryptosporidium* in calves in Kafr El Sheikh Province, Egypt, was made by Amer et al. (162). They sequenced the SSU rDNA gene and COWP gene of *C. parvum* as well as the high polymorphic 60-kDa glycoprotein gene. In Beni-Suef Province, Egypt, Ibrahim et al. (163) identified *C. parvum* in cattle and buffaloes as well as *C. hominis* and *C. parvum* in humans using RFLP analysis of the COWP fragments. Moreover, the sequence analysis of the gp60 gene showed the *C. parvum* IIdA20G1 subtype in animals and humans. Helmy et al. (18) demonstrated through molecular techniques that the prevalence rate of cryptosporidiosis among 165 diarrheal children in Ismailia Province was 49.1% (60.5% *C. hominis* and 38.3% *C. parvum*). Other molecular techniques such as fluorescence in situ hybridization (FISH) and loop-mediated isothermal amplification (LAMP), a nucleic acid amplification method, are used for the molecular detection of *Cryptosporidium* species (164, 165).

Few studies were done on different avian species regarding the molecular characterization of *Cryptosporidium* (36, 43, 55, 28, 15, 24). In Algeria, the PCR analysis of the intestine showed prevalence rates of *C. meleagridis* of 34% (26/90) and 44% (25/57) in the intestines of chickens and turkeys, respectively (24). However, *C. parvum* DNA was detected in feces in 86% of the chickens in Brazil using PCR (15). In Germany, Helmy et al. (20) demonstrated that the PCR analysis of chickens and turkeys' fecal specimens revealed an overall 7.0% prevalence rate of *C. parvum*, while in China, the prevalence was 10% in 90-day-old broiler chickens (25).

Zoonotic importance

Cryptosporidium species in humans are regarded as one of the most infectious zoonotic protozoan parasites since 1976 during an outbreak of cryptosporidiosis in the United States due to contamination of water (166). The different routes of cryptosporidiosis transmission to humans are listed in Table 2.

Humans can be infected with cryptosporidiosis predominantly through the consumption of contaminated food or water with infective oocysts or through direct contact with infected animals (167-169). Amer et al. (19) found that the dominant genotypes IIa and IId of *C. parvum* in Egyptians which were similar to those in contact calves suggests calves can be potential reservoirs of zoonotic cryptosporidiosis. Some outbreaks have been reported among veterinary students as a result of contact with animals (168-171). Birds are considered an important source of infection as they are mechanical disseminators and shedders of oocysts for long distances in the environment (172, 54, 173, 93, 55, 174, 20). Moreover, infection of pigeon handlers with *C. meleagridis* in China has been reported (74). Workers in farms that handle with birds can contaminate water, feed, or litter in the poultry houses with *Cryptosporidium* oocysts of mammalian/human origin (37). Pet birds are also regarded as an important source of infection

to humans (87), but the literature related to this point is very rare. Traveling, living in villages, drinking underground water (lakes, etc.), and contact with animals are risk factors associated with human cryptosporidiosis (18, 175).

Individuals can get infection of the gastrointestinal tract through different *Cryptosporidium* species. Cryptosporidiosis can affect immunocompetent HIV-infected individuals, children, and healthy persons, causing asymptomatic carrier status or severe lethal diarrhea (176). Previously, *C. parvum* in ruminants was the only cause of human cryptosporidiosis for many years (177), while *C. hominis* was not recognized as a separate species till 2002 (178). Nowadays, both *C. parvum* and *C. hominis* cause more than 90% of human infection, while other species or genotypes of animal origin can cause sporadic infection. Meanwhile, *C. meleagridis* is the only avian species with public health concern and causes zoonotic infection of humans (179, 25, 26). Xiao (180) stated that *C. meleagridis* is the third most common species causing a serious public health hazard in humans as it has a wider host spectrum. Two subtypes of *C. meleagridis* detected in AIDS patients were shared by chicken, ducks, and pigeons in the same location in Peru (25, 26). *C. meleagridis* was reported to be similar to *C. parvum* as both were responsible for 10%-20% of human cryptosporidiosis (181-184). In Bangladesh, in urban regions, the prevalence rate of *C. meleagridis* was 13%, which was more than *C. parvum* with a prevalence rate of 2% in children without diarrhea. However, in rural regions, the prevalence rates of *C. meleagridis* and *C. parvum* were 90% and 4%, respectively, causing subclinical cryptosporidiosis (185). Stensvold et al. (186) and Wang et al. (26) demonstrated that the phylogenetic analysis of multiple loci of *C. meleagridis* isolates showed that these isolates may be related to those in humans, and that constitutes evidence of human infection by *C. meleagridis*. Furthermore, in Sweden, *C. meleagridis* isolates of layer and broiler chickens were found to be identical in nucleotide sequences (18S rRNA and HSP-70 genes) to isolates of human origin (91). Perhaps, this may be due to the anthropozoonotic nature of cryptosporidiosis as it is transmitted from chickens kept in households to the person in contact (187). It is important to note that a reverse zoonotic transmission (zooanthroponosis, from humans to animals) of *C. parvum* has also been reported (188). In addition, cryptosporidiosis is regarded as a largely anthroponotic disease that is transmitted from person to person, especially *C. hominis* or *C. parvum* (189). Cryptosporidiosis in humans is regarded as an acute life-threating disease especially in immunocompetent hosts (179). It is characterized by the sudden onset of clinical signs a week after infection in the form of prolonged and persistent diarrhea. Severe infection can be seen in very young, malnourished, and immunocompromised persons. It has been documented that cryptosporidiosis is a major cause of mortality in infants and children under two years of age

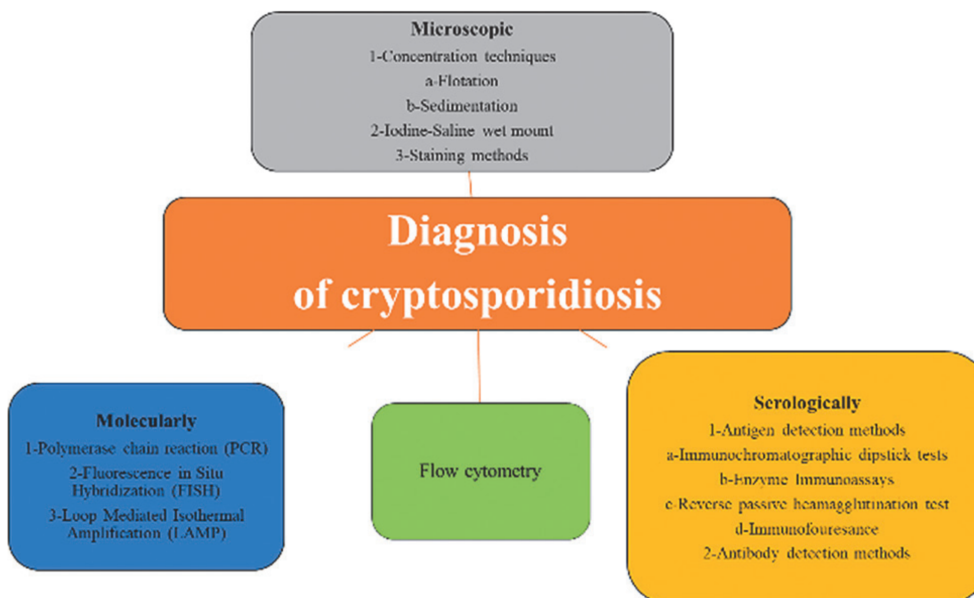

Figure 1: Methods of diagnosis of cryptosporidiosis

Table 2: Transmission methods of cryptosporidiosis in humans in developing countries (190).

Source of infection	Reference (s)
Food and water contamination	[168, 169]
Livestock animals	[20, 167]
Calves	[19, 170, 171, 177]
Domestic poultry (chickens and turkeys)	[20, 25, 26, 37, 93, 172, 179, 187]
Pigeons	[70, 74]
Geese	[54]
Captive pet birds	[55, 87]
Free range birds (mallard duck, graylag goose, common merganser, mute swan, grey heron, white stork, carrion crow, and rook)	[93]
Aquatic birds (ducks, geese, coots, and cormorants)	[174]

In Egypt, the prevalence rate of *Cryptosporidium* infection varies according to the locality. For example, Gabr et al. (191) demonstrated that *Cryptosporidium* infection was prevalent in diarrheic persons in Minia Province, as they showed that the overall prevalence rate of infection in 300 stained fecal samples was 44.7%. However, a previous study conducted by Gabr et al. (146) found that the prevalence rate was 61%. In immunocompetent and immunosuppressed children in Minia Province, the prevalence rates of *Cryptosporidium* infection were 42.2% and 60.2%, respectively (192). In another study conducted in Ismailia Province, the overall prevalence rate of *Cryptosporidium* species in humans was 49.1%, of which 60.5% were C. *hominis*, 38.2% were *C. parvum*, and 1.2% were C. parvum and *C. bovis* (193). In other provinces of Egypt, the prevalence rates were 23.5% in Abou El-Rish Hospital using nanogold-beads-based ELISA (194), 31.1% in Greater Cairo after staining of stool samples (195), 19.5% in Benha using microscopical and immunological techniques (196), 15% in Zagazig among chronic renal failure patients after conducting microscopical staining examination (197), and 33.3% in Ismailia among children (198). Moreover, ELISA results revealed that the incidences of *Cryptosporidium* were 37.7% and 91% in immunodeficient children and adult patients with cancer, respectively (199). In addition, the study of Antonios et al. (200) revealed that the prevalence rate of *Cryptosporidium* species among immunocompromised Egyptians was 33.3%.

The differences in the infection rates may depend on the immune status of individuals, age of the host, environmental habitats, season, sample size, and the virulence of the parasite strain (201).

Prevention and control

Elimination of *Cryptosporidium* infection through the destruction of the parasite is difficult perhaps due to the resistant and persistent nature of the oocysts as well as the wide distribution of the infection (202). Household hygiene practices are recommended to prevent transmission of the different *Cryptosporidium* species causing infection with cryptosporidiosis (203). Hygienic measures include regular and thorough cleaning and disinfection of birds' drinkers, feeders, cages, and premises, in addition to keeping separate cloths during contact with birds and washing hands with disinfectants before and after contact with the birds (67, 9, 135). A concentration of 6% or 7.5% hydrogen peroxide, chlorine dioxide, ozone, and ultraviolet light have been found to inactivate oocysts (204). Moreover, aluminum sulfate, iron sulfate, and iron chloride can coagulate oocysts present in water (205). In addition, reverse osmosis, filtration, and electronic/radiation methods have been used to counteract oocysts (206, 207). Halofuginone showed variable efficiency against cryptosporidiosis in animals (208, 23, 209). Lately, Hassan et al. (210) demonstrated the effect of silver nanoparticles as a water disinfectant against *C. parvum*, as oocysts showed considerable resistance to the traditional water treatment processes. Silver nanoparticles in a concentration of 1 ppm for 30 min and 0.1 ppm for 1 hr reduced oocysts by 97.2% and 94.4%, respectively.

Until now, there is no specific treatment of cryptosporidiosis in animals and humans. However, the United States Food and Drug Adminis-

tration (FDA) licensed and approved treatment of the infection with nitazoxanide after a trial conducted on mice (211). Nitazoxanide treated diarrhea in buffaloes within three to four days and reduced the shedding of oocysts (212). In humans, this treatment is recommended especially for patients aged one year or older in good health condition and with immune status (213, 214). Treatment with other drugs such as paromomycin, spiramycin, rifaximin, and azithromycin is unsatisfactory or inconsistent (215). Nutritional and supportive therapy is very important for complete recovery. Oral fluids and electrolyte replacement using sodium, potassium, bicarbonate, and glucose as well as starch-based oral rehydration solutions can provide calories with lower osmolarity, which can help in restoring mucosal function, enhance immune responses, and help rehabilitate the intestinal mucosal barrier following mucosal injury.

Due to the development of drug resistance, there is a demand to search for alternatives to control cryptosporidiosis. Some studies were conducted to evaluate the effect of using different herbs and their extracts on controlling cryptosporidiosis (216). Oil extracts can be effective in the complete elimination of oocyst shedding on the ninth day postinfection (217). The anti-cryptosporidiosis effects of pine-bark, garlic, onion, cinnamon, blueberry, and curcumin extracts showed successful results (218-222, 212). In chickens, Wahba et al. (219) demonstrated that garlic extract induced a mild reduction in *C. baileyi* oocyst shedding in experimentally infected birds, but there was a significant difference with control non-treated birds. However, both garlic and nitazoxanide completely eradicated *Cryptosporidium* oocyst shedding in the treated buffaloes (212). Moreover, black seeds or black cumin (Nigella sativa) can be used for the treatment of *C. parvum* in experimentally infected calves (223). Zaki and El-Amir (224) stated that phenyl vinyl sulfone (cysteine protease inhibitor) with black seeds altered *Cryptosporidium* oocyst shedding compared with paromomycin treatment in mice. Another recent Egyptian study conducted by Sadek et al. (225) showed that both garlic and black seed extracts were greatly effective in reducing *Cryptosporidium* oocyst excretions in mice (75.4%) compared with treatment with nitazoxanide. Interestingly, after the treatment of cryptosporidiosis HIV patients with high doses of garlic, some patients showed recovery from chronic diarrhea and complete healing (226).

In conclusion, cryptosporidiosis is considered an important disease in animals and birds as it induces severe economic loses, in addition to the public health significance of the disease in humans. Therefore, several studies, especially in developing countries, should be conducted on cryptosporidiosis infection in various hosts and its relation with humans.

Conflict of interests

No conflict of interests is declared.

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KRIPTOSPORIDIOZA PTIC: POMEMBNA PARAZITSKA BOLEZEN, NEVARNA ZA JAVNO ZDRAVJE

W. A. A. El-Ghany

Izvleček: Kriptosporidioza je ena najpomembnejših zoonotskih parazitskih bolezni, ki prizadene številne gostiteljske vrste. Bolezen je razširjena po vsem svetu. Vrste *Cryptosporidium* lahko prizadenejo različne ptičje gostitelje in povzročijo veliko gospodarsko škodo. Resnost simptomov kriptosporidioze pri pticah je različna, od asimptomatične bolezni do hudih črevesnih in/ali dihalnih znakov z visoko smrtnostjo. Diagnoza okužbe s parazitom *Cryptosporidium* temelji predvsem na mikroskopskem odkrivanju oocist, seroloških metodah ali molekularnih tehnikah za identifikacijo različnih vrst povzročitelja. Ljudje in živali so zelo dovzetni za okužbo z različnimi vrstami *Cryptosporidium*, ki so posledica zaužitja kontaminirane hrane ali vode z oocistami ali neposrednega stika z okuženimi gostitelji. Za preprečevanje in nadzor bolezni pri okuženih živalih, pticah in ljudeh ali v njihovi okolici so bile uporabljene različne strategije. Namen tega preglednega članka je bil zato razjasniti vrste povzročiteljev in razširjenost kriptosporidioze ptic, dovzetnost za okužbo in način prenosa ter klinično sliko, laboratorijsko diagnostiko, zoonotski pomen in strategije preprečevanja in nadzora bolezni.

Ključne besede: ptičji; *Cryptosporidium*; diagnoza; človeški

Slov Vet Res 2022; 59 (1): 31–45
DOI 10.26873/SVR-1150-2022
UDC 636.7.09:616-007.43-089:616-036

Original Research Article

ANTIOXIDANT EFFECT OF *Buchholzia coriacea* ETHANOL LEAF-EXTRACT AND FRACTIONS ON FREUND'S ADJUVANT-INDUCED ARTHRITIS IN ALBINO RATS: A COMPARATIVE STUDY

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Abstract: Several studies have implicated reactive oxygen species in perpetuation of inflammation and subse-quent destruction of joints in patients with arthritis. Antioxidant effects of *Buchholzia coriacea* ethanol leaf-extract, aqueous and ethyl acetate fractions on oxidative stress indices in rheumatoid arthritic female Wistar albino rats were examined. 180 rats were randomly divided into 12 groups. Fifteen rats were placed in each group. Rats without arthritis were placed in Group 1. Rheumatoid arthritis was induced in groups 2 to 12 by intradermal injection of 0.1 ml complete Freund's adjuvant into the left hind paws of rats. Group 2 (arthritic rats) did not receive any treatment but rather were given normal saline while group 3 (arthritic rats) received 5 mg/kg indomethacin™ (a standard drug). Rats in Groups 4 to 12 were administered the samples at doses of 200, 400 and 800 mg/kg body weight. Freund's ad-juvant administration led to inflammation and oxidative stress which were marked by significant ($P<0.05$) increase in paw sizes, oxidative stress markers and reduced body weight of the rats. Arthritic rats were treated with standard drug and samples (at varied doses) and this resulted to reversal of the trend of those parameters in a time and dose-dependent manner. Rats that received 800 mg/kg of the aqueous fraction displayed the best desirable result which was similar to the effect of indomethacin. Thus, *Buchholzia coriacea* ethanol extract and fractions may be useful in the management of oxidative stress which is very common among individuals with rheumatoid arthritis.

Key words: rheumatoid arthritis; *Buchholzia coriacea*; free radicals; oxidative stress; reactive oxygen species

Introduction

Rheumatoid arthritis (RA) is an autoimmune disorder. In individuals with autoimmune disorders, their immune system attack joints and some other tissues (1). Patients with these disorders have antibodies in their blood that target their own body tissues, resulting to inflammation (2). RA is a systemic (body-wide) disease, involving many organs of the body (3). Symptoms of RA include morning stiffness, joint pain, limited range of motion in the affected joints, fatigue, occasional redness, fever, firm bumps (nodules) under the skin and symptoms are always worse in the morning (4). RA is the most common rheumatic disease, affecting about 1 % of individuals world-wide (5). Male to female prevalence is 1:3 (6). Increased risk in family members of patients with RA may suggest that it can be hereditary (7). Increased risk among smokers and women may justify the role of environmental pollution and hormonal factors at perpetuating the inflammatory process and joint destruction (8).

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Received: 16 June 2020
Accepted for publication: 18 February 2022

is paramount. This is because free radicals can be deposited into joint cartilage, attacking its proteoglycan and hence inhibits its synthesis (9).

RA is diagnosed by measurement of acute phase reactants, full blood count, and auto-antibody such as rheumatoid factor and anti-cyclic citrullinated peptide assay (14).

The purpose of RA treatments is to inhibit disease severity, reduce symptoms and delay the onset of joint damage and other associated functional limitations (15).

Some RA medications include non-steroidal anti-inflammatory agents, steroids and anti-tumor necrosis factor therapy (2, 3). Though these drugs reduce pain but they are unable to repair damaged tissues. They are mainly used for managing the pain and slowing the progression of RA. Therefore, there is no known drug for curing RA completely (16).

About 80 % of world's population depends on use of various plant parts in the prevention and treatment of diseases (17, 18). The adverse reaction and toxicity associated with the use of anti-inflammatory drugs, in addition to the high cost of the drugs have expeditiously promoted the use of natural plant products or procedures to manage RA locally (19).

Buchholzia coriacea (B. coriacea) is in the family of *Capparidaceae* (20, 21). It is commonly called Wonderful kola, Musk tree, Cola pime, and Elephant cola. It has multiple medicinal values. It is useful in treatment of hypertension and also prevents premature aging. Its methanol seed extract has hypoglycemic, hypolipidemic, anti-lipid peroxidation and anti-ulcer effects (22, 23, 24, 25, 26, 27). It has anti-microbial, antihelmintic and antifungal properties (28, 29, 30, 31).

B. *coriacea* has been in use for quite some time by rural dwellers but there is scarce information on its antioxidant potentials. Therefore, this study was aimed at investigating the effect of *B. coriacea* ethanol leaf extract and fractions on oxidative stress markers in Freund's adjuvant-induced arthritis in albino rats

Material and methods

Materials

Chemicals and reagents used were of analytical standard. Freund's adjuvant was purchased from Sigma Aldrich Company, USA.

Biological materials: Biological materials used for this study were *B. coriacea* leaves and female Wistar albino rats.

Methods

Collection and Identification of Biological Materials: Leaves of *B. coriacea* were collected from Ngodo Village in Afikpo North Local Government Area of Ebonyi State, South-Eastern Nigeria. *B. coriacea* leaf was identified by a Taxonomist in Department of Applied Biology, Ebonyi State University, Abakaliki. Female albino rats (Wistar strain) weighing 121–146 g were obtained from the Department of Animal Science, University of Nigeria, Nsukka, Enugu. The rats were acclimatized for a period of two weeks in the Animal House of Divine Analytical Laboratory, Nsukka under standard laboratory conditions and fed with commercial rat feed and were allowed free access to clean water. The study was approved by the Departmental Institutional Ethical Committee of Biochemistry Department, Ebonyi State University Abakaliki, Nigeria with the Ethical approval number: EBSU/BCH/ET/ 19/010. The guidelines agree with world standard for care and use of laboratory animals in research.

Preparation of extract and fractions: *B. coriacea* leaves were washed and shade dried and later pulverized in a grinder and sifted to obtain powdered sample. Eight hundred grammes of the sample were soaked in 2000 ml of ethanol for 48 hours with intermittent rocking. Thereafter, it was filtered and filtrate was dried. The extracts were stored in airtight container. The dried crude ethanol leaf extract was fractionated in a glass column by eluting in succession with 500 ml water, 500 ml ethyl acetate to obtain aqueous and ethyl acetate fractions, respectively. The crude ethanol leaf extract, aqueous and ethyl acetate fractions were subsequently used for other analyses.

Acute Toxicity Study: Modified Lorke (32) method was used in acute toxicity study. The modification was in the use of albino rats instead of mice. This is because the animal model of this work is albino rats. Thirty-six (36) rats were used for the acute toxicity test. The rats were weighed and fasted overnight before the acute toxicity testing. They were assigned to two experimental groups A and B. Group A which had four rats and served as the normal control group was

administered normal saline. The B group received crude ethanol leaf-extracts of *B. coriacea*. Group B animals were further sub-divided into eight groups with each group having four rats. The sub-groups (B_1-B_8) were administered orally with *B. coriacea* ethanol leaf-extracts at 200, 400, 800, 1200, 1800, 2000, 3000 and 5000 mg/Kg body weight, respectively. All the experimental rats were allowed to have access to food and water and were observed for a 24 hours period for possible signs of toxicity and possible death.

Induction of arthritis in albino rats: Pearson method (33) was employed in induction of arthritis by intradermal injection of 0.1 ml Complete Freund's adjuvant (CFA) into the left hind paws of rats in groups 2 to 12, according to their body weights. The paw sizes of all rats were checked with the aid of vernier caliper and this was done before and after administration of the adjuvant. It was observed that by day 10, arthritis had completely set in.

Rats and experimental groups: Female albino rats (Wistar) were used in this study and a total of 180 rats were utilized. Rats were distributed into 12 groups comprising fifteen rats in each group. The study lasted for 31 days and route of administration of extract and fractions was via oral intubation. The rats were grouped as follows: Group 1 comprises of non-arthritic rats that were given normal saline (1 ml/kg) while Group 2 are arthritic rats that received normal saline (1 ml/kg). Arthritic rats treated with 5 mg/kg indomethacine™ (standard drug) were placed in Group 3 and this served as standard control. Rats in Groups 4-6 are arthritic rats treated with 200, 400 and 800 mg/kg body weight of *B. coriacea* ethanol leaf-extract, respectively. Arthritic rats administered with 200, 400 and 800 mg/kg body weight of aqueous fraction of the crude ethanol leaf extract of *B. coriacea* were placed in Groups 7-9, respectively. Groups 10-12 were arthritic rats treated with 200, 400 and 800 mg/kg body weight of ethyl acetate fraction of ethanol leaf extract of *B. coriacea.* Administration of drugs and samples was through oral intubation.

Determination of Body Weight and Paw Size: The changes in body weight and paw size were measured before and after adjuvant-induced arthritis with the aid of a weighing balance and vernier caliper, respectively. This was done daily. However, records of 10th, 17th, 24th and 31st day of study were used in data analysis.

Collection of Blood Samples for Biochemical Evaluation: Blood samples were humanely collected from three rats each from the groups by cardiac puncture via cervical dislocation on days 10, 17, 24, and 31 into plain sterile bottles. Thereafter, the blood samples were centrifuged at 3,000 rpm for 15 min and serum obtained for biochemical studies.

Determination of Oxidative stress indices: Malondialdehyde (MDA) was evaluated spectrophotometrically by measuring thiobarbituric acid reactive substance (TBARS) as outlined by Wallin *et al.* (34). Nitric oxide (NO) concentration was determined following the procedure described by Bories and Bories (35) based on the Griess reaction in which nitrite is reacted with sulfanilamide (diazotizing reagent) and N-(1-naphthyl) ethylendiamine (a coupling reagent) to produce an azo dye. NO being a labile compound with a brief half-life is rapidly converted to nitrite and nitrate, in oxygenated aqueous solutions. Nitrite levels were measured after the enzymatic reduction of nitrate to nitrite with nitrate reductase. Reduced glutathione (GSH) was assessed following the procedure outlined by Ellman (36). The mechanism was based on the fact that thiols react with Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid) or DTNB), cleaving the disulfide bond to give 2-nitro-5-thiobenzoate (TNB^-), that in turn ionizes to the TNB^{2-} dianion in water at neutral and alkaline pH. The concentration of Tocopherol was determined according to the method described by Desai (37). The activity of superoxide dismutase (SOD) was determined as outlined by McCord and Fridovich (38) based on the production super-oxide radicals produced by xanthine and xanthine oxidase, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. Catalase was spectrophotometrically determined using the method explained by Sinha (39).

The activity of glutathione peroxidase was assayed as outlined by Paglia and Valentine (40) based on the fact that GPX catalyzed the oxidation of glutathione by cumene hydroperoxide. In presence of the glutathione reductase and nicotinamide adenine dinucleotide phosphate (NADPH), the oxidized glutathione was immediately converted to the reduced form with concomitant oxidation of NADPH to $NADP^+$.

Statistical analysis

All results were expressed as Mean ± Standard deviation (SD) and data were subjected to one-way analyses of variance (ANOVA) with Duncan multiple range test for assessment of significant differences between means. A significance threshold of P<0.05 was taken for the analyses. Data were analyzed using IBM statistical package for social sciences (IBM-SPSS), version 20 (IBM, Corp., Atlanta, GA). Value of (P<0.05) was considered statistically significant.

Results

Acute toxicity study

The extracts were subjected to acute toxicity study in Wistar albino rats and the rats were monitored for 24 hours. No mortality was recorded and this revealed that the extracts were not toxic even at a high dose of 5,000 mg/kg. This formed the basis of our dose selection for the study.

Effect of Ethanol leaf-extract, Aqueous and Ethyl acetate Leaf Fractions of B. coriacea on Body weight and Paw Size of Adjuvant-induced Arthritic Rats

A significant (P<0.05) increase in body weight of rats in the treated groups was observed relative to negative control as shown in Table 1. There were significant (P<0.05) differences in body weight of rats treated with standard drug (indomethacin) relative to those treated with the extract, aqueous and ethyl acetate fractions. However, rats treated with ethyl acetate fraction had highest increase in body weight.

There was increase in paw size in the feet of rats injected with Freund's adjuvant. A significant (P<0.05) reduction in paw size of rats treated with crude ethanol leaf-extract, aqueous and ethyl acetate leaf fractions of *B. coriacea* at 200, 400 and 800 mg/kg body weight, relative to normal control was observed. The effect was both dose and time-dependent, as shown in Table 2. There were significant (P<0.05) differences in paw size of rats in all the treated groups. Maximum reduction of paw size with aqueous and ethyl acetate fractions at 200 mg/kg and 800mg/kg on day 31, respectively, occurred, relative to normal control. This effect was similar to that of the standard drug.

Effect of Ethanol leaf-extract, Aqueous and Ethyl acetate leaf fractions of B. coriacea on Oxidative Stress Indices in Adjuvant-induced Arthritic Rats

The results showed that the levels of MDA and NO increased in RA-induced rats relative to normal control as shown Tables 3 and 4, respectively. Treatment with varied doses of the ethanol extract and fractions of the sample at 200, 400 and 800 mg/kg body weight significantly (P<0.05) reduced the levels of MDA and NO in a time-dependent manner. This reduction was comparable to that of indomethacin™. Treatment with aqueous fraction at doses 200 and 400 mg/kg on day 31 yielded the highest reduction in the level of NO, and this was better than the effect of the indomethacin™. Activities of SOD, CAT and GPx were lowered in adjuvant-induced arthritic rats (Tables 5-7), respectively. Treatment with standard drug and varied doses of the ethanol extract, aqueous and ethyl acetate fractions at 200, 400 and 800 mg/kg body weight caused a significant (P<0.05) increase in the activities of SOD, CAT and GPx, likewise an increase in the level of reduced GSH. The observed rise in the activity of SOD during treatment was time-dependent but there was no difference among the treated groups. Aqueous and ethyl acetate fractions at 800 mg/kg on day 31 yielded a significantly (P<0.05) higher value in the activity of catalase and this effect was better than that of standard drug. Treatment with ethanol extract and aqueous fraction on days 17 and 24 yielded highest increase in the activity of GPx, when compared with indomethacin™ and ethyl acetate fraction. Treatment with aqueous fraction produced the highest increase in the level of GSH especially on day 31.

Table 1: Effect of Ethanol Leaf-extracts, Aqueous and Ethyl acetate Fractions of *B. coriacea* on Body Weight (g) of Adjuvant-induced Arthritic Rats

Groups	Day 10	Day 17	Day 24	Day 31
1	145.25 ± 9.53^{a}	146.33 ± 7.57^{a}	147.33 ± 0.58^{a}	147.00 ± 5.29^{a}
2	145.67 ± 8.72^{a}	140.33 ± 4.04^{q}	132.67 ± 5.13^{e}	131.75 ± 3.79^{e}
3	127.17 ± 3.86^{i}	131.00 ± 7.00^{g}	134.00 ± 3.46^{c}	135.00 ± 2.00^{c}
4	125.17 ± 3.33^{k}	127.00 ± 2.65^{i}	132.00 ± 2.65^{e}	136.33 ± 2.08^{b}
5	133.00 ± 3.46^{d}	136.67 ± 4.04^{b}	137.67 ± 1.53^{b}	138.67 ± 2.52^{b}
6	127.50 ± 2.28^{i}	131.67 ± 3.06^{f}	133.00 ± 3.00^{d}	136.00 ± 3.00^{b}
7	126.67 ± 2.57^{k}	130.67 ± 3.06^{h}	136.00 ± 2.65^{b}	132.33 ± 1.53^{e}
8	132.83 ± 4.09^{e}	135.67 ± 1.53^{b}	138.00 ± 2.65^{b}	139.67 ± 3.51^{b}
9	131.92 ± 2.39^{f}	137.00 ± 1.00^{b}	138.33 ± 1.53^{b}	142.00 ± 1.00^{q}
10	121.08 ± 2.94^{m}	122.67 ± 2.52^{l}	131.67 ± 2.08^{f}	134.33 ± 1.53^{c}
11	123.08 ± 2.75^{l}	127.67 ± 1.15^{i}	132.33 ± 4.93^{e}	135.67 ± 5.03^{b}
12	124.50 ± 2.47^{l}	127.33 ± 3.06^{i}	132.33 ± 3.21^{e}	136.00 ± 2.65^{b}

The degree of significance was set at P<0.05. Means (on the same column and row) with different superscripts are significantly different at P<0.05. This implies that comparison was done on both rows (point of sampling) and columns (among experimental groups). Values are mean ± standard deviation of 3 results obtained from 3 rats (n=3). BCE=*B. coriacea* Crude Ethanol extract, BCA=*B. coriacea* Aqueous fraction, BCZ=*B. coriacea* Ethyl acetate fraction. 1=normal control, 2= positive control, 3= standard control, 4=200mg/Kg BCE, 5=400mg/Kg BCE, 6=800mg/Kg BCE, 7=200mg/Kg BCA, 8=400mg/Kg BCA, 9=800mg/Kg BCA, 10=200mg/Kg BCZ, 11=400mg/Kg BCZ, 12=800mg/kg BCZ

Table 2: Effect of Ethanol Leaf-extract, Aqueous and Ethyl acetate Fractions of *B. coriacea* on Paw size (mm) of Adjuvant-induced Arthritic Rats

Groups	Before induction	Day 10	Day 17	Day 24	Day 31
1	2.14 ±0.02^{a}	2.14 ± 0.01^{a}	2.14 ± 0.02^{a}	2.14 ±0.01^{a}	2.14 ± 0.02^{a}
2	2.20 ±0.03^{a}	5.39 ±0.20^{d}	6.59 ±0.22^{q}	7.33 ±0.07^{x}	7.78 ± 0.04^{z}
3	2.14 ±0.07^{a}	4.75 ±0.55^{e}	3.89 ±0.59^{g}	2.86 ±0.32^{gh}	2.16 ± 0.22^{a}
4	2.16 ±0.02^{a}	5.60 ±0.20^{cd}	4.53 ±0.42^{def}	3.48 ±0.28^{efg}	3.01 ± 0.14^{bc}
5	2.14 ±0.03^{a}	5.61 ±0.30^{cd}	4.47 ±0.32^{def}	4.00 ±0.22^{bcd}	3.30 ± 0.35^{b}
6	2.21 ±0.04^{a}	5.57 ±0.33^{cd}	4.44 ±0.27^{def}	3.44 ±0.28^{fg}	3.00 ± 0.45^{bc}
7	2.16 ±0.02^{a}	5.80 ±0.20^{bc}	4.48 ±0.38^{def}	3.36 ±0.30^{g}	2.16 ± 0.34^{a}
8	2.15 ±0.02^{a}	5.73 ±0.24^{bc}	4.64 ±0.23^{cdef}	4.13 ± 0.10^{b}	3.34 ± 0.28^{b}
9	2.16 ±0.03^{a}	5.97 ±0.40^{ab}	5.03 ±0.67^{b}	3.66 ±0.48^{efg}	3.25 ± 0.27^{b}
10	2.14 ±0.03^{a}	5.68 ±0.32^{c}	4.70 ±0.26^{bcde}	3.73 ±0.34^{def}	3.10 ±0.23^{bc}
11	2.20 ±0.04^{a}	5.68 ±0.16^{c}	4.26 ±0.42^{f}	3.80 ±0.43^{cde}	3.08 ±0.41^{bc}
12	2.16 ±0.03^{a}	5.70 ±0.40^{ac}	4.33 ±0.55^{ef}	3.53 ±0.40^{efg}	2.16 ± 0.44^{a}

The degree of significance was set at P<0.05. Means (on the same column and row) with different superscripts are significantly different at P<0.05. This implies that comparison was done on both rows (point of sampling) and columns (among experimental groups). Values are the mean ± standard deviation of 3 results obtained from 3 rats (n=3). BCE=*B. coriacea* Crude Ethanol extract, BCA=*B. coriacea* Aqueous leaf fraction, BCZ=*B. coriacea* Ethyl acetate leaf fraction. 1=normal control, 2=positive control, 3=standard control, 4=200 mg/Kg BCE, 5=400 mg/Kg BCE, 6=800 mg/Kg BCE, 7=200 mg/Kg BCA, 8=400 mg/Kg BCA, 9=800 mg/Kg BCA, 10=200 mg/Kg BCZ, 11=400 mg/Kg BCZ, 12=800 mg/Kg BCZ

Table 3: Effect of Ethanol leaf-extract, Aqueous and Ethyl acetate leaf fractions of *B. coriacea on* Malondialdehyde (MDA) level (Nmol/g) of Adjuvant-induced Arthritic Rats

Groups	Day 10	Day 17	Day 24	Day 31
1	6.60 ± 0.27^{a}	6.81 ± 0.19^{a}	6.61 ± 0.58^{a}	6.62 ± 0.42^{a}
2	6.62 ± 0.40^{a}	6.83 ± 0.55^{a}	7.70 ± 0.54^{a}	8.27 ± 0.52^{a}
3	6.68 ± 0.34^{a}	6.20 ± 0.66^{b}	5.68 ± 0.25^{h}	5.70 ± 0.25^{c}
4	6.11 ± 0.61^{a}	6.03 ± 0.83^{b}	5.84 ± 0.08^{b}	5.33 ± 0.30^{k}
5	6.29 ± 0.65^{a}	6.43 ± 0.41^{a}	5.74 ± 0.14^{c}	5.57 ± 0.47^{f}
6	6.35 ± 0.20^{a}	5.68 ± 0.24^{h}	5.70 ± 0.17^{c}	5.54 ± 0.23^{g}
7	6.50 ± 0.34^{a}	5.89 ± 0.16^{b}	5.83 ± 0.11^{b}	5.55 ± 0.17^{f}
8	6.82 ± 0.37^{a}	6.08 ± 0.28^{b}	5.92 ± 0.20^{b}	5.48 ± 0.08^{i}
9	6.93 ± 0.15^{a}	5.76 ± 0.13^{c}	5.80 ± 0.12^{b}	5.59 ± 0.10^{f}
10	6.62 ± 0.43^{a}	5.71 ± 0.13^{h}	5.80 ± 0.07^{b}	5.38 ± 0.23^{j}
11	6.76 ± 0.13^{a}	5.77 ± 0.03^{c}	5.74 ± 0.04^{c}	5.67 ± 0.27^{d}
12	6.66 ± 0.09^{a}	5.80 ± 0.29^{b}	5.71 ± 0.11^{c}	5.52 ± 0.21^{h}

The degree of significance was set at $P<0.05$. Means (on the same column and row) with different superscripts are significantly different at $P<0.05$. This implies that comparison was done on both rows (point of sampling) and columns (among experimental groups). Values are the mean ± standard deviation of 3 results obtained from 3 rats (n=3). BCE=*B. coriacea* Crude Ethanol extract, BCA=*B. coriacea* Aqueous fraction, BCZ=*B. coriacea* Ethyl acetate fraction. 1=normal control, 2=positive control, 3=standard control, 4=200 mg/kg BCE, 5=400 mg/kg BCE, 6=800 mg/kg BCE, 7=200 mg/kg BCA, 8=400 mg/kg BCA, 9=800 mg/kg BCA, 10=200 mg/kg BCZ, 11=400 mg/kg BCZ, 12=800 mg/kg BCZ

Table 4: Effect of Ethanol leaf-extract, Aqueous and Ethyl acetate leaf fractions of *B. coriacea on* Nitric oxide (NO) level (Nmol/ml) of Adjuvant-induced Arthritic Rats

Groups	Day 10	Day 17	Day 24	Day 31
1	20.32 ± 1.02^{a}	19.78 ± 0.68^{a}	20.35 ± 0.07^{a}	19.21 ± 0.91^{b}
2	17.41 ± 0.95^{e}	18.23 ± 1.00^{c}	19.42 ± 0.15^{b}	22.01 ± 0.07^{a}
3	19.02 ± 2.10^{b}	18.65 ± 0.48^{c}	17.92 ± 1.13^{d}	17.91 ± 2.20^{d}
4	18.71 ± 0.48^{c}	18.38 ± 0.65^{c}	18.35 ± 0.45^{c}	16.81 ± 0.47^{h}
5	18.45 ± 1.54^{c}	18.31 ± 0.82^{c}	18.39 ± 0.46^{c}	16.63 ± 0.59^{i}
6	18.88 ± 0.46^{d}	18.19 ± 1.08^{c}	17.61 ± 0.43^{c}	16.45 ± 0.16^{j}
7	18.79 ± 0.27^{b}	18.66 ± 0.38^{c}	17.99 ± 0.53^{d}	16.34 ± 0,58^{k}
8	18.41 ± 0.55^{c}	18.31 ± 0.83^{c}	17.86 ±0.70^{d}	16.95 ± 0.27^{g}
9	17.92 ± 0.36^{d}	17.72 ± 0.94^{d}	17.91 ± 0.87^{d}	17.86 ± 0.21^{d}
10	18.64 ± 0.38^{c}	18.30 ± 0.42^{c}	17.98 ± 0.67^{d}	16.72 ± 0.50^{h}
11	18.19 ± 1.70^{c}	18.28 ± 0.86^{c}	17.89 ± 1.59^{d}	17.05 ± 0.36^{f}
12	20.97 ± 0.35^{a}	18.34 ± 9.42^{c}	18.11 ± 1.18^{c}	18.14 ± 0.43^{c}

The degree of significance was set at $P<0.05$. Means (on the same column and row) with different superscripts are significantly different at $P<0.05$. This implies that comparison was done on both rows (point of sampling) and columns (among experimental groups). Values are the mean ± standard deviation of 3 results obtained from 3 rats (n=3). BCE=*B. coriacea* Crude Ethanol extract, BCA=*B. coriacea* Aqueous fraction, BCZ=*B. coriacea* Ethyl acetate fraction. 1=normal control, 2=positive control, 3=standard control, 4=200 mg/kg BCE, 5=400 mg/kg BCE, 6=800 mg/kg BCE, 7=200 mg/kg BCA, 8=400 mg/kg BCA, 9=800 mg/kg BCA, 10=200 mg/kg BCZ, 11=400 mg/kg BCZ, 12=800 mg/kg BCZ

Table 5: Effect of Ethanol leaf-extract, Aqueous and Ethyl acetate leaf fractions of *B. coriacea* on SOD activity (u/mg) of Adjuvant-induced Arthritic Rats

Groups	Day 10	Day 17	Day 24	Day 31
1	23.10 ± 0.98^a	23.11 ± 2.08^a	23.11 ± 1.29^a	23.21 ± 1.91^a
2	22.25 ± 1.67^c	20.72 ± 0.57^d	19.50 ± 0.79^e	18.47 ± 1.22^e
3	22.23 ± 0.92^c	23.07 ± 0.56^a	24.56 ± 1.16^a	26.00 ± 0.48^a
4	21.91 ± 1.01^c	22.56 ± 1.04^c	22.80 ± 0.45^b	24.21 ± 0.70^a
5	20.91 ± 1.93^d	21.96 ± 0.77^c	21.41 ± 0.52^c	25.21 ± 0.43^a
6	22.13 ± 1.51^c	21.51 ± 0.18^c	21.64 ± 0.83^d	22.88 ± 0.73^b
7	22.16 ± 0.22^c	22.53 ± 1.41^c	23.42 ± 1.36^a	25.88 ± 0.72^a
8	21.14 ± 1.17^d	21.67 ± 0.75^c	22.30 ± 0.38^d	23.48 ± 0.23^a
9	21.88 ± 0.66^c	22.20 ± 1.09^c	24.21 ± 0.56^a	26.17 ± 0.32^a
10	21.44 ± 1.54^d	22.17 ± 0.14^c	24.28 ± 0.70^a	26.41 ± 1.09^a
11	20.91 ± 1.61^d	21.55 ± 0.89^d	23.41 ± 0.16^a	26.48 ± 0.42^a
12	20.47 ± 1.18^d	20.63 ± 0.09^d	23.03 ± 1.65^a	25.28 ± 0.26^a

The degree of significance was set at P<0.05. Means (on the same column and row) with different superscripts are significantly different at P<0.05. This implies that comparison was done on both rows (point of sampling) and columns (among experimental groups). Values are the mean ± standard deviation of 3 results obtained from 3 rats (n=3). BCE=*B. coriacea* Crude Ethanol extract, BCA=*B. coriacea* Aqueous fraction, BCZ=*B. coriacea* Ethyl acetate fraction. 1=normal control, 2=positive control, 3=standard control, 4=200 mg/kg BCE, 5=400 mg/kg BCE, 6=800 mg/kg BCE, 7=200 mg/kg BCA, 8=400 mg/kg BCA, 9=800 mg/kg BCA, 10=200 mg/kg BCZ, 11=400 mg/kg BCZ, 12=800 mg/kg BCZ

Table 6: Effect of Ethanol leaf-extract, Aqueous and Ethyl acetate leaf fractions of *B. coriacea* on Catalase activity (u/mg) of Adjuvant-induced Arthritic Rats

Groups	Day 10	Day 17	Day 24	Day 31
1	67.67 ± 3.02^a	67.84 ± 1.26^a	66.52 ± 2.03^a	66.63 ± 5.57^a
2	58.49 ± 2.73^c	55.79 ± 0.41^d	52.21 ± 4.34^f	45.62 ± 3.16^g
3	57.48 ± 2.46^d	59.21 ± 1.14^b	58.42 ± 2.66^c	61.60 ± 1.41^a
4	53.25 ± 2.77^f	54.19 ± 1.66^e	57.31 ± 0.80^d	59.84 ± 1.12^a
5	54.59 ± 3.25^d	55.63 ± 2.35^d	57.92 ± 4.32^d	59.66 ± 3.22^a
6	55.16 ± 2.51^d	56.04 ± 1.10^d	55.68 ± 1.25^d	60.44 ± 0.63^a
7	55.44 ± 2.20^d	57.57 ± 0.74^d	57.58 ± 2.16^d	57.99 ± 2.41^d
8	55.56 ± 3.13^d	57.52 ± 2.52^d	56.52 ± 1.50^d	58.21 ± 2.28^c
9	55.59 ± 0.54^d	55.32 ± 2.38^d	56.56 ± 4.36^d	62.14 ± 1.55^a
10	53.90 ± 3.64^e	55.95 ± 0.30^d	55.61 ± 3.06^d	59.65 ± 1.96^a
11	54.20 ± 2.74^e	54.61 ± 1.32^d	56.24 ± 0.97^d	59.35 ± 4.88^a
12	57.25 ± 2.84^d	57.30 ± 2.39^d	58.97 ± 2.54^c	59.81 ± 1.76^a

The degree of significance was set at P<0.05. Means (on the same column and row) with different superscripts are significantly different at P<0.05. This implies that comparison was done on both rows (point of sampling) and columns (among experimental groups). Values are the mean ± standard deviation of 3 results obtained from 3 rats (n=3). BCE=*B. coriacea* Crude Ethanol extract, BCA=*B. coriacea* Aqueous fraction, BCZ=*B. coriacea* Ethyl acetate fraction. 1=normal control, 2=positive control, 3=standard control, 4=200 mg/kg BCE, 5=400 mg/kg BCE, 6=800 mg/kg BCE, 7=200 mg/kg BCA, 8=400 mg/kg BCA, 9=800 mg/kg BCA, 10=200 mg/kg BCZ, 11=400 mg/kg BCZ, 12=800 mg/kg BCZ

Table 7: Effect of Ethanol leaf-extract, Aqueous and Ethyl acetate leaf fractions of *B. coriacea* on glutathione peroxidase (GPx) activity (u/l)) of Adjuvant-induced Arthritic Rats

Groups	Day 10	Day 17	Day 24	Day 31
1	23.64 ± 1.18[b]	24.37 ± 0.72[a]	24.43 ± 2.66[a]	23.89 ± 0.24[b]
2	21.61 ± 2.16[b]	20.76 ± 0.41[d]	19.51 ± 2.70[e]	18.21 ± 0.66[f]
3	20.43 ± 0.69[c]	21.38 ± 0.51[b]	22.26 ± 2.47[b]	22.31 ± 0.85[b]
4	20.63 ± 0.42[c]	21.42 ± 1.11[b]	22.31 ± 1.04[b]	21.58 ± 0.17[b]
5	19.82 ± 1.23[e]	20.71 ± 0.56[c]	21.83 ± 1.02[b]	21.55 ± 0.59[b]
6	20.19 ± 0.21[d]	20.63 ± 0.66[c]	21.16 ± 0.38[b]	22.05 ± 1.21[b]
7	20.73 ± 1.02[c]	21.07 ± 0.55[b]	21.96 ± 0.62[b]	22.21 ± 0.39[b]
8	20.91 ± 0.64[b]	21.08 ± 0.54[b]	21.30 ± 0.52[b]	22.09 ± 0.61[b]
9	20.15 ± 0.98[d]	21.23 ± 0.46[b]	21.52 ± 1.42[b]	22.84 ± 0.98[b]
10	21.23 ± 0.78[b]	20.51 ± 0.18[c]	21.64 ± 1.35[b]	21.64 ± 0.90[b]
11	21.72 ± 1.67[b]	20.76 ± 0.55[c]	21.26 ± 0.13[b]	22.70 ± 1.06[b]
12	21.31 ± 0.62[b]	21.04 ± 0.47[b]	21.36 ± 0.80[b]	22.50 ± 0.59[b]

The degree of significance was set at P<0.05. Means (on the same column and row) with different superscripts are significantly different at P<0.05. This implies that comparison was done on both rows (point of sampling) and columns (among experimental groups). Values are the mean ± standard deviation of 3 results obtained from 3 rats (n=3). BCE=*B. coriacea* Crude Ethanol extract, BCA=*B. coriacea* Aqueous fraction, BCZ=*B. coriacea* Ethyl acetate fraction. 1=normal control, 2=positive control, 3=standard control, 4=200 mg/kg BCE, 5=400 mg/kg BCE, 6=800 mg/kg BCE, 7=200 mg/kg BCA, 8=400 mg/kg BCA, 9=800 mg/kg BCA, 10=200 mg/kg BCZ, 11=400 mg/kg BCZ, 12=800 mg/kg BCZ

Table 8: Effect of Ethanol leaf-extract, Aqueous and Ethyl acetate leaf fractions of *B. coriacea* on reduced glutathione (GSH) level (Umol/l) of Adjuvant-induced Arthritic Rats

Groups	Day 10	Day 17	Day 24	Day 31
1	26.45±2.37[a]	26.82±0.59[a]	26.760±0.03[a]	25.99±0.08[a]
2	22.88±0.71[d]	19.18±0.62[h]	16.36±0.18[i]	10.17±1.43[d]
3	18.57±0.86[h]	21.99±0.39[f]	22.03±0.41[e]	22.38±0.76[c]
4	17.09±0.24[i]	20.42±0.50[g]	23.80±0.59[c]	24.82±1.21[a]
5	15.60±0.25[j]	19.31±0.33[h]	22.94±0.58[d]	25.54±0.33[a]
6	15.91±0.61[j]	20.12±1.54[g]	22.34±1.80[d]	25.62±0.70[a]
7	15.26±0.55[e]	22.53±0.34[d]	23.54±0.40[c]	24.51±0.57[a]
8	16.27±0.97[i]	22.72±0.32[d]	22.85±0.14[d]	25.16±1.46[a]
9	16.27±0.20[i]	21.77±0.89[f]	24.02±0.38[b]	25.12±0.88[a]
10	15.46±0.66[j]	23.22±1.48[c]	25.14±0.15[a]	25.48±0.53[a]
11	15.22±0.43[j]	22.40±1.63[d]	22.95±0.73[d]	23.70±1.11[b]
12	15.29±0.90[j]	21.91±1.03[f]	23.60±0.70[c]	24.36±0.65[a]

The degree of significance was set at P<0.05. Means (on the same column and row) with different superscripts are significantly different at P<0.05. This implies that comparison was done on both rows (point of sampling) and columns (among experimental groups). Values are the mean ± standard deviation of 3 results obtained from 3 rats (n=3). BCE=*B. coriacea* Crude Ethanol extract, BCA=*B. coriacea* Aqueous fraction, BCZ=*B. coriacea* Ethyl acetate fraction. 1=normal control, 2=positive control, 3=standard control, 4=200 mg/kg BCE, 5=400 mg/kg BCE, 6=800 mg/kg BCE, 7=200 mg/kg BCA, 8=400 mg/kg BCA, 9=800 mg/kg BCA, 10=200 mg/kg BCZ, 11=400 mg/kg BCZ, 12=800 mg/kg BCZ

Discussion

In the first week following induction of arthritis, all adjuvant-induced arthritic rats showed a significant (P<0.05) decrease in the body weight relative to rats in normal control group. A significant (P<0.05) increase in the body weight of rats was observed in all the treated groups while progressive weight loss was observed in the untreated-arthritic group till the end of the study. Our present results are in agreement with previous studies that showed that Complete Freund's Adjuvant (CFA)-injected rats showed decreases in body weight relative to non-arthritic rats (41). Administration of CFA leads to increase in leptin level, anorexia and weight loss (42). In this study, we speculate that the cause of the decrease in body weight on injection of CFA might be due to increase in leptin level in the arthritic rats. Leptin is a hormone secreted by fat cells and is known for suppressing hunger signals, also has influences on the immune system. Elevated level of leptin contributes to chronic inflammation by up-regulating inflammatory cytokines (like tumor necrosis factor-alpha, TNF-α; interleukins, IL-1β, and IL-6) (43). Elevated levels of pro-inflammatory cytokines could exert a strong effect on protein and energy metabolism by promoting muscle breakdown. Increased catabolism leads to resting energy expenditure culminating to weight loss and reduced lean body mass (44).

Inflammation can also cause a decrease in absorption capacity of the intestine. Elmali *et al.* (2005) (45) reported a restoration of absorption capacity of the intestine upon treatment with anti-inflammatory drugs. Thus, increased body weight of the arthritic rats during the course of treatment with an anti-inflammatory drug (indomethacin) and varied doses of the extract and fractions could be due to the reduction of the inflammatory cytokines and subsequent decrease in protein and muscle breakdown. It could also be due to the restoration of absorption capacity of the intestine.

Edema is one of the fundamental actions of acute inflammation and is an essential parameter to be considered when evaluating compounds with potential anti-inflammatory activity (46, 47). In this study, there was a two-fold increase in paw size in the feet of rats injected with Freund's adjuvant. A significant (P<0.05) reduction in paw size of rats treated with standard drug, ethanol leaf-extract, aqueous and ethyl acetate leaf fractions of *B. coriacea* at varied, was observed. Previous authors have also reported a significant reduction in paw size of rats on treatment with medicinal plants (48, 49).

Oxidative stress is a situation in the biological science in which there is an imbalance between oxidants and antioxidants in favor of the oxidants, culminating in interference of redox signaling and control and/or molecular injury (50). In normal physiological processes, reactive oxygen species (ROS) are formed and they play crucial functions in cell signaling and tissue homeostasis (51). Nevertheless, their excessive production culminates in severe alterations to cell components and augment various pathogenesis, such as lipids, proteins, and DNA damage (52). Sequel to high level of polyunsaturated fatty acids (PUFAs) in cellular membranes or organelle membrane, they are prone to ROS damage, which is referred to as lipid peroxidation. Thus, lipid peroxidation is a process that involves the removal of electrons from lipids by free radical species such as oxyl radicals, peroxyl radicals, and hydroxyl radicals leading subsequently to the production of reactive intermediates that can undergo further reactions. This causes damage to phospholipids and function as cell death signal which induces programmed cell death. Hence, oxidized phospholipid mediates important function in several inflammatory disorders and frequently mediate proinflammatory alteration (53).

There exist three major classes of antioxidant enzymes in all body cells which include the catalases, superoxide dismutases (SOD), and glutathione peroxidases (GPX). These enzymes play vital functions in cells' homeostasis. Their induction is a reflection of specific response to pollutant oxidative stress (54). These enzymic and non-enzymic antioxidants such as glutathione (GSH) have the ability to prevent or retard the oxidation of macromolecules. These antioxidants retard or terminate these chain reactions by mopping up free radicals or via inhibition of other oxidation reactions by being oxidized themselves (55).

Chemical composition analysis of *B. coriacea* has been investigated by previous studies. Phytochemicals present in *B. coriacea* ethanol leaf-extract and fractions are terpenoids, phenols, alkaloids, flavonoids, tannins, saponins and steroids (56). Other authors have also corroborated this finding (57, 58, 59, 60, 61). Phytochemicals can act as antioxidants (e.g, flavonoids, alkaloids, tan-

nins, saponins and terpenoids) antiproliferative and anti-inflammatory compounds for prevention of chronic diseases (62, 63).

The results of our study revealed increased oxidative stress in adjuvant-induced arthritic rats as evidenced by increased lipid peroxidation product-MDA and NO production, and impaired enzymatic and non-enzymatic antioxidant defense system of the body (Tables 3- 8). The raised MDA (a consequence of increased extent of lipid peroxidation) might be due to the increased formation of ROS which tends to increase abundantly during chronic inflammation and could result to damage to tissues. Other authors have also reported increase in MDA in liver and brain of rats with arthritis (64, 65, 66). Elevated level of MDA has been found in the serum, plasma and erythrocytes of RA patients (13, 67). Administration of standard drug and samples reduced levels of MDA as shown in Table 3.

Some functions of NO include immune response, neural communication and blood pressure maintenance (68). In this study, increased NO level portends oxidative stress and this corroborates previous findings as reported by other authors (64, 13, 67). However, Veselinovic *et al. (*69) reported unaltered level of NO in the plasma of RA patients. The increase in the level of NO in the plasma of arthritic rats might be due to the hyperactivity of the NO forming enzyme, nitric oxide synthase (70). The observed reduced SOD activity might be due to its depletion caused by increased oxidative stress that occur during inflammatory process.

This decreased erythrocyte SOD activity is in agreement with other studies as well (13, 71, 72, 73, 74, 75). However, increased (69, 76), or even unaltered SOD activity (77) has also been reported by some groups.

Catalase is an enzyme that catalyses the conversion of hydrogen peroxide into water and oxygen. This protects cells from harmful effects that accumulated hydrogen peroxide could have caused. From our result, catalase activity is lowered in arthritic rats and this might be as a result of catalase being used up by hydrogen peroxide.

Diminished catalase activity in brain and liver of arthritic rats have been reported by previous authors (65, 66). This result is in tandem with other findings using human beings (54, 13, 71, 74). However, some groups have also reported unaltered catalase activity in RA patients (64, 75, 78, 72).

We also observed a significant decrease in GPx activity in the adjuvant-induced arthritic rats (Table 7). This is in line with the findings of other studies using humans (79, 73, 72). However, two studies reported an increase on GPx activity in RA patients (80, 76). However, three study groups did not report any differences between cases and controls in GPx activity in RA patients (75, 78, 74).

We noticed significantly low levels of GSH in arthritic rats as compared to the control rats (healthy rats) (Table 8). GSH is a sulfhydryl molecule that acts as a defense system in the body. It functions as an intracellular reductant in redox reactions taking place in the human body by protecting cellular components from damage caused by ROS. The observed low level of GSH in plasma of arthritic rats has also been reported by previous authors (64, 75, 79, 74, 81, 82, 13). Veselinovic *et al.* (69) reported that GSH level was unchanged in RA patients. This contradicting report by various authors could be due to differences in RA severity and response to treatments by rats/or patients.

ROS are highly reactive chemical species that have the potential to damage lipids, proteins and deoxyribonucleic acid (DNA) in joint tissues. ROS are required in maintaining redox state of cells. Other functions include cell signaling, differentiation, proliferation, growth, apoptosis and phagocytosis. However, if the concentrations of ROS are increased beyond physiological conditions they can damage macromolecules like lipids in the cell membranes, proteins and nucleic acids (83, 81). Oxidative stress results if the concentration of oxidants is higher than antioxidants (84). Under this condition, redox signaling is disrupted leading to macromolecular damage (85, 86, 87). The damaging ROS is annulled by the action of antioxidants. Enzymatic antioxidant response is carried out by SOD, catalase, GPx, glutathione reductase and transferase while non-enzymatic antioxidant includes the action of vitamins (e.g. A, C, and E), β-carotene, some minerals (e.g. copper, zinc, manganese, and selenium), GSH and some phytochemicals (e.g. flavonoids, terpenoids, alkaloids) (85, 64).

A large number of authors reported that ROS are implicated in inflammation and destruction in the joints of arthritic animals and RA patients (64, 88, 13, 85, 89, 90, 91). Oxidative stress is implicated in damaging of joints due to RA (87). There is strong evidence that ROS are highly involved in cartilage degradation in experimental arthritic rats (92).

Oxidative stress can also impair DNA mismatch repair mechanism which may result to an increase in the formation of DNA adducts in the joints thereby aggravating the disease symptoms (93). Phytochemicals serve as sources of natural compound for developing novel drugs. This is because of their antioxidant and anti-inflammatory roles in management of inflammatory diseases such as RA. The presence of antioxidant minerals (e.g., copper, zinc, manganese, and selenium) and vitamins (A, C and E) in *B. coriacea* is well-documented (56, 94). Thus, the anti-RA effect exerted by the samples could be attributed to the action of these antioxidants present in the plant. However, limitation of this study is the small number of rats sampled at each point.

Conclusion

Induction of RA caused oxidative stress evidenced by elevation of MDA and NO and diminished activities of antioxidants: SOD, GPx, Catalase and GSH. Administration of samples to rats led to decrease in oxidative stress and boosting of the antioxidants. This study has demonstrated *in vivo* the therapeutic potential of leaf extract and ethyl acetate fraction of *B. coriacea* at curbing oxidative stress and hence a potential alternative to synthetic drugs in the management of oxidative stress in RA patient.

Ethics approval and consent to participate was adequately sought.

Acknowledgements

Authors acknowledge the management of Divine Analytical Laboratory, Nsukka, Nigeria, where this research was carried out.

Consent for publication: All authors granted consent for this work to be published. Availability of data and material: Adequate. Competing interests: None. Funding: None.

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ANTIOKSIDATIVNI UČINEK ETANOLNEGA IZVLEČKA IN FRAKCIJ LISTOV *Buchholzia coriacea* NA FREUNDOV ADJUVANTNI ARTRITIS PRI ALBINO PODGANAH: PRIMERJALNA ŠTUDIJA

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Izvleček: V več študijah je bil nakazan pomen reaktivnih kisikovih zvrsti pri ohranjanju vnetja in poznejšem uničenju sklepov pri pacientih z osteoartritisom. Proučevali smo antioksidantivne učinke etanolovega izvlečka ter vodnih in etil acetatnih frakcij listov *Buchholzia coriacea* na kazalce oksidativnega stresa pri samicah albino podgan Wistar z revmatoidnih artritisom. 180 podgan smo naključno porazdelili v 12 skupin. V vsaki skupini je bilo 15 podgan. Podgane brez artritisa so bile uvrščene v skupino 1. V skupinah 2 do 12 so bile podgane z revmatoidnim artritisom, povzročenim z intradermalno injekcijo 0,1 ml Freundovega kompletnega adjuvansa v zadnjo levo taco podgan. Skupina 2 (podgane z artritisom) ni bila zdravljena, prejela je fiziološko raztopino, skupina 3 (podgane z artritisom) pa je prejela 5 mg/kg indometacina™ (standardno zdravilo). Podgane v skupinah 4 do 12 so prejele vzorce adjuvansa v odmerkih 200, 400 in 800 mg/kg telesne mase. Aplikacija Freundovega adjuvansa je povzročila vnetje in oksidativni stres, kar se je kazalo v značilnem ($p<0.05$) povišanju velikosti tac in kazalcev oksidativnega stresa ter zmanjšanju telesne teže podgan. Podgane z artritisom so bile zdravljene s standardnim zdravilom in vzorci adjuvansa (v različnih odmerkih), kar je vodilo v obrat trenda teh parametrov v odvisnosti od časa in odmerka. Najboljši rezultat, podoben učinku indometacina, je bil pri podganah, ki so prejele 800 mg/kg vodne frakcije adjuvansa. Etanolni izvleček in frakcije listov *Buchholzia coriacea* bi zato lahko bili uporabni pri obvladovanju oksidativnega stresa, ki se zelo pogosto pojavlja pri posameznikih z revmatoidnim artritisom.

Ključne besede: revmatoidni artritis; *Buchholzia coriacea*; prosti radikali; oksidativni stres; reaktivne kisikove zvrsti

Slov Vet Res 2022; 59 (1): 47–57
DOI 10.26873/SVR-1295-2022
UDC 636.7.09:616-007.43-089:616-036

Original Research Article

HERNIORRHAPHY AND SURGICAL OUTCOMES OF DIAPHRAG-MATIC HERNIA IN CATS

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Abstract: The aim of this study was to evaluate the surgical outcome and mortality of diaphragmatic hernia in cats and present herniorrhaphy results. Diagnostic and surgical data from 25 cats with diaphragmatic hernias were analyzed retrospectively. The cats were between the ages of 2 months and 4 years, included 9 cases of males (5/9 neutered) and 16 cases of females (9/16 spayed). Acute cases were 12/25 and chronic cases were 13/25. Diaphragmatic rupture locations were defined according to the points that are determined based on a clockwise scale. Location was classified as multiple in 10 (40 %), ventral in 7 (28 %), right in 5 (20 %), and left in 3 (12 %) cases. The three most frequently herniated organs were small intestine (20, 80 %), liver (16, 64 %), and omentum (15, 60 %). Postoperative survival rates were 83.3 % for acute cases and 69.2 % for chronic. Full recovery was achieved in 19 (76 %) of the cats. Results suggest that the location and size of diaphragm ruptures, the organs herniated, and the presence of concurrent pathologies directly affect mortality. Mortality is higher in cases with concurrent injuries, large diaphragmatic ruptures, and excessively herniated organs.

Key words: prognosis; feline diseases; hernia; rupture; survival

Introduction

A diaphragmatic hernia occurs when organs in the abdominal cavity pass into the thoracic cavity due to an abnormal opening occurring in the diaphragm (1). Acquired diaphragmatic hernias may occur as a result of blunt trauma such as traffic accidents or falling from a high-rise (1, 2). The most common clinical finding is dyspnea (3). Other clinical findings vary depending on the herniated organs and other factors.

According to the guidance of clinical findings, the most important and simple method of diaphragmatic hernia detection is radiography. Plain radiography shows loss of the diaphragmatic border, shadowing of the heart, increased density of the thorax, and gas deposits of the stomach or small intestine if herniated into the thoracic cavity (4). In some cases, if plain radiography does not ensure a diagnosis with a clear diaphragmatic border, contrast radiography or ultrasonography may be necessary (4, 5).

In the treatment of diaphragmatic hernias, many factors before, during, and after surgery affect the survival rate. It is a disease with a high risk of mortality because of leading significant disruptions in systems such as breathing and

Received: 4 February 2021
Accepted for publication: 21 January 2022

digestion that directly affect the metabolism (3, 6, 7). The aim of this study, was to provide retrospective evaluations of diaphragmatic hernia in cats, according to stage of the disease, the size and localization of the diaphragm rupture, the relationship between herniated organs and other concurrent pathologies, surgical outcome, and mortality of the disease.

Material and methods

Medical records of the Hatay Mustafa Kemal University College of Veterinary Medicine (Turkey) between January 2015 and January 2020 were searched for diaphragmatic hernia cases in cats. Twenty-five cats whose owners accepted surgery and received surgical treatment were included in the study. Three cats whose owners did not accept surgery were excluded from the study. Diagnostic data such as age, gender, clinical findings, and duration of the diaphragmatic hernia were collected. Patients with a period of 14 days or less from the known trauma were considered acute, and others were considered chronic. Causes of diaphragmatic hernia were classified as motor vehicle accidents, falls from high-rise, other traumas, and unknown/trauma suspected.

For a definitive diagnosis, plain radiographs were examined including the thorax and abdomen. In cases where the diagnosis could not be confirmed by plain radiography, iohexol (30–60 mg/kg PO) (Omnipaque® GE Healthcare) was used as a contrast agent for contrast radiography. Before herniorrhaphy, diaphragm rupture lines were recorded by intraoperative determination of the clockwise direction points. According to this definition, which has not been previously given in the literature, the leftmost and most ventral tear-point of the diaphragm in the dorsal recumbency position were determined as the beginning and the rightmost and the most ventral point as the end. Accordingly, the line of tear was specified in a clockwise direction manner (Fig 1). Organs that herniated and other concurrent pathologies were determined and recorded intraoperatively. Descriptive data were reported as range, median, and mean ± SD values.

For the perioperative effect, before surgery, a cefazolin sodium (20 mg/kg IM) was administered as an antibiotic, and meloxicam (0.3 mg/kg SC) as an analgesic. For general anesthesia, sedation was provided with xylazine hydrochloride (1–2 mg/kg IM), and induction was provided with ketamine hydrochloride (10 mg/kg IM) (Ketasol 10 %® İnterhas). Following endotracheal intubation (No. 3–3.5), anesthesia was maintained with isoflurane (1–3 % inhalation)) in 100 % O_2. In all patients, 6–8 mL/kg tidal volume (20–30 times/min, 10–15 cm H_2O) was performed. Positive end-expiratory pressure (PEEP) was 4–8 cm H_2O. A Lactated Ringer's solution (10–15 ml/kg/hr, IV) was used for perioperative fluid management.

Surgical access was provided with ventral midline celiotomy in the dorsal recumbency position. After exploration, the herniated organs were pulled out from the thoracic cavity with careful, slow, and repetitive atraumatic pulling motions. In no case was sternotomy or enlarging of the diaphragmatic rupture required to bring the herniated organs from the thoracic cavity to the abdomen. Diaphragmatic ruptures were repaired with a simple, continuous pattern of sutures made of polyglycolic acid (2/0 or 3/0) multifilament absorbable suture material. Next, the air and fluids remaining in the thorax were aspirated with a cannula and a syringe in order to provide negative pressure. Anesthesia was ended after the incision site was closed routinely. Positive pressure automatic ventilation was terminated and the patient was allowed to breathe spontaneously.

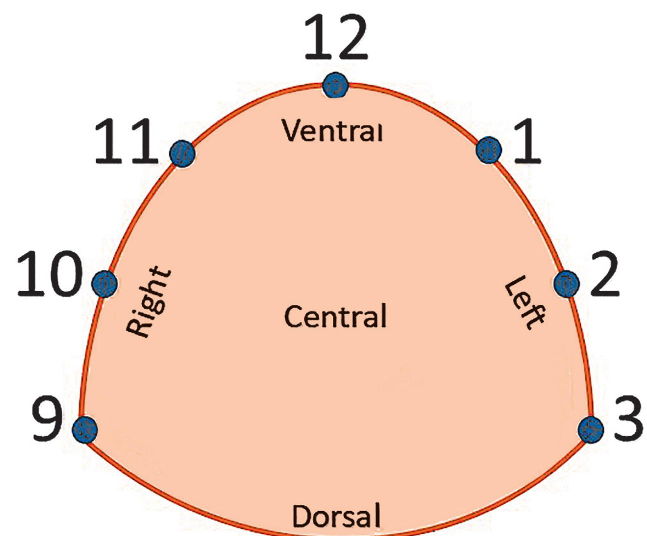

Figure 1: Diagram of the clockwise determination points in diaphragm rupture localization. The animal was assumed to be in a dorsal recumbency position in surgery

The integrity of the diaphragm line and appearance of the thorax and abdomen were checked with postoperative radiographs. For postsurgical analgesia and anti-inflammatory purposes, tolfenamic acid (2–4 mg/kg PO) was given for 5 days as NSAID. In cases with high WBC, cefazolin (20 mg/kg IM) was administered for 7 days postoperatively. For supportive treatment, a supplement containing iron along with cobalt, vitamin B12 and vitamin B3 (1–2 drops / kg PO, Fercobsang, Novakim®, Turkey) was given for 5 days in cases that resulted in low hematocrit and hemoglobin values of complete blood count analysis. Clinical improvements, complications, and survival were recorded for at least 10 days postoperatively.

Results

All of the patients diagnosed with diaphragmatic hernia were domestic shorthair cats. The age range was 2 months to 4 years old (median 7 months). The gender distribution was 16/25 (64 %) females (9/16 spayed) and 9/25 (36 %) males (5/9 neutered). The time between trauma and diagnosis ranged from 3 hours to 5 months (median 7 days, mean 20.7 days, SD±34.4). The stage of the disease was acute in 12 cases (48 %) and chronic in 13 cases (52 %). It was determined that 11 (44 %) of the cases were caused by trauma not seen by the owners, 9 (36 %) by motor vehicle accident, 3 (12 %) by other physical trauma, and 2 (8 %) by falls from a high-rise (Table 1). Typical findings such as dyspnea, tachypnea, open-mouth breathing, exercise intolerance, abdominal breathing, anorexia, and vomiting were seen in clinical examinations.

Table 1: Demographic and diagnostic information of cases

Case No	Sex	Age (Months)	Time[a] (Days)	Cause	Category
1	F	9	150[b]	Unknown /suspected trauma	Chonic
2	F	2	2	Other trauma	Acute
3	F	7	60[b]	Motor vehicle accident	Chronic
4	F	4	14[b]	Unknown /suspected trauma	Chronic
5	F	6	30[b]	Unknown /suspected trauma	Chronic
6	F	2	1	Motor vehicle accident	Acute
7	M	12	0	High-rise fall	Acute
8	F	3	9	Unknown /suspected trauma	Chronic
9	M	10	2	Unknown /suspected trauma	Acute
10	M	10	14[b]	Motor vehicle accident	Chronic
11	F	9	60[b]	Motor vehicle accident	Chronic
12	F	3	14[b]	Unknown /suspected trauma	Chronic
13	F	3	14[b]	Unknown /suspected trauma	Chronic
14	M	48	1	Other trauma	Acute
15	M	24	14[b]	Unknown /suspected trauma	Chronic
16	M	18	90	Motor vehicle accident	Chronic
17	M	12	1	High-rise fall	Acute
18	F	12	14[b]	Motor vehicle accident	Chronic
19	F	2	2	Motor vehicle accident	Acute
20	M	7	1	Unknown /suspected trauma	Acute
21	F	12	5	Other trauma	Chronic
22	F	4	1	Unknown /suspected trauma	Acute
23	F	6	5	Motor vehicle accident	Acute
24	F	6	1	Unknown /suspected trauma	Acute
25	M	4	7	Motor vehicle accident	Acute

F: Female, M: Male, a: From trauma to admission; b: It is known to be more than

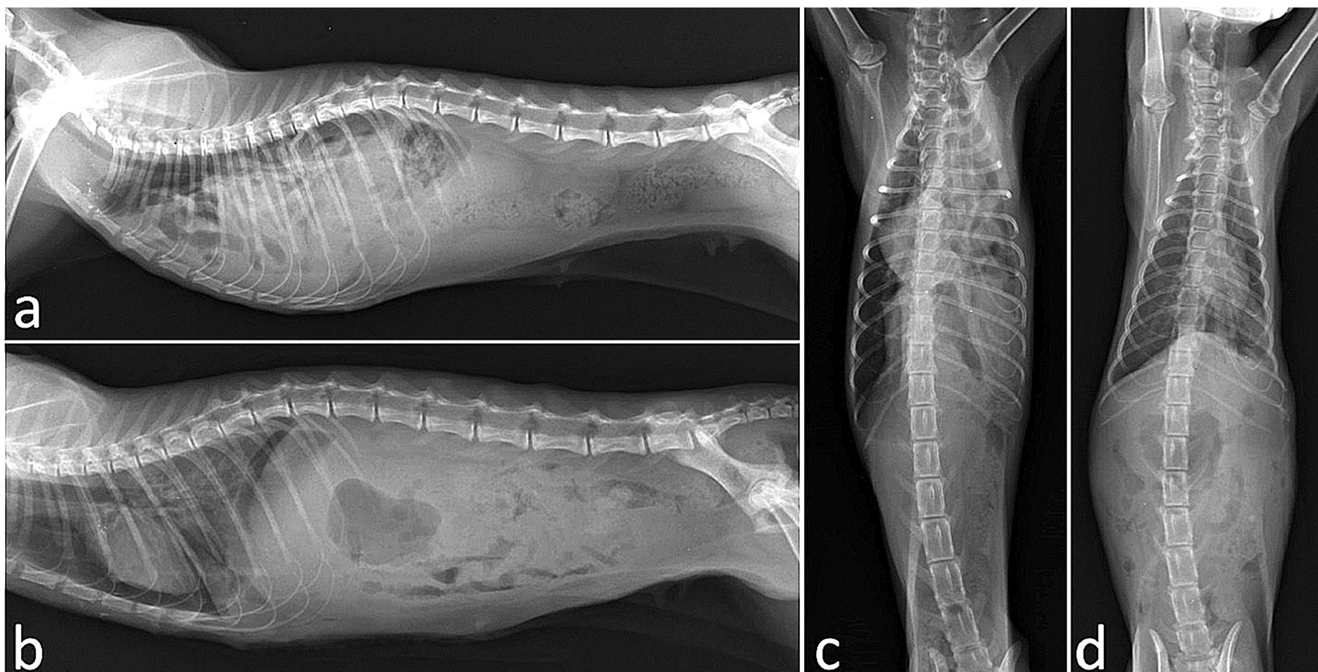

Figure 2: Plain radiographic images of a cat with a diaphragmatic hernia. Preoperative (a, c) and postoperative (b, d) images show the abdominal organs filled in the thorax before surgery, and in their normal position postoperatively

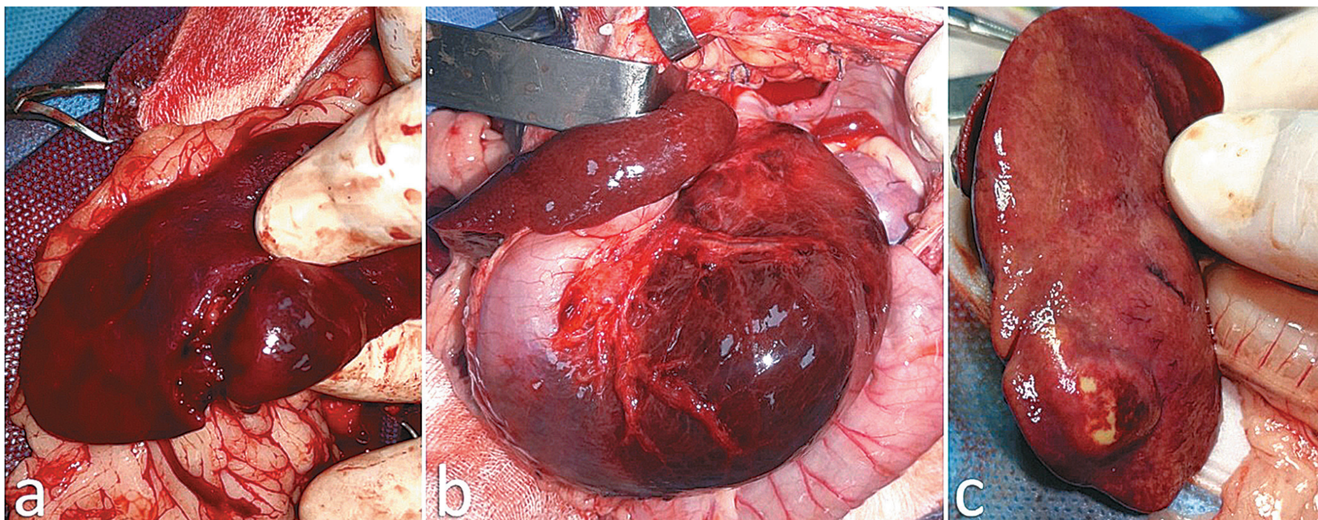

Figure 3: Samples of intraoperative images of organ pathologies associated with diaphragmatic herniated cats: spleen rupture (a); gastric torsion (b); and hepatic abscess (c)

In radiographic examinations, a definitive diagnosis was achieved in 21 cases by plain radiography. In 4 cases where definitive diagnosis could not be determined by plain radiography, the diagnosis was confirmed with contrast (gastrointestinal) radiography. Radiographic examinations revealed that the diaphragm line disappeared, and the intestines, liver, stomach, and other abdominal organs were in the thoracic cavity displaced (Fig 2). Other concurrent disorders or damages were determined by clinical, physical, and radiographic examinations, or during surgery.

In addition to the diaphragmatic hernia, 7 cases of orthopedic damage were detected in 6 cats, and 12 cases soft-tissue damage or pathology in 10 cats (some examples can be seen in Fig 3 and all of concurrent damages/disorders are presented in Table 2). The most common starting points of tearing in the diaphragm were noted towards 9 o'clock in 14 (56 %) cases, 10 o'clock in 5 (20 %) cases, and 12 o'clock in 3 (12 %) cases.

Table 2: Herniated organs and numbers, diaphragm rupture lines (clockwise), concurrent damages, and surgical outcomes of cases

Case No	Organs herniated		Diaphragm rupture line (clockwise)				Concurrent injuries or pathologies	Surgical Outcome
	Organs	Number	Region	Start	End	Width[a]		
1	L, GB, SI, LI	4	Multiple	9	2	5		Survived
2	O, SI	2	Left	1	3	2	Enlarged GB, Femoral F	Survived
3	L, SI, LI	3	Left	12	3	3		Survived
4	O, SI	2	Right	9	12	3		Died (13th day)
5	RK, L (one lobe), O, SI	4	Right	9	11	2	Spleen displaced (to right)	Survived
6	St, O, SI	3	Ventral	10	12	2	Neck (soft tissue) injury	Survived
7	O, Sp	2	Rright	9	12	3	Spleen rupture, Femoral F	Survived
8	St, O, SI	3	Ventral	11	2	3	Hepatic abscess, Femoral F	Survived
9	St, O, SI, LI, Sp	5	Right	9	12	3	Gastric tortion	Survived
10	L, GB, SI, LI	4	Left	12	3	3	Megaesophagus	Died (8th day)
11	L, GB, St, P, Sp, SI, LI	7	Multiple	9	1	4	Splenomegaly	Survived
12	L, St	2	Multiple	9	3	6		Died (on 10th day)
13	St, SI, LI	3	Ventral	10	12	2		Survived
14	L, St, O, SI	4	Ventral	10	1	3	Pelvic F	Survived
15	L, O, SI	3	Multiple	9	1	4		Survived
16	L, St, O, SI, Sp	5	Multiple	11	3	4	Splenomegaly, Enlarged GB	Survived
17	St, O	2	Ventral	12	1	1		Died (in 24 hours)
18	L, GB, O, SI	4	Multiple	9	1	4	SIL, Pelvic MF	Died (in 24 hours)
19	L, St, O, SI	4	Multiple	9	1	4	Abdominal hernia	Survived
20	L, St, Sp	3	Multiple	9	1	4	Spleen rupture	Died (in 3 h)
21	L, GB, SI	3	Multiple	9	2	5		Survived
22	St, O	2	Ventral	10	1	3		Survived
23	L, SI, LI	3	Right	9	12	3		Survived
24	L, O, Sp, SI	4	Ventral	10	1	3		Survived
25	L, SI	2	Multiple	9	3	6		Survived

a: Units were given in hours, L: Liver, GB: Gall bladder, SI: Small intestine, LI: Large intestine, O: Omentum, RK: Right kidney, St: Stomach, Sp: Spleen, P: Pancreas, SIL: Sacroiliac luxation, F: Fracture, MF: Multiple fractures

Table 3: Distribution of herniated organs according to diaphragmatic rupture localization.

Organs	Right	Left	Ventral	Multiple	Total
Small intestines	4/20 (20 %)	3/20 (15 %)	5/20 (25 %)	8/20 (40 %)	20 (80 %)
Liver	2/16 (12,5 %)	2/16 (12,5 %)	2/16 (12,5 %)	10/16 (62,5 %)	16 (64 %)
Omentum	4/15 (26,7 %)	1/15 (6,7 %)	6/15 (40 %)	4/15 (26,7 %)	15 (60 %)
Stomach	1/12 (8,3 %)	-	6/12 (50 %)	5/12 (41,7 %)	12 (48 %)
Large intestines	2/7 (28,6 %)	2/7 (28,6 %)	1/7 (14,3 %)	2/7 (28,6 %)	7 (28 %)
Spleen	2/6 (33,3 %)	-	1/6 (16.7 %)	3/6 (50 %)	6 (24 %)
Gall bladder	-	1/5 (20 %)	-	4/5 (80 %)	5 (20 %)
Pancreas	-	-	1/1 (100 %)	-	1 (4 %)
Kidney (Right)	1/1 (100 %)	-	-	-	1 (4 %)

Table 4: Complete blood count and serum biochemistry results of the cases (N: Normal)

Parameter/ Case No	Referenge Ranges	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9
WBC	5,5-19,5	20,1	19,6	28,5	25,4	22,8	21,7	30,4	27,9	N
Lymphocyte	1-7	7,8	N	12,7	8,9	8,9	10,3	11,8	13,9	N
Monocyte	0,07-1,9	2,4	N	3	2,9	2,5	N	N	N	N
Eosinophils	0-4	N	N	N	5,4	N	N	N	7,2	N
Granulocytes	35-85	N	87,8	N	87,6	N	N	N	N	N
RDW	14-18	N	N	N	N	N	19,9	N	N	N
HGB	9.3-15,3	N	N	N	7,9	8,5	N	7,7	N	N
HCT	28-49	N	N	N	25,9	25,3	N	24,2	N	N
PLT	100-514	N	N	N	85	N	N	N	N	75
ALT	10-101	136	N	245	N	N	N	N	N	458
AST	7-80	87,6	N	95,7	N	N	N	N	N	91,3
GLU	60-150	N	N	N	155	N	N	N	N	172
CRE	0,9-2,20	N	N	N	N	N	N	N	0,8	N
Fe	68-215	N	N	N	54,9	N	N	N	N	N
ALB	2,1-4,6	N	N	N	2	N	N	N	N	N
Ca	8,8-11,7	N	N	N	N	N	N	N	N	N

Parameter/ Case No	Referenge Ranges	Case 10	Case 11	Case 12	Case 13	Case 14	Case 15	Case 16	Case 17
WBC	5,5-19,5	N	20,6	35,8	N	32	N	21,9	35,7
Lymphocyte	1-7	N	9,8	8,9	N	N	8,3	9,3	7,3
Monocyte	0,07-1,9	N	3,1	3,6	N	N	2,5	3,3	2,7
Eosinophils	0-4	N	N	N	10,3	N	N	N	N
Granulocytes	35-85	N	N	95,7	N	N	N	N	N
RDW	14-18	N	N	19,5	N	N	N	N	19,3
HGB	9.3-15,3	N	7,6	7,3	N	8	N	7,6	7,3
HCT	28-49	N	23,9	22,9	N	27,5	N	22,7	22
PLT	100-514	N	N	87,6	N	N	N	N	79,6
ALT	10-101	N	N	129,3	135	322	141	N	155,7
AST	7-80	N	N	98,3	N	209	90,4	N	89,5
GLU	60-150	N	N	N	410	N	N	N	162,4
CRE	0,9-2,20	N	N	0,7	0,9	0,3	N	N	N
Fe	68-215	N	N	53,9	N	61,7	N	N	N
ALB	2,1-4,6	N	N	1,9	N	1,6	N	N	N
Ca	8,8-11,7	N	N	N	N	7,4	7,4	N	N

Parameter/ Case No	Referenge Ranges	Case 18	Case 19	Case 20	Case 21	Case 22	Case 23	Case 24	Case 25
WBC	5,5-19,5	N	22,9	N	25,3	N	19,9	20,1	23,6
Lymphocyte	1-7	N	12,3	N	8,5	7,6	7,4	7,9	8,3
Monocyte	0,07-1,9	2,1	N	N	2,9	N	N	N	N
Eosinophils	0-4	11,1	N	4,7	N	N	N	N	N
Granulocytes	35-85	N	86,7	N	89,5	N	N	N	N
RDW	14-18	22,4	N	N	19,3	N	N	N	N
HGB	9.3-15,3	6,1	8,3	6,1	N	9,1	N	N	N
HCT	28-49	22,9	25,4	21,7	N	25,9	N	N	N
PLT	100-514	72	N	N	85	N	96,2	94,7	95,4
ALT	10-101	N	N	120,9	N	126,3	105,7	N	N
AST	7-80	N	N	89,6	N	N		N	N
GLU	60-150	N	N	N	155,6	N	156,7	N	N
CRE	0,9-2,20	0,9	N	0,8	N	N	N	N	N
Fe	68-215	59,3	N	54,9	N	N	N	N	N
ALB	2,1-4,6	N	N	N	N	N	N	N	N
Ca	8,8-11,7	N	N	N	N	8,5	N	N	N

The most common end points were towards 1 o'clock in 9 (36 %) cases, 12 o'clock in 6 (24 %) cases, and 3 o'clock in 6 (24 %) cases (Table 2). The extent of the diaphragm ruptures was in the range of 1–6 units according to the starting and ending points in a clockwise direction (median 3, mean 3.4, SD±1.2). The distribution of the herniated organs according to diaphragmatic rupture localization is presented in Table 3.

Surgical repair of diaphragmatic hernias resolved clinical signs of tachypnea, dyspnea, exercise intolerance, vomiting, and anorexia in 17/25 (68 %) cats. Respiratory or gastrointestinal tract signs were detected after surgery in 2/25 cats (8 %). Despite all the interventions (intravenous fluid support, postoperative care that supports oxygen and body temperature in the intensive care unit, and cardiopulmonary resuscitation treatment), death occurred due to postanesthetic cardiac arrest in the first 24 hours in 3/25 cases. In addition, 3/25 cats died on the eighth, tenth, and thirteenth days owing to the owners' postoperative care neglect leading to septicemia. Therefore, the perioperative and postoperative mortality rates were both determined as 12 %. Concurrent disruptions such as megaesophagus, spleen rupture, and multiple fractures were determined in these cases. From a complete blood count, WBC and lymphocyte was high in 18 cases, monocyte and eosinophils in 8 cases, and granulocytes and RDW in 5 cases. Low values of HGB and HCT were found in 12 cases, and of PLT in 9 cases. Biochemistry values were high in ALT in 11, AST in 8, and glucose in 6 of the patients. Low values of creatinine were found in 6 cases, of Fe in 5 cases, and of ALB and Ca in 3 cases (The anormal complete blood count and serum biochemistry results are presented in Table 4). Complete recovery was achieved in 19 (76 %) of the 25 cats treated surgically. Survival rates in acute and chronic cases were 83.3 % and 69.2 %, respectively.

Discussion

It has been reported in previous scientific studies that 77–85 % of diaphragmatic hernias in cats are due to trauma (8, 9, 10). In our study, all cases (100 %) were traumatic diaphragmatic hernias, and none were congenital. Various studies have reported that diaphragmatic hernia is more common in male cats, but in our study most (64 %) of the cases were female (9/16 of females were spayed and 5/9 of males were neutered; 21/25 were indoor cats and 4/21 were outdoor) (4, 6, 7, 11, 12, 13). Although the gender of the patients is stated in the studies, sterilization information and life circumstances (indoor/outdoor) seems to be lacking. It has been stated in other studies that contrast gastrointestinal radiographs, celiography, and ultrasonography are also useful (5, 13, 14, 15). Contrast gastrointestinal radiographs are advantageous in that the stomach and intestines are clearly visible in the thorax. However, they may not be sufficient in a situation such as partial obstruction of the gastrointestinal system which prevents the passage of the contrast agent. Positive contrast celiography has the disadvantage of giving false negative results if the defect in the diaphragm is occluded with abdominal organs (5, 15). In the present study, plain and contrast gastrointestinal radiographs were found to be adequate. Clinical and radiographic signs of patients in the present study were similar to those in other reports (1, 6). Recently, researchers have considered diaphragmatic hernia cases that last over 2 weeks as chronic (3, 6).

Many risk factors are known as causes of postoperative surgical site infection under the headings of patient, environmental, and therapeutic factors to be (16, 17). Perioperative use of antibiotics is known to reduce the risk of infection (16, 18). Postoperative antimicrobial use is known to be unnecessary for clean surgeries, but is required when patients show signs of infection in the presence of major risks of breakage in sterility of surgery or other factors (16). However, it has been controversial in surgeries classified as clean-contaminated (17). Eugster et al. (16) reported that, although 84.2 % of 836 cases in their study were surgeries performed under clean or clean-contaminated conditions, postoperative antibiotics were used in 77.5 % of cases. In some studies, it has been reported to be unnecessary except for contaminated and dirty wounds (16). Another study suggests antimicrobial therapy in the surgical patient for decreased short-term morbidity and prevention of devastating infection risk (19). In the present study, perioperative and postoperative antibiotics were used, taking into account WBC in complete blood count analysis. The preference for cefazolin was due to its broad spectrum and the local supply facilities for the patients' owners.

Some authors may resort to additional techniques such as median sternotomy, lateral thoracotomy, or widening of the diaphragmatic rupture during the retracting of herniated organs from the thorax to the abdomen (6, 11, 13, 20, 21). In this study, as in recent studies, the ventral median celiotomy had provided adequate access for the repair of the diaphragmatic rupture (21). However, these additional methods should not be avoided if the organs are likely to be damaged during replacement. In standard herniorrhaphy repairs it is not necessary to place a thoracostomy tube. However, it is necessary if the patient has pneumothorax or respiratory distress, or if there any extra-routine conditions such as sternotomy during herniorrhaphy, damage to the pericardium, or chylothorax in the pleural space. Thoracostomy tube application can be performed for drainage or to give intrapleural analgesics (22). Nevertheless, it is not recommended if not necessary due to its complication risks, increased hospitalization time, and morbidity (23, 24). In our study, no additional technique was performed except for ventral midline celiotomy. Therefore, there was considered to be no need to insert a thoracostomy tube.

The use of polydioxanone, glycomer, polypropylene, polygluconate, polyglycolic acid, and polyglactin 910 has been reported as suture material in diaphragmatic hernias (6, 25, 26, 27). Some authors find the use of polydioxanone and glycomer appropriate and avoid the use of others (26). In some studies, the use of polyglycolic acid and polyglactin 910 suture materials is reported as a suitable choice (1, 25, 27). In this study, the absorption time was considered sufficient for healing. Polyglycolic acid, an absorbable material, was preferred in suture application because of the advantages of being pliable, soft, and easy to handle (28). There was no complication in the results related to breakdown of the integrity of the tear repair.

Diaphragmatic hernia is a disease with a high risk of mortality in cats. Various factors before, during, and after surgery affect the survival rate (3, 6, 7). It has been reported that mortality is higher when there are complications such as pneumothorax, lobe torsion, necrosis of the liver or lung, and strangulation of the intestines during the chronicization process (6, 13). Besalti et al. (6) reported that mortality rates in diaphragmatic hernias in cats are related to the location of the diaphragm rupture as well as the stage of the disease, and mortality is higher in ruptures in the center of the diaphragm. Studies evaluating localization of the diaphragm rupture in diaphragmatic hernias of cats are limited (6, 29). Besalti et al. (6) reported that their study was the first in which localization and mortality were evaluated together. They described the localization of diaphragmatic ruptures as right, left, central, ventral, dorsal, and multiple. In our study, we determined the localization and quantity of ruptures according to the clockwise direction for the first time, which we considered a precise method of determining of the rupture line. It has been reported that in diaphragmatic hernias, the herniation is mostly seen on the right side (4, 6, 12, 30). In our study, herniation was largely (40 %) on both sides. One study has suggested that if the rupture is on the right side, the liver acts as a barrier to prevent herniation (11), however, Besalti et al. (6) stated that this hypothesis is not valid. Our study also weakens the validity of this hypothesis by showing that the liver and other organs accompany diaphragmatic hernias frequently.

In diaphragmatic hernias of cats and dogs, the most herniated organs have been identified as the liver, small intestines, stomach, omentum, spleen, pancreas, and large intestines, respectively (4, 6, 7, 30). In this study, the most herniated organs were identified as the small intestines on the left and right sides, the stomach and omentum on the ventral side, and the liver on multiple-sided ruptures.

Schmiedt et al. (7) suggested in their study that mortality does not have a significant relationship with herniated organs. Besalti et al. (6) noted that among 21 chronic cases, the commonality point of 5 cases that died was herniation of the liver. Nevertheless, in many of the healed cats, the liver is among the herniated organs and could not be directly associated with mortality. However, they stated that the number of herniated organs has a significant effect on mortality (6). In our study, due to the fact that there were 2 or more organs herniated in all cases, a direct correlation between the number of herniated organs and mortality could not be proven. Although there was no statistical evidence in this study, it is suggested based on the authors' observations that impairment of the volume or function of the organ may increase mortality and affect the

surgical outcome. Another study stated that, if the intestines or stomach herniated, undesirable conditions such as motility disorders and vomiting occurred before or after surgery. In addition, many problems such as megaesophagus, heart disease, pulmonary disease, or liver disease can occur in the pre- or postoperative periods (22). These facts supports the hypothesis that complications caused by diaphragmatic herniated organs affect mortality.

In previous studies, it has been stated that prolonged surgical procedures and anesthesia, perioperative oxygen needs, and concurrent injuries are significantly associated with mortality. Mortality rates have been reported to be 4.3 times as high in cases with concurrent soft-tissue injury, and 7.3 times as high if there are soft tissue and orthopedic injuries occur together (3,7). In our study, there were concurrent pathologies in 3 (50 %) of 6 cases in which death occurred. However, 9 (47.37 %) of the 19 recovered cases also had concurrent damage or pathologies. Similarly, in other studies, concurrent injuries did not significantly change the mortality (6, 31). Therefore, the main factor in the increase in mortality is the extent to which concurrent injuries or pathologies. In previous studies, chronic diaphragmatic hernia of cats have been reported with worse surgical outcomes (8). This is because diaphragmatic hernia cases with a history of more than 1 year were considered chronic. In recent studies, diaphragmatic hernia cases have been considered as chronic if patients have had them for 15 days or more (13, 32), thus making the results difficult to compare with the former studies.

Perioperative mortality rates in different studies range from 8–18 % in different studies (7, 31). In our study, perioperative mortality rate were 2/25 of cases as similar to previous studies. The reported postoperative survival rates are between 54 % and 90 % (3, 6, 7, 8, 9, 29, 33, 34). Mortality rates in cats with acute and chronic traumatic diaphragmatic hernia have been reported as 16.1–20 % and 11.8–19 %, respectively (6, 7). In our study, mortality rates in acute and chronic cases were 2/12 and 4/13, respectively. The overall survival rate was 19/25 (10/12 acute, 9/13 chronic). The majority of cases were with a large ruptured diaphragm, which is an important factor that reduces survival rates, but these results are close to those in the aforementioned studies.

In some studies, it has been reported that the risk of mortality increases significantly in patients with diaphragmatic hernia operated on within 24 hours following trauma (8, 33). For this reason, it is recommended to postpone surgery (8). However, because of the development of surgical equipments and care conditions in the over the years, recent studies have suggested that there is no relation between the timing of the surgery and survival (3, 32). Based on this, it has been suggested that preoperative stabilization and perioperative support would be sufficient and the surgery should not be postponed even for 24 hours (3). In our study, only one patient was operated on less than 24 hours after trauma. The surgery was performed on the same day as the proper preoperative stabilization. In spite its concurrent orthopedic and soft-tissue injuries, this patient survived.

In cats with acquired diaphragmatic hernia, the location, size, type, and concurrent injuries and pathologies directly affect the perioperative mortality rate. The risk of mortality is higher in cats with a diaphragmatic hernia in the presence of different concurrent pathologies, orthopedic injuries, or soft-tissue injuries. Further studies are needed to investigate the surgical outcomes and prognostic factors with detailed measurements in diaphragmatic hernias of cats and dogs.

Acknowledgments

The authors would like to thank Dr. Halil Alakuş and Dr. Ömer Kirgiz for their help in the clinical work.

The authors declare that there are no conflicts of interest.

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HERNIORAFIJA IN KIRURŠKI IZIDI DIAFRAGMALNE KILE PRI MAČKAH

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Izvleček: Namen te študije je bil ovrednotiti kirurški izid in smrtnost diafragmalne kile pri mačkah ter predstaviti rezultate herniorafije. Retrospektivno smo analizirali diagnostične in kirurške podatke pri 25 mačkah z diafragmalno kilo. Mačke so bile stare od 2 mesecev do 4 let, med njimi je bilo 9 samcev (5/9 kastriranih) in 16 samic (9/16 steriliziranih). Akutnih primerov je bilo 12/25 in kroničnih 13/25. Mesta pretrganja diafragme so bila opredeljena glede na točke, določene na podlagi lestvice v smeri urinega kazalca. Pretrganje je bilo določeno kot večmestno v 10 (40 %) primerih, ventralno v 7 (28 %), desno v 5 (20 %) in levo v 3 (12 %) primerih. Trije najpogosteje vrinjeni organi so bili tanko črevo (20, 80 %), jetra (16, 64 %) in omentum (15, 60 %). Preživetje po operaciji je bilo pri akutnih primerih 83,3 %, pri kroničnih pa 69,2 %. Popolno okrevanje je bilo doseženo pri 19 (76 %) mačkah. Rezultati kažejo, da na umrljivost neposredno vplivajo mesto in velikost raztrganine diafragme, vrinjeni organi in prisotnost sočasnih patologij. Smrtnost je večja pri sočasnih poškodbah, velikih raztrganinah diafragme in prekomernem vrinjenju organov.

Ključne besede: prognoza; bolezni mačk; kila; raztrganina; preživetje

Slov Vet Res 2022; 59 (1): 59–69
DOI 10.26873/SVR-1348-2022
UDC 639.2.09:579.841:579.25:615.015.8(620.1)

Original Research Article

ANTIMICROBIAL RESISTANCE AND VIRULENCE-ASSOCIATED GENES OF AEROMONADS ISOLATED FROM LAKE MANZALA WATER AND WILD NILE TILAPIA: IMPLICATIONS TO PUBLIC HEALTH AND THE LAKE MICROBIAL COMMUNITY

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Abstract: This study was conducted to investigate the prevalence, antimicrobial susceptibility, and molecular characterization of *Aeromonas* spp. from wild Nile tilapia from Lake Manzala and the lake water. Swabs from the surface, gills, and internal organs of apparently healthy Nile tilapia (n =100) and lake water (n = 25) were collected and examined bacteriologically for the presence of *Aeromonas* spp. The isolates obtained were tested for their susceptibility to 11 antimicrobial agents using the disk diffusion method. The presence of antibiotic resistance genes (bla_{TEM}, *sul1, tetA(A)*, and *aadA1*) and virulence genes (enterotoxins) (*alt, ast,* and *act*) was determined using conventional polymerase chain reaction. Overall, *Aeromonas* spp. were recovered from 69% of Nile tilapia (*Oreochromis niloticus*) samples and 80% of water samples. Four types of aeromonads were detected in all the samples examined, namely, *A. hydrophila*, *A. sobria, A. caviae,* and *A. schubertii*, with *A. hydrophila* predominating in both the fish and the lake water samples. The antimicrobial resistance profiles of the isolates showed very high resistance to ampicillin, penicillin, sulfamethoxazole/trimethoprim, and oxytetracycline and considerable resistance to streptomycin. However, all isolates were sensitive to cefotaxime. Molecular characterization revealed the presence of the *act* (100%) and *alt* (37.5%) genes, but *ast* was not found in any of the isolates. Specific amplification bands of the antimicrobial resistance genes bla_{TEM}, *sul1,* and *tetA(A)* were detected in all the tested isolates, whereas *aadA1* (12.5%) was found only in one isolate of *A. hydrophila* from Nile tilapia. The presence of these enterotoxigenic and resistant *Aeromonas* spp. in the fish and water samples from Lake Manzala could pose a potential public health threat to human consumers and fish handlers in the study area; moreover, these species carry a risk for the transfer of resistance genes to other microbial communities in the lake.

Key words: *Aeromonas hydrophila* complex; enterotoxin genes; antibiotic resistance genes; *Oreochromis niloticus*; Lake Manzala

Introduction

Lake Manzala is the largest and most important of Egypt's northern coastal lakes and is considered the most productive lake for fishing. The lake accounts for 32% of the total fish production from the lakes of Egypt (1), with the production of tilapia species alone accounting for >65% of the total Egyptian fish production (2). Over the last six decades, Lake Manzala has been subjected to various threats and has been contaminated with residues from domestic, industrial, mining, and agricultural effluents. The lake has evolved from a predominantly aquatic or estuarine ecosystem to an almost eutrophic freshwater system, which may have serious health, environmental,

Received: 2 June 2021
Accepted for publication: 7 January 2022

economic, and social consequences (3). Hence, poor water quality and the consequent decline in fish production in the lake have been reported (4).

Oreochromis niloticus is the most widely distributed freshwater fish species and one of the best known, cheapest, and most commonly available fish in Egypt. Owing to its high disease resistance, it can survive in harsh environmental conditions. Moreover, this fish species is preferred by aquaculture entrepreneurs because it can withstand a wide range of environmental factors, has active reproductive strategies, can feed at different trophic levels, and develops rapidly (5). The genus *Aeromonas* includes >30 different species, of which *A. hydrophila, A. caviae*, and *A. sobria* are of particular clinical importance (6). In humans, these species can cause several diseases, including septicemia, meningitis, wound infections, gastroenteritis, and pneumonia (7, 8). The virulence of *Aeromonas* spp. is multifactorial. Several factors, including endotoxins, enterotoxins, adhesins, hemolysins, cytotoxins, proteases, and lipases, have been detected in systemic and intestinal infections caused by aeromonads (9, 10). Remarkably, *Aeromonas* spp. can take up and transfer many genes located in genetic components such as plasmids, transposons, IS elements, genomic pathogen islands, and integron-associated gene cassettes. These genes may encode toxic elements, virulence factors, and antimicrobial resistance (11). These components are critical for the rapid transfer of genetic material between microbial communities. Therefore, environmental contamination is thought to provide an ideal medium for the selection of resistant species and the exchange of resistant genes by mobile genetic elements (12). Enterotoxins (also called exotoxins) secreted by aeromonads are one of the most important virulence factors (13); cytotoxic heat-labile enterotoxin (*act*), cytotonic heat-labile enterotoxin (*alt*), and cytotonic heat-stable enterotoxin (*ast*) are a few examples (14, 15). The presence of virulence genes (enterotoxins) in *Aeromonas* spp. determines their degree of virulence (16). Owing to the frequent consumption of fish by people in our study area, much attention has been paid to the occurrence of antibiotic-resistant pathogens in fish and the associated risks to consumer health. Based on the existing data on this bacterial species, this study attempted to further investigate the antibiotic resistance profiles and virulence genes in extensively resistant aeromonads from apparently healthy Nile tilapia and water from Lake Manzala. We aimed to provide an up-to-date overview of the currently circulating isolates and to widen our knowledge of the likely public health implications.

Material and methods

Ethical statement

The collection and processing of samples for this study were reviewed and approved by the Scientific Research Committee and Bioethics Board of Suez Canal University, Faculty of Veterinary Medicine, Ismailia, Egypt.

Sampling

In total, 100 apparently healthy Nile tilapia were randomly netted at different locations in Lake Manzala. Swab samples (surface, gills, and internal organs) were collected from each fish. In addition, water samples (n = 25) were obtained in sterile, labeled, plastic screw-capped vials from the same areas where the fish were collected. All samples were immediately transported to the laboratory and processed under fully aseptic conditions for microbiological examination.

Isolation and identification of Aeromonas spp.

The isolation and identification of *Aeromonas* spp. from fish and water samples were performed in accordance with the standard procedures described previously (13, 17). Briefly, the samples were enriched in alkaline peptone water at 28°C for 18 h before inoculation onto Aeromonas agar medium (Oxoid, Hampshire, UK). Typical colonies suspected to be *Aeromonas* spp. were selected and purified for further identification. The isolates were identified to belong to the genus *Aeromonas* on the basis of Gram staining, motility tests, oxidase, glucose fermentation, catalase, and resistance to 2,4-diamino-6,7-diisopropylpteridine (O/129) (150 µg). A series of biochemical tests was used to identify the aeromonads at the species level. Pure colonies of the identified isolates were preserved on nutrient agar plates for further study.

Antimicrobial susceptibility and multiple antibiotic resistance (MAR) index value

Aeromonas spp. isolates were tested for susceptibility to the following 11 antimicrobial agents on Mueller–Hinton agar (Oxoid, Hampshire, UK) using the disk diffusion method (18) at the indicated concentrations: Ampicillin (AM, 10 µg); penicillin (P, 10 µg); streptomycin (S, 10 µg), sulfamethoxazole/ trimethoprim, (SXT, 25 µg); oxytetracycline (T, 30 µg); norfloxacin (NOR, 10 µg); cefotaxime (CTX, 30 µg); amikacin (AK, 30 µg); nalidixic acid (NA, 30 µg); gentamycin (CN, 10 µg); and chloramphenicol (C, 30 µg) (Bioanalysis®, Turkey). The tested strains were classified as sensitive or resistant based on the diameters of the inhibition zones around the disk. The MAR index was calculated using the following equation: MAR index = a/b; where, (a) is the number of antibiotics to which the isolate is resistant and (b) is the number of antibiotics to which the isolate has been exposed) (19).

Molecular characterization of Aeromonas spp. Isolates

Pure isolates were enriched by cultivating them in alkaline peptone water at 37°C for 24 h, and the QIAamp DNA mini kit was used to extract the genomic DNA in accordance with the manufacturer's instructions. For confirmation of the *Aeromonas* isolates, conventional polymerase chain reaction (PCR) was performed using *16S rRNA* primers, followed by the detection of enterotoxin genes (*alt*, *act*, and *ast*) and antibiotic resistance genes (bla_{TEM}, *sul1*, *tetA(A)*, and *aadA1*) corresponding to the agents to which the isolates were phenotypically resistant (Table 1). In all protocols, the reaction was performed in a total volume of 25 µL, which contained 12.5 µL dreamTaq Master Mix (Green PCR Master Mix (2×), (Thermo Scientific), 1 µL of each primer, 2 µL of DNA template, and 9.5 µL of DNase/RNAse-free water. The PCR cycling conditions were initial denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 30 s, annealing for 40 s at the temperature indicated for each gene (50°C for 16S rRNA; 55°C for *alt*, *act*, and *ast*; 54°C for bla_{TEM} and *aadA1*; 50°C for *tetA(A)*; 60°C for *sul1*), and extension at 72°C for 30 s; and a final extension step at 72°C for 10 min. The amplified products were photographed after electrophoresis using an agarose gel (1.5%) stained with ethidium bromide (0.5 µg/m^{1}) against a 100-bp DNA ladder (Invitrogen, San Jose, CA).

Statistical analysis

The data were processed and analyzed using SPSS (IBM-SPSS Inc., Chicago, IL, USA). Examination of the data revealed that the Shapiro–Wilk P value was significant ($P < 0.00$*); hence, non-parametric data analysis was performed. The difference between the groups was considered significant when the *P* value of the chi-square test was <0.05.

Results

Prevalence of Aeromonas spp. in Nile tilapia and Lake Manzala water samples

Aeromonas bacterial spp. were found in 69% of *O. niloticus* (69/100) and 80% (20/25) of water samples. In total, 119 isolates belonging to the Aeromonas spp. were recovered from *O. niloticus*; *A. hydrophila* was the predominant species (54.62%), followed by *A. sobria* (26.05%), *A. caviae* (15.97%), and *A. schubertii* (3.36%). The same order was observed in the isolates from Lake Manzala too (n = 36). The predominant *Aeromonas* sp. was *A. hydrophila* (52.87%) (19/36), followed by *A. sobria* (27.78%) (10/36), *A. caviae* (16.76%) (6/36), and *A. schubertii* (2.78%) (1/36) (Table 2). The occurrence of *Aeromonas* spp. in different swabbing sites from Nile tilapia revealed that the surface showed the highest recovery rate (53.78%), followed by the gills (31.93%) and the internal organs (14.29%). The order of distribution of the different *Aeromonas* spp. followed the same pattern as in *O. niloticus* and lake water, with *A. hydrophila* being the predominant strain (Table 2). Statistically significant ($P < 0.05$) differences were found in the prevalence of *Aeromonas* spp. from Nile tilapia and lake water and from different swabbing sites.

Antimicrobial susceptibility and MAR index value of the identified Aeromonas isolates

As shown in Table 3, all of the 45 representative isolates of *Aeromonas* spp. tested from fish and lake water sources were multidrug resistant, i.e., resistant to ≥1 antibiotic falling under ≥3 antimicrobial classes. The isolates were highly resistant (100%) to ampicillin, penicillin, and sulfamethoxazole/

Table 1: Target genes, oligonucleotide primer sequences, and amplified product size specific for *Aeromonas* spp.

Target gene	Primer sequence (bp)	Amplified product size	Reference
16S rRNA	CTACTTTTGCCGGCGAGCGG TGATTCCCGAAGGCACTCCC	953 bp	(20)
act	GGGTGACCACCACCAAGAACA AACTGACATCGGCCTTGAACTC	232 bp	
ast	TCTCCATGCTTCCCTTCCACT GTGTAGGGATTGAAGAAGCCG	331 bp	(21)
alt	TGACCCAGTCCTGGCACGGC GGTGATCGATCACCACCAGC	442 bp	
bla_{TEM}	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516 bp	(22)
sul1	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433 bp	(23)
aadA1	TATCAGAGGTAGTTGGCGTCAT GTTCCATAGCGTTAAGGTTTCAT	484 bp	(24)
tetA(A)	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	576 bp	

Table 2: Prevalence of *Aeromonas* spp. in the examined water samples and Nile tilapia, with special reference to their recovery rate from different swabbing sites of fish.

Aeromonas species	Water samples	Nile tilapia	Distribution in different swabbing sites of fish		
			Surface	Gills	Internal organs
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
A. hydrophila	19 (52.78)	65 (54.62)	34 (53.13)	21 (55.26)	10 (58.8)
A. sobria	10 (27.78)	31 (26.05)	17 (26.56)	10 (26.32)	4 (23.53)
A. caviae	6 (16.67)	19 (15.97)	11 (17.19)	6 (15.79)	2 (11.76)
A. schubertii	1 (2.78)	4 (3.36)	2 (3.13)	1 (2.6)	1 (5.88)
Total	36	119	64	38	17

Percent calculated in relation to the total of each column.

trimethoprim, followed by oxytetracycline (86.7%) and streptomycin (66.7%). However, all isolates were sensitive to cefotaxime (100%), with varying sensitivities to gentamycin (84.4%), chloramphenicol (80%), amikacin (75.6%), norofloxacin, and nalidixic acid (73.3%) (Table 3). These differences in the susceptibility of the four *Aeromonas* spp. to the different antimicrobial agents were statistically significant ($P < 0.05$). The MAR index values of the *Aeromonas* spp. ranged from 0.27–0.45 (Table 4).

Molecular confirmation and detection of enterotoxin genes and antibiotic resistance genes from isolates of the Aeromonas spp.

All tested *Aeromonas* isolates from the fish and water sources showed specific amplification bands at 953 bp when universal *Aeromonas* bacterial *16SrRNA* primers were used (Fig. 1 A). The detection of enterotoxin genes and antibiotic resistance genes for eight isolates, which showed an extensive drug resistance pattern, revealed the presence of the cytotonic heat-labile gene (*alt*) in three isolates (37.5%) and the cytotoxic enterotoxin gene (*act*) in all isolates. None of the isolates were positive for the cytotonic heat-stable enterotoxin (*ast*) gene (Table 5, Fig. 1 B, C, and D). With regard to the presence of the antibiotic resistance genes, bla_{TEM}, *sul1*, and *tetA(A)* were detected in all isolates of the *Aeromonas* spp. examined; however, the streptomycin-resistant gene (*aadA1*) was detected in only one *A. hydrophila* (12.5%) isolate from *O. niloticus* (Table 5, Fig. 2 A, B, C, and D).

Table 3: Antimicrobial resistance of *Aeromonas* spp. isolated from *O. niloticus* and Lake Manzala water samples

Antimicrobial agent (Conc.)	*A. hydrophila* n=18	*A. sobria* n=13	*A. caviae* n=9	*A. schubertii* n=5	Total n=45
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Ampicillin (AM) (10 µg)	18 (100.0)	13 (100.0)	9 (100.0)	5 (100)	45 (100.0)
Penicillin (P) (10 µg)	18 (100.0)	13 (100.0)	9 (100.0)	5 (100)	45 (100.0)
Streptomycin (S) (10 µg)	12 (66.7)	9 (69.2)	6 (66.7)	3 (60)	30 (66.7)
Sulphamethoxazole - trimethoprim (SXT) (25 µg)	18 (100.0)	13 (100.0)	9 (100.0)	5 (100)	45 (100.0)
Oxytetracyclin (T) (30 µg)	15 (83.3)	11 (84.6)	8 (88.9)	5 (100)	39 (86.7)
Cefotaxime (CTX) (30 µg)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Norofloxacin (NOR) (10 µg)	4 (22.2)	3 (23.1)	3 (33.3)	2 (40)	12 (26.7)
Amikacin (AK) (30 µg)	4 (22.2)	5 (38.5)	2 (22.2)	0 (0.0)	11 (24.4)
Gentamycin (CN) (10 µg)	5 (27.8)	1 (7.7)	1 (11.1)	0 (0.0)	7 (15.6)
Nalidixic acid (NA) (30 µg)	6 (33.3)	4 (30.8)	1 (11.1)	1 (20)	12 (26.7)
Chloramphenicol (C) (30 µg)	4 (22.2)	2 (15.4)	2 (22.2)	1 (20)	9 (20.0)

Table 4: MAR index value of *Aeromonas* species isolatedfrom Nile tilapia and Lake Manzala water samples

Strains	No. of isolates	Resistant antibiotics	MAR index	%
A. hydrophila	12	AM, P, SXT, S,T	0.45	66.6
	3	AM, P, SXT, T	0.36	16.6
	3	AM, P, SXT	0.27	16.6
A. sobria	9	AM, P, SXT, S,T	0.45	69.23
	2	AM, P, SXT, T	0.36	15.38
	2	AM, P, SXT	0.27	15.38
A. caviae	6	AM, P, SXT, S,T	0.45	66.6
	2	AM, P, SXT, T	0.36	16.6
	1	AM, P, SXT	0.27	16.6
A. schubertii	3	AM, P, SXT, S,T	0.45	60
	2	AM, P, SXT, T	0.36	30

AM: ampicillin, P: penicillin, S: streptomycin, SXT: sulphamethoxazole - trimethoprim, and T: oxytetracyclin

Table 5: Occurrence of *16SrRNA*, virulence (enterotoxin) genes and antimicrobial resistance genes in the examined *Aeromonas* species isolated from *O. niloticus* and Lake Manzala water samples

Aeromonas species	Enterotoxin genes			Antibiotic resistance genes			
	alt	*ast*	*act*	bla_{TEM}	*sul1*	*tetA(A)*	*aadA1*
A. hydrophila	-	-	+	+	+	+	+
A. hydrophila	+	-	+	+	+	+	-
A. hydrophila	+	-	+	+	+	+	-
A. sobria	-	-	+	+	+	+	-
A. sobria	-	-	+	+	+	+	-
A. sobria	-	-	+	+	+	+	-
A. caviae	-	-	+	+	+	+	-
A. schubertii	+	-	+	+	+	+	-
Total (%)	3(37.5%)	0 (0%)	8 (100%)	8 (100%)	8 (100%)	8 (100%)	1 (12.5%)

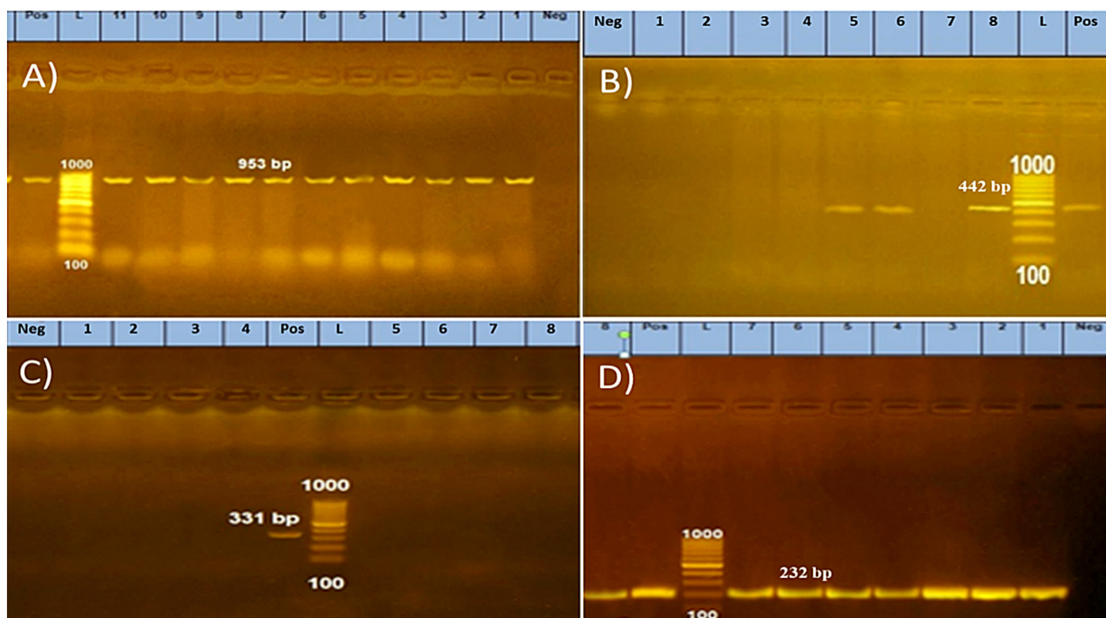

Figure 1: Agarose gel electrophoresis showing specific amplification product size of **A**) *16SrRNA* of *Aeromonas* species isolated from fish and water samples. Lane 1-8 are positive to genus *Aeromonas*, L: 100 -1000 bp Molecular ladder, Pos: Positive control, Neg: Negative control. **B**) Cytotonic heat- labile enterotoxin (*alt*) gene. Lane 5, 6,and 8: positive for *(alt)* gene with a specific band at 442 bp. (*A. Schubertii* from *O. niloticus*, *A. hydrophila* from *O. niloticus* and *A. hydrophila* from lake water), L: 100 -1000 bp Molecular ladder, Pos: Positive control, Neg: Negative control. **C**) Cytotoxic heat-stable enterotoxin *(ast)* gene. Lane 1-8: Negative for *(ast)* gene L: 100 -1000 bp Molecular ladder, Pos: Positive control, Neg: Negative control. **D**) Cytotonic heat-stable enterotoxin *(act)* gene. Lane 1-8: positive with a specific *act* band at 232 bp. for *(act)* gene. L: 100 -1000 bp Molecular ladder, Pos: Positive control, Neg: Negative control

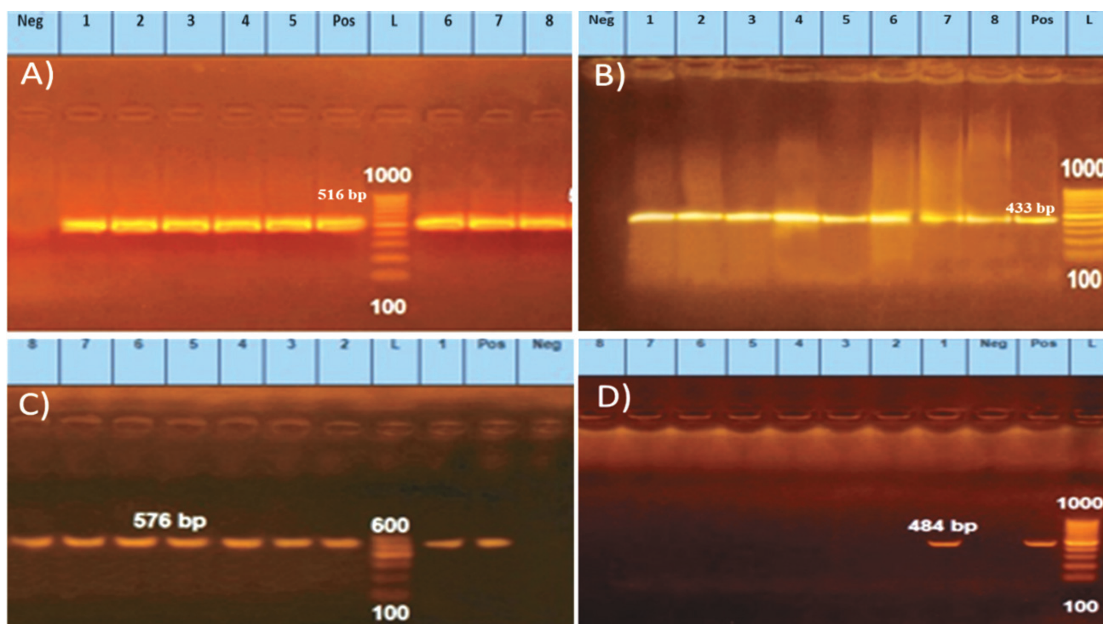

Figure 2: Agarose gel electrophoresis showing specific amplification product size of **A**) β- lactamase ampicillin resistance gene (bla_{TEM} gene). Lane 1-8: Positive for (bla_{TEM}) resistance gene. L: 100 -1000 bp Molecular ladder, Pos: Positive control. Neg: Negative control. **B)** Sulfonamide resistance gene (*sul1* gene). Lane 1-8: Positive for (*sul1*) resistance gene. L: 100 -1000 bp Molecular ladder, Pos: Positive control. Neg: Negative control. **C)** Tetracycline resistance gene (*tet*A(A*)*gene). Lane 1-8: Positive for (*tet*A(A) resistance gene. L: 100 -1000 bp Molecular ladder, Pos: Positive control. Neg: Negative control. **D)** Streptomycin resistance gene (*aadA1*gene). Lane 1: positive for (*aadA1*) resistance gene of *A. hydrophila* from *O. niloticus*, Lane 2-8: negative for (*aadA1*) resistance gene, L: 100 -1000 bp Molecular ladder, Pos: Positive control. Neg: Negative control.

Discussion

O. niloticus is one of the most common and valuable freshwater fish species in Egyptian commercial fisheries (25). Despite their wide distribution in the aquatic environment, aeromonads can cause disease in humans and fish depending on several environmental and host factors as well as the virulence of the organisms involved (26).

Similarly, *Aeromonas* spp. (84%) were isolated from Lake Manzala water (28). This high frequency of aeromonads may be attributed to the contamination of Lake Manzala water by large amounts of domestic sewage, agricultural, and industrial effluents (29). Motile aeromonads (*A. sobria*, *A. vernoii*, *A. jandae*, *A. cavae*, and *A. hydrophila*) have been described as the most common pathogens of carp and Nile tilapia in Egypt (30). The results of the present study showed that among the 119 aeromonad isolates obtained from *O. niloticus* and water, *A. hydrophila* was the most abundant species, followed by *A. sobria*, *A. caviae, and A. schubertii* (Table 2). This observation is in line with the results of a previous study on aeromonads from Lake Manzala (31). Similar results have been reported for Nile tilapia in Uganda where *A. hydrophila* (43.8%) and *A. sobria* (20.8%) were isolated (32). In addition, a high prevalence of *A. hydrophila* (47%) was found in 170 fish (100 from freshwater, 30 from the marine environment, and 40 from brackish water) from different farms in Alexandria, El-Behera, and Kafr Elsheikh in Egypt (33). Notably, *A. hydrophila* and *A. sobria*, which are most commonly associated with human infections (34, 35), were the most frequently isolated species from fish and water in this study.

In the current report, the highest recovery rate of aeromonads was recorded from the surface of the fish (53.78%, 64/119), followed by the gills (31.93%, 38/119) and internal organs (14.92%, 17/119), with *A. hydrophila* being the most common species (Table 2). Similar results have been reported previously when the recovery of *Aeromonas* spp. was higher in the skin of fish samples than in the internal organs and the gills (36). However, in a recent study in Egypt, the recovery rate of aeromonads was lower in the gills than in the internal organs (37). This finding may be related to the health status of the fish studied because the fish from the more recent study were clinically ill. In general, the isolation of different aeromonad species from fish and water provides information on the pollution rate as these species are widely distributed in surface waters and sewage systems (36).

The contamination of fish with resistant bacteria poses a serious threat to public health because genes encoding resistance can spread to other bacteria that are clinically relevant to humans. Consequently, antibiotic options for the treatment of common human infectious diseases have become increasingly limited, expensive, and ineffective owing to the emergence of antibiotic-resistant bacteria (29). A previous study has concluded that the genus *Aeromonas* can be used as an indicator bacterium to detect antibiotic resistance in the aquatic environment (38). In the present study, the examination of the susceptibility of 45 identified *Aeromonas* spp. isolates to 11 different antibiotics revealed that they were highly resistant to five antibiotics, namely, ampicillin, penicillin, sulfamethoxazole/trimethoprim (100% each), and oxytetracycline (86.7%) and considerably resistant to streptomycin (66.7%) (Table 3). However, in a recent report from Dakahlia, Egypt, *Aeromonas* spp. from farmed fish in private farms had high resistance to chloramphenicol (67.4%), amikacin (51.9%), and gentamicin (47.1%) but were sensitive to amoxicillin/clavulanic acid (73.3%) and trimethoprim/sulfamethoxazole (64.2%) (37). Since Lake Manzala is located in the northeastern part of the Nile Delta, between the Mediterranean Sea and the Suez Canal, it receives untreated and/or pretreated wastewater from the Bahr El-Baqar drain (100 km long), which is considered one of the most polluted drains in Egypt. This canal receives wastewater from two tributary canals (Bilbeis Canal and Qalubeya Canal) (39).

MAR is chiefly attributed to the improper use of antimicrobials. When the MAR index is >0.2, there is a high risk of bacterial contamination when antibiotics are used extensively (27). In the present study, the MAR index values for the four *Aeromonas* spp. described (Table 4) ranged from 0.27 to 0.45, which indicates the indiscriminate use of antibiotics. Such indiscriminate use may lead to the transfer of antibiotic resistance genes to other bacterial species and affect humans and fish, especially in areas with a high population density.

In this study, the eight isolates from Nile tilapia and lake water samples that showed extensive resistance patterns were screened for the presence

of antibiotic resistance genes corresponding to the observed phenotypic resistance. Three genes (*bla*$_{TEM}$, *sul1*, and *tetA(A)*) were amplified from all eight *Aeromonas* spp. (Table 5). Similarly, *bla*$_{TEM}$ antibiotic resistance genes were amplified from all *Aeromonas* isolates (40). However, in another study on Nile tilapia from Lake Temsah, Egypt, the *bla*$_{TEM}$ resistance gene was detected to a lesser extent in *A. hydrophila* (73.68%, 14/19) and *A. sobria* (5.3%, 1/19) and to a much lesser extent in *A. hydrophila* (25%, 1/4) isolated from Nile tilapia from brackish water aquaculture (41). This difference in the prevalence of *bla*$_{TEM}$ resistance genes may be due to the variations in the environments from which the *Aeromonas* spp. were isolated. The presence of β-lactamase genes in *Aeromonas* spp. from environmental sources is a complicated issue because it limits the therapeutic options for *Aeromonas* infections (42). In addition, the expression of the *bla*$_{TEM}$ gene results in concurrent resistance to penicillins and broad-spectrum cephalosporins (43).

In the present study, the sulfonamide resistance gene (*sul1*) was detected in all *Aeromonas* isolates examined. However, the same gene was present at lower levels of 29.2% (44) and 11.5% (45) in other studies. Moreover, the presence of the *Sul1* gene has been reported to be different in *A. hydrophila* (80%, 8/10) and *A. caviae* (66.67%, 4/6) (46). These differences may be due to the presence of different Sul genes, such as *Sul1*, *Sul2*, and *Sul3*.

A specific amplification band of the *tetA(A)* gene was detected in all *Aeromonas* isolates tested, which agrees with the findings from another study (46). The streptomycin resistance gene, *aadA1*, was detected in only one strain of *A. hydrophila* (6.2%) from *O. niloticus*, which is less than that reported in other studies (30, 47). However, *aadA1* was detected in one strain of *A. hydrophila* from crayfish in Iran (48) and in seven of ten *A. hydrophila* isolates and in three of six *A. caviae* isolates in Egypt (30). These variations in the occurrence of the *aadA1* resistance gene could be due to the presence of other streptomycin resistance genes that were not investigated in this study, such as *aadA2* and *aadA5*.

Enterotoxin genes are very important for the pathogenicity of aeromonads. In the present study, the *act* gene was successfully amplified from the eight extensively drug resistant isolates from fish and water samples, but the *alt* gene was confirmed in only three isolates (Table 5). The prevalence of the *act* gene in this study was 100%, which agrees with another study (49). In contrast, other studies have shown different prevalence rates of 76.92% (50) and 65% (26) for the *act* gene in *Aeromonas* spp. The *act* gene was detected in 55% of *A. hydrophila* isolates, but it was not detected in *A. caviae* in another study (51). In this report, none of the isolates carried the cytotonic (*ast*) gene. This finding is in agreement with a previous result (26). However, the *ast* gene has been detected in the isolates of *Aeromonas* spp. in other studies at lower rates of 4% (52), 6% (50), and 10.25% (26). A higher occurrence of *alt* has previously been found in isolates of *A. hydrophila* (75%) but not in *A. caviae* (50) and in *A. sobria* but not in *A. caviae* (51). In general, biological tests are important for the evaluation of the virulence of spp. and to determine the pathogenicity of *Aeromonas* spp., which may pose a public health risk (53).

Conclusion

The ability of aeromonads to adapt to their environment is determined by their diverse virulence profiles. The presence of certain Aeromonas isolates with virulence and antimicrobial resistance properties in the same environment with other potentially pathogenic species poses a potential risk associated with the transfer of these virulence genes and antimicrobial resistance genes to other pathogenic microorganisms in Lake Manzala. Hence, these genes may reach humans and other animals through the food chain. The presence of such enterotoxigenic virulent strains on the surface and organs of fish poses a potential public health risk to fish handlers and human consumers in the study area.

Acknowledgments

The authors declare that they have no conflict of interest.

This research was not financially supported by any public, commercial, or nonprofit entity.

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PROTIMIKROBNA ODPORNOST IN Z VIRULENCO POVEZANI GENI BAKTERIJE *AEROMONAS* IZOLIRANIH IZ JEZERA MANZALA IN DIVJE NILSKE TILAPIJE: POSLEDICE NA JAVNO ZDRAVJE IN NA JEZERSKO MIKROBNO ZDRUŽBO

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Izvleček: Namen te študije je bil raziskati razširjenost in protimikrobno občutljivost ter molekularno karakterizirati bakterijo *Aeromonas spp.*, izolirane iz divje nilske talapije in vode iz jezera Manzala. Zbrali smo brise površine, škrg in notranjih organov navidezno zdrave nilske tilapije (n = 100) in vzorce vode (n = 25), ki smo jih bakteriološko pregledali na prisotnost bakterije *Aeromonas spp.* Pridobljene izolate smo testirali na občutljivost za 11 protimikrobnih sredstev z metodo difuzije diska. Prisotnost genov za odpornost proti antibiotikom (bla_{TEM}, *sul1*, *tetA(A)* in *aadA1*) in genov za virulenco (enterotoksini; *alt*, *ast* in *act*) smo določili z običajno verižno reakcijo s polimerazo. Skupno smo *Aeromonas spp.* odkrili v 69% vzorcev nilske tilapije in 80% vzorcev vode. V vseh pregledanih vzorcih smo odkrili štiri vrste bakterij in sicer *A. hydrophila, A. sobria, A. caviae*, in *A. schubertii*, od katerih je *A. hydrophila* prevladovala v vzorcih rib in jezerske vode. Profili protimikrobne odpornosti izolatov so pokazali zelo visoko odpornost na ampicilin, penicilin, sulfametoksazol/trimetoprim in oksitetraciklin ter znatno odpornost na streptomicin. Izolati so bili občutljivi na cefotaksim. Molekularna karakterizacija je pokazala prisotnost genov *act* (100%) in *alt* (37.5%). Gena *ast* nismo našli v nobenem izolatu. Geni za protimikrobno odpornost bla_{TEM}, *sul1* in *tetA(A)* so bili prisotni pri vseh testiranih izolatih, medtem ko je bil gen *aadA1* (12.5%) najden samo pri enem izolatu *A. hydrophila* iz nilske tilapije. Prisotnost enterotoksičnih in odpornih bakterij *Aeromonas spp.* v vzorcih rib in vode iz jezera Manzala bi lahko predstavljala nevarnost za javno zdravje potrošnikov in oseb, ki rokujejo z ribami na območju študije; poleg tega te vrste predstavljajo tveganje za prenos genov za odpornost na druge mikrobne skupnosti v jezeru.

Ključne besede: *Aeromonas hydrophila* kompleks; geni za enterotoksin; geni za rezistenco na antibiotike; *Oreochromis niloticus*; jezero Manzala

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