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DETECTION OF *SALMONELLA* AND *HELICOBACTER* SPP. IN CAPTIVE WILD FELIDS

Running title: *Salmonella* and *Helicobacter* Spp. in Captive Wild Felids.

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ABSTRACT

The current study aimed to investigate the presence of *Salmonella* and *Helicobacter* species in captive wild felids in addition to perform serotyping, antibiotic sensitivity test to the isolated *Salmonella* spp. and detection of antibiotic resistance and virulence genes. A total of 60 fecal samples were collected from 30 captive wild felids from Giza zoo and private zoo in Egypt. All animals were apparent healthy except eight African lions (*Panthera Leo*) have a history of vomiting. Samples were examined bacteriologically for the presence of *Salmonella* spp., followed by biochemical and serological tests. Moreover, obtained isolates were subjected to antimicrobial sensitivity testing and detection of antibiotic resistance and virulence genes. Fecal samples from lions with history of vomiting, were subjected to direct molecular identification for detection of *Helicobacter* spp. Overall, *Salmonella* spp. were isolated from 3 wild cats (*Felis chaus)*. Two serovars of *Salmonella* were detected; *S. Bovismorbificans* and *S. Southampton* while *Helicobacter felis* was isolated from one African lion. Isolates of *Salmonella* spp. showed complete resistance to cefaclor (100%), cefoxitin (100%), and cefadroxil (100%); and very high resistance to tobramycin (66.7%), while it completely sensitive to Azithromycin (100%), Sulfa/trimethoprim (100%), Nitrofurantoin (100%), Doxycycline (100%), Amoxicillin-Clavulanic acid (100%), Fosfomycin (100%) and Oxytetracycline (100%). bla_{TEM} and bla_{SHV} were confirmed in *Salmonella* isolates showing resistant to Cefaclor and Cefoxitin, and *aadA2* in *S. Bovismorbificans* that showing resistant to tobramycin. *S. Southampton* and *S. Bovismorbificans* have *invA, stn, sopB*, and *hilA* genes while *S. Bovismorbificans* carry also *pefA* gene as a virulence genes.

Key words: Wild felids, *Salmonella*, *Helicobacter*, antibiotic resistance, virulence genes.

INTRODUCTION

Wild felids are strict carnivorous occupy the top of the food chain and considered as the most famous predator animals (Wang *et al.*, 2012). They have an ecological role in regulating prey populations size and shaping animal communities (Sarasola, 2016). Furthermore, providing food for other species like scavengers, detritivores animals, and microorganisms (Marker, 2002). So, they play a critical role as a keystone species for animal community structure, function,

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distribution, population dynamics, and affecting behavior of interacting specie (Hopcraft *et al.,* 2010). Loss of apex predators has negative impact on terrestrial ecosystems resulting in destabilization of herbivore-plant interactions, reduction of diversity, and loss of flexibility within ecosystems (Loveridge *et al.,* 2016). Also in zoo and circus, it is considered as the most popular species that attract visitors and good source of income (Ripple *et al.,* 2014).

In captivity, wild felids are susceptible to many bacterial diseases as *Helicobacter* gastritis, *Salmonella*, *E. coli, Collinsella, Shigella*, *Proteus* and *Fusobacterium*; however, it may be considered as a source of disease not only for other animals but also for human such as veterinarian, workers, and visitors. (Daszak *et al.,* 2001).

Salmonella species have been isolated from the digestive contents of birds and mammals, and are capable of infecting a wide range of domestic and wild animal species (Rubini *et al.,* 2016). In some animals, it is assumed to be an opportunistic pathogen or potentially a component of the natural gut microbiota (Dróżdż 2021) and it is considered as one of the most serious zoonotic pathogen causing several outbreaks in human around the world (Brandwagt *et al.,* 2018, Gilsdorf *et al.,* 2005 and Stafford *et al.,* 2002).

Helicobacter infection in captive wild felids like cheetah is associated with progressive gastritis which result in vomiting, weight loss, and failure to thrive. While in human, the infection is usually linked to gastrointestinal problems, cancer, and the immunocompromised persons (Heilmann and Borchard 1991).

The current study aimed to investigate the presence of *Salmonella* and *Helicobacter* spp. in captive wild felids in addition to perform serotyping, antibiotic sensitivity test, and detection of antibiotic resistance and virulence genes for the isolated *Salmonella* spp.

MATERIALS AND METHODS

Sampling:

The current study was performed on 30 animals belonged to family Felidae, including (16 African lions; *Panthera Leo*, 2 Bengal tiger; *Panthera Tigris Tigris* and 2 Cheetah; *Acinonyx jubatus*) from Giza Zoo and (5 African lions; *Panthera Leo* and 5 wild cats; *Felis chaus*) from private zoo in Egypt. A total of 60 fecal samples were collected, 2 samples from each animals; one during summer and the other during winter. Each animal was housed in separate enclosure. All animals were apparent healthy and showing no signs of diseases except eight lions in Giza zoo had a previous history of vomiting.

Collection of fecal samples:

Fresh fecal samples were collected aseptically from the floor of the animal's enclosure by removing the superficial layer of the feces and a cotton swab was inserted in the core of feces then put the swab in tube contain 10 ml peptone water.

Bacterial isolation and identification for *Salmonella*

Tubes of peptone water containing the fecal samples were incubated at 37º C for 18 hours before being plated onto Rappaport Vassiliadis (Himedia) broth and incubated at 42°C for 24 hours for *Salmonella* enrichment. A loop full from Rappaport Vassiliadis broth was streaked onto Xylose Lysine Deoxycholate media (Himedia), Hektoen enteric agar (LabM) and *Salmonella-Shigella* media (Himedia) then the inoculated plates were incubated at 37º C for 18-24 hours. Purification was done on the above-mentioned media tell obtaining separate, clear, and pure colonies for studying the cultural characters. (Gelaw *et al.,* 2018)

Biochemical identification of bacterial isolates was carried out by using oxidase, citrate utilization, urease, indole, methyl red, H_2S on TSI, lysine decarboxylase, D-glucose–acid and gas production and

Voges–Proskaure test. (Bullock and Aslanzadeh, 2013)

Serological identification of *Salmonella* spp. A single pure colony of isolated bacteria was picked up into TSI slant. Three confirmed isolates of *Salmonella* were subjected to serological identification in Animal health research institute, Dokki, Giza, Egypt. The recovered *Salmonella* isolates were serotyped based on their polyvalent and monovalent (O) to detect somatic antigen, and polyvalent and monovalent (H) antisera to detect phase one and two flagellar antigen.

Antimicrobial sensitivity of *Salmonella*

The disk diffusion method was used to test antibiotic susceptibility of isolates on Mueller-Hinton agar (LabM) using 15 different antibiotic discs (Table 1). Into 5 mL of Mueller-Hinton broth (LabM), a pure colonies from 24 hours old culture were inoculated and incubated for 4-5 h until the turbidity was observed. Then, the bacterial suspension was adjusted to a density equivalent to 0.5 McFarland standard. The surfaces of Mueller-Hinton agar plates was streaked with a sterile cotton swab containing the bacterial suspension, and the plates were left for 30 min at room temperature. Then, by using an antibiotic dispenser and sterile forceps, the antibiotic discs were placed on the surface of the plate (Hudzicki 2009). The recommended diameter for the inhibition zone of the National Committee for Clinical and Laboratory Standards Institute was used to classify the isolates as resistant, intermediate, or sensitive (CLSI 2017)

Molecular identification for *Helicobacter* spp. and detection of antibiotic resistance and virulence genes for *Salmonella* spp. Fecal samples from eight lions that had a previous history of vomiting were subjected directly to the molecular identification by using QIAamp DNA stool Mini Kit (Qiagen, Germany, GmbH), Catalogue no.51504 for DNA extraction of *Helicobacter* spp.

QIAamp DNA Mini Kit (Qiagen, Germany, GmbH), Catalogue no.51304 was used for DNA extraction of *Salmonella* spp.

Emerald Amp GT PCR mastermix (Takara, Japan) Code No. RR310A was used. Oligonucleotide primers (Metabion, Germany) sequences for detection of the antibiotic resistance genes and virulence genes in *Salmonella* spp. and for identification of *Helicobacter* spp. were showed in table (2). PCR cycling condition were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec denaturation, annealing for 40 sec (annealing temperatures (Table 2) and 72 °C for 45 sec extension, with a final elongation at 72 °C for 10 min. All PCR reactions were performed in Master cycler Gradient thermocycler (Eppendorf, Hamburg, Germany). The amplified products were run in 0.1-0.5 μg/ml ethidium bromide-stained agarose gel 1.5% with a 100 bp DNA ladder (GeneDirex, USA and Taiwan) in 1× TBE buffer at 100V/30min, then recorded using the SynGene Gel Documentation System.

Ethical approval:

This study was approved by the Scientific Research Ethics Committee at the Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt.

RESULTS

Based on cultural characteristics and biochemical reactions, *Salmonella* was isolated from wild cat (*Felis chaus*) while the other species of wild felids appeared free from *Salmonella* infection. Three wild cat from total five were infected with prevalence rate 60%.

From the three isolates of *Salmonella*, two serotypes were detected; one isolate was S. *Southampton* with antigenic formula (O4,12,27;r,Z6) and two isolates were S. *Bovismorbificans* with antigenic formula (O6,8,20;r[i],1,5).

Regarding to the antimicrobial sensitivity test, *Salmonella* isolates showed complete resistance to Cefaclor (100%), Cefoxitin (100%), Cefadroxil (100%) and very high resistance to Tobramycin (66.7%), while it completely sensitive to Azithromycin (100%), Sulfa/trimethoprim (100%), Nitrofurantoin (100%), Doxycycline (100%), Amoxicillin-Clavulanic acid (100%), Fosfomycin (100%), Oxytetracycline (100%), Colistin (100%) and highly sensitive to Neomycin (66.7%) (Table 1).

The detection of virulence genes in isolated *Salmonella* revealed that, the three isolates have *inva, stn, sopB, and hilA* genes and two isolates have *pefA* gene while *integron* gene was not detected. (Table 3 and Figure 1)

The detection of antibiotic resistant genes confirmed the presence of bla_{TEM} and bla_{SHV} in *Salmonella* isolates (*S. Bovismorbificans* and *S. Southampton)* that showing resistant to Cefaclor and Cefoxitin, and the presence of the *aadA2* in isolate of *S. Bovismorbificans* that showing resistant to tobramycin as shown in photos (Figure 2)

Eight lions in Giza zoo had a previous history of vomiting and not showed any clinical signs of diseases during the study. The fecal samples from these lions were subjected directly to molecular identification for *Helicobacter* infection. Only one sample was positive for *Helicobacter felis* with a percent of 12.5% (Figure 3).

Table 1: Antimicrobial sensitivity of *Salmonella.*

Antibiotic disc	Disc potency	Resistance %	Intermediate %	Sensitive %
Azithromycin (AZM)	15 mcg	-	-	100
Cefaclor (CEC)	30 mcg	100	-	-
Sulfa/trimethoprim (COT)	23.75/1.25 mcg	-	-	100
Nitrofurantoin (F)	300 mcg	-	-	100
Ciprofloxacin (CIP)	5 mcg	-	100	-
Doxycycline (DO)	30 mcg	-	-	100
Amoxicillin-Clavulanic acid (AMC)	20/10 mcg	-	-	100
Cefoxitin (FOX)	30 mcg	100	-	-
Gentamycin (CN)	10 mcg	-	66.7	33.3
Tobramycin (TOB)	10 mcg	66.7	33.3	-
Fosfomycin (FO)	200 mcg	-	-	100
Oxytetracycline (T)	30 mcg	-	-	100
Cefadroxil (CFR)	30 mcg	100	-	-
Neomycin (N)	10 mcg	-	33.3	66.7
Colistin (CL)	10 mcg	-	-	100

Table 2: Oligonucleotide primers sequences and annealing temperatures

Bacterial spp.	Gene	Sequence	Amplified product	Annealing temp.	Reference
Salmonella	*invA*	GTGAAATTATCGCCA CGTTCGGGCAA / TCATCGCACCGTCAA AGGAACC	284 bp	55	**(Oliveira et al., 2003)**
	stn	TTG TGT CGC TAT CAC TGG CAA CC / ATT CGT AAC CCG CTC TCG TCC	617 bp	59	**(Murugkar et al., 2003)**
	pefA	TGT TTC CGG GCT TGT GCT / CAG GGC ATT TGC TGA TTC TTC C	700 bp	55	**(Murugkar et al., 2003)**
	sopB	TCA GAA GRC GTC TAA CCA CTC / TAC CGT CCT CAT GCA CAC TC	517 bp	58	**(Huehn et al., 2010)**
	hilA	CATGGCTGGTCAGTT GGAG / CGTAATTCATCGCCT AAACG	150 bp	60	**Yang et al.,) (2014**
	Integron	TGCGGGTYAARGAT BTKGATTT / CARCACATGCGTRTA RAT	491 bp	55	**(White et al., 2000)**
Helicobacter spp.	*16S rRNA*	AAG GAT GAA GCT TCT AGC TTG CTA / GTG CTT ATT CGT GAG ATA CCG TCA T	398 bp	54	**(Shojaee Tabrizi et al., 2015)**
H. felis	*urea, ureB*	GTG AAG CGA CTA AAG ATA AAC AAT / GCA CCA AAT CTA ATT CAT AAG AGC	241 bp	62	**(Camargo et al., 2003)**
H. heilmanni	*ureB*	GGG CGA TAA AGT GCG CTT G	580 bp	58	**(Arfaee et al., 2014)**

Table 3: Occurrence of virulence genes in isolated *Salmonella*.

Isolates No. and its serotype.	*inva*	*Stn*	*pefA*	*sopB*	*hilA*	*Integron*
1. S. *Bovismorbificans*	+	+	+	+	+	-
2. S. *Bovismorbificans*	+	+	+	+	+	-
3. S. *Southampton*	+	+	-	+	+	-

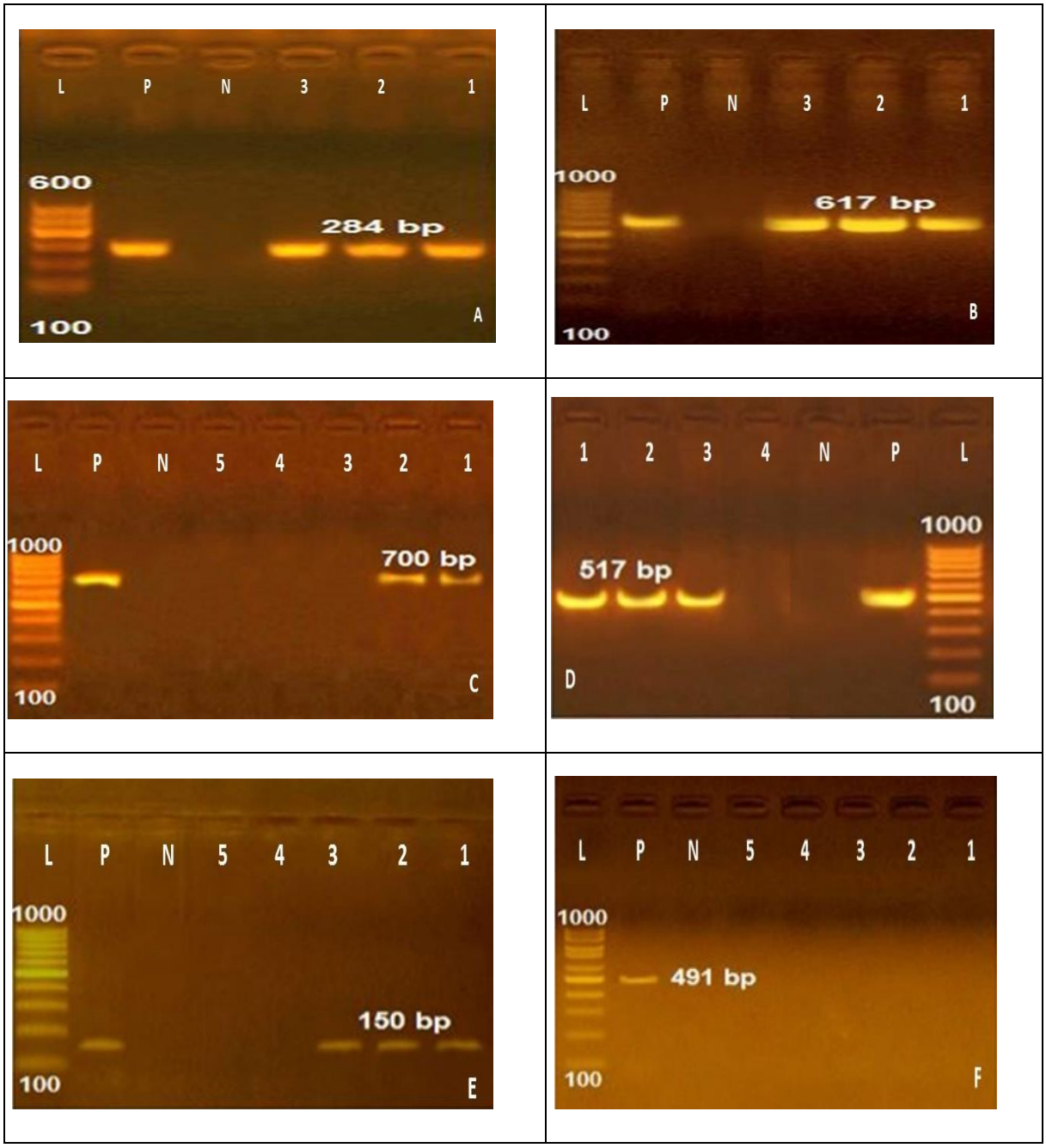

Figure (1): Molecular identification of virulence genes of *Salmonella* isolates, Lane (L): DNA marker, lane (**P**): positive control, lane (**N**): negative control, **(A)**: 1, 2, 3 lanes are positive for *inva* gene, **(B)**:1, 2, 3 lanes are positive for *stn* gene, **(C)**: 1, 2 lanes are positive for *pefA* gene, **(D)**: 1, 2, 3 lanes are positive for *sopB* gene. **(E)**: 1, 2, 3 lanes are positive *Salmonella* for *hilA* gene, **(F)**: 1, 2, 3 lanes are negative for *integron* gene.

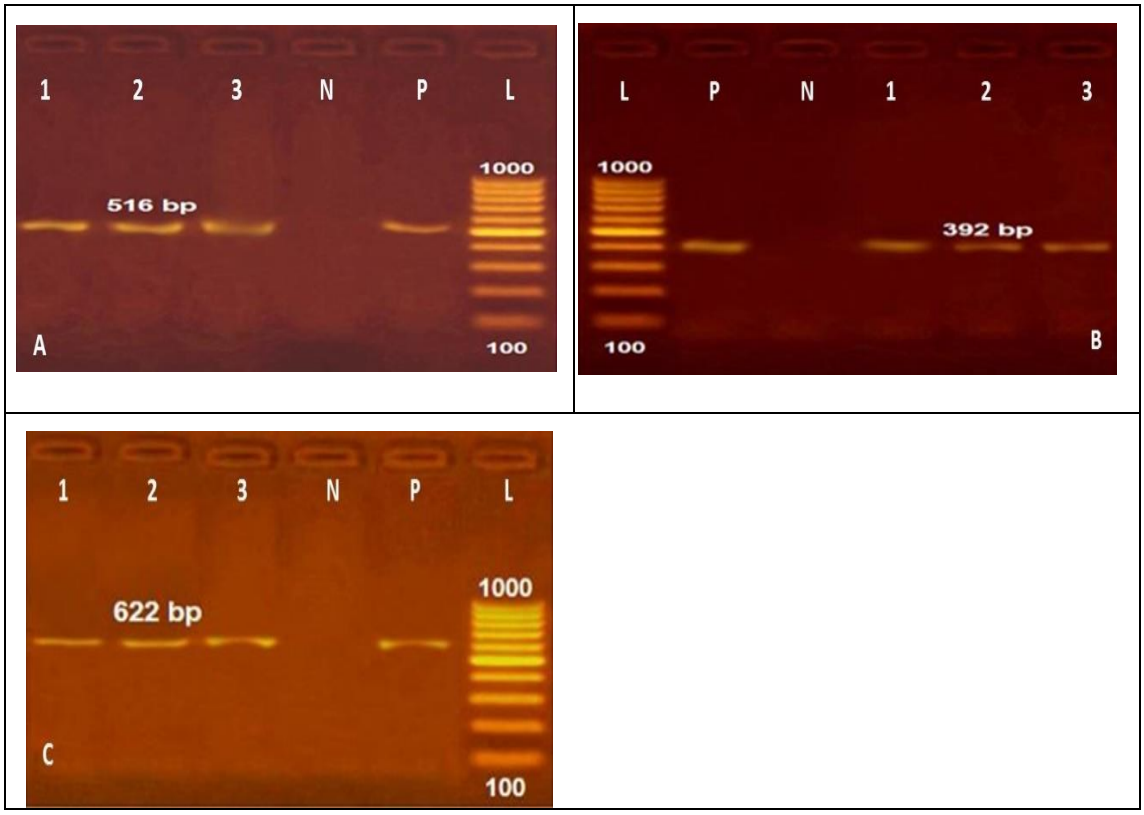

Figure (2): Molecular identification of Antibiotic resistance genes of *Salmonella* isolates, Lane (L): DNA marker, lane (**P**): positive control, lane (**N**): negative control, **(A)**: 1, 2, 3 lanes are positive for *bla*$_{TEM}$ gene, **(B)**:1, 2, 3 lanes are positive for *bla*$_{SHV}$ gene. **(C)**: 1, 2, 3 lanes are positive for *aadA* gene.

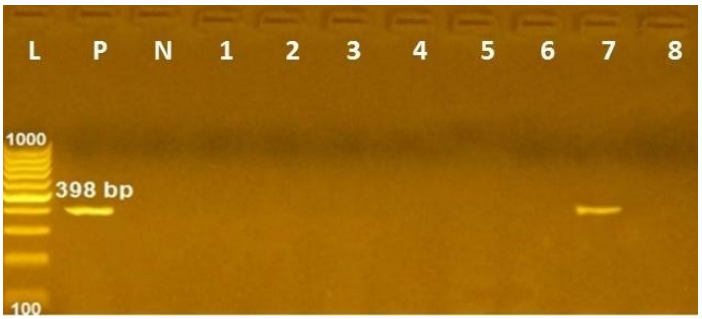

Figure (3): Agarose gel electrophoresis of *Helicobacter felis*.

L lane: 100 bp DNA ladder P lane: control positive N lane: control negative
Lane (7) is positive for *Helicobacter felis.*

DISCUSSION

Wild animals act as a reservoir of many infectious and zoonotic diseases. Diseases transmit to people when animals are in close contact with human, such close contact can occur in zoo and captive breeding centers (Green *et al.*, 2020).

In the present study, the rate of isolated *Salmonella* from wild felids were (5%) which is lower than Clyde *et al.* (1997) who isolated *Salmonella* at percent (95%) from (leopard, snow leopard, cougars, serval and caracal) and Venter *et al.* (2003) who isolated *Salmonella* at percent of (39.5%) from lions and cheetahs. This difference may returns to the type and quality of diet fed to animals in each study as the food

contamination may consider as a major source for *Salmonella* affection where Harrison *et al.* (2006) isolated *Salmonella* at percent 28% from carcass meat fed to zoo carnivores.

From all examined felids in the current study, *Salmonella* was isolated only from wild cat (60%) (*Felis chaus*) and that may return to their diet, where the wild cat is the only species in this study fed on poultry meat while the other species fed on donkey and beef meat. Poultry meat is considered as the most common source of transmission of *Salmonella* (Percival and Williams, 2014) or the transmission may occur via workers due to the zoonotic nature of isolated serovars.

In the current study, two servars of *Salmonella* were detected from wild cats; *S. Southampton* with antigenic formula (O4,12,27;r,Z6) and *S. Bovismorbificans* with antigenic formula (O6,8,20;r[i],1,5). Both serovars are zoonotic and considered as a potential pathogen for human.

Salmonellosis is among the most frequent zoonotic infections in many countries and serovar *Bovismorbificans* is the primary cause of *Salmonella* outbreak infection in human in Netherlands (Brandwagt *et al.,* 2018), Germany (Gilsdorf *et al.,* 2005) and in Queensland (Stafford *et al.,* 2002)

S. Bovismorbificans was previously isolated from pet cats (Van Immerseel *et al.,* 2004) and captive reptiles (Pedersen *et al.,* 2009) while little knowledge about the isolation of *S. Southampton* and *S.* Bovismorbificans from wild felidae is available.

In the present study, *Helicobacter* spp. was isolated from one lion of total 8 lions (12.5%) which had a previous history of vomiting. *Helicobacter* spp. was previously isolated from domestic and feral cat (Ghil *et al.,* 2009) and from wild felids like cheetah (Terio *et al.,* 2005), lynx (Mörner *et al.,* 2008), and in Bengal tiger (Tegtmeyer *et al.,* 2013). *Helicobacter felis* has zoonotic importance and is considered as a potential pathogen in humans (Heilmann and Borchard, 1991). The fecal oral route is the main route of transmission in both cat and human (Ghil *et al.,* 2009). The fecal contamination for water and soil play a role in spreading of the infection (Hopkins *et al.,* 1993). The source of the infection for *Helicobacter* in the current study was unknown, may from the feral cat which roaming inside the zoo or from the zoo keepers due to the zoonotic nature of the isolated sample.

Bacterial adaptation to antibiotics has been extremely increased and causes significant medical problems in the last decade. Apex predators are at the top of the food chain and occupy higher trophic level. Trophic accumulation of Pollutants, and toxins can occur, in such way, the antimicrobial resistance can follow this trophic accumulation pattern from low to high trophic level (Jobbins and Alexander, 2015).

Salmonella isolates in the current study were sensitive to Sulfa/trimethoprim, Amoxicillin-Clavulanic acid, Gentamycin and Oxytetracycline, this agree with the Van Immerseel *et al.* (2004)

In this study, bla_{TEM} gene was detected in three isolates of *Salmonella,* this agree with Van Immerseel *et al.* (2004) who found bla_{TEM} gene in *Salmonella* isolated in cat. Also, bla_{SHV} and *aadA2* antibiotic resistant genes were detected in three isolates of *Salmonella.*

The severity of disease caused by genus *Salmonella* depends on virulence genes such as *invA* gene which enables it to invade, penetrate and cause infection in host epithelial cells (Mubita *et al.,* 2020). Such essential gene was recorded in three isolates of genus *Salmonella* from wild cats in the current study.

There are many virulence genes in *Salmonella* responsible for pathogenicity such as *sop* gene that encode *Salmonella* outer proteins and the *hilA* gene, these genes

are important for penetration of cells and survival of *Salmonella* in macrophage (Ammar *et al.,* 2016). All of these gene were recorded in all *Salmonella* isolates in the current study, this agree with Van Immerseel *et al.* (2004) who detected *SopB* gene in all *Salmonella* isolates in his study and with Pathmanathan et al. (2003) who found *hilA* gene in *Salmonella bovismorbificans.*

CONCLUSION:

Data in the current study highlight on the zoonotic nature of *Salmonella* and *Helicobacter* spp. which may infect the captive wild felids and cause a hazard for veterinarians and zoo keepers which routinely dealing with these animals in addition to detection for the antibiotic resistance and virulence genes that may pose a risk for failure of treatment of the captive wild felids.

Further studies are recommended to determine the source of infection for both zoonotic *Salmonella* and *Helicobacter* species in the captive wild felids.

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الكشف عن أنواع السالمونيلا والهليكيوباكتر في القطط البرية الاسيرة

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تعتبر القطط البرية هي الحيوانات الأكثر شعبية في حديقة الحيوان ولكنها تعتبر أيضا مصدرللأمراض ليس فقط للحيوانات الأخرى ولكن أيضا للإنسان مثل الأطباء البيطريين والعاملين والزوار. تهدف الدراسة الحالية الي التحقق من وجود اصابات للقطط البرية بالسالمونيلا والهليكيوباكتر بالإضافة إلي عمل السيرولوجي وإختبار الحساسية للمضادات الحيوية وتحديد جينات مقاومة المضادات الحيوية وجينات الضراوة في معزولات السالمونيلا. تم تجميع ٦٠ عينة براز من ٣٠ حيوان تابع للعائلة القطية البرية من حديقة الحيوان بالجيزة وحديقة حيوان خاصة في مصر. كانت جميع الحيوانات بصحة جيدة فيما عدا ثمانية أسود أفريقية كانت لها تاريخ سابق من القئ. تم فحص عينات البراز للكشف عن السالمونيلا بالطرق البكتريولوجية والإختبارات البيوكيميائية والسيرولوجية بالاضافة الي عمل اختبار الحساسية للمضادات الحيوية والكشف عن جينات مقاومة المضادات الحيوية وجينات الضراوة في معزولات السالمونيلا. أما عن عينات البراز المجمعة من الاسود التي لديها تاريخ سابق من القئ فتم الكشف عن الهليكيوباكتر فيها بالطريقة الجزيئية بشكل مباشر. تم عزل السالمونيلا من عدد ثلاث قطط برية وبالكشف السيرولوجي على المعزولات وجدت انها تنتمي إلي نوعين *S.Bovismorbificans* و *S. Southampton* بينما اصيب نوع واحد من الاسود الأفريقية بالهليكيوباكتر *Helicobacter felis*. أظهرت عزلات السالمونيلا مقاومة كاملة للسيفاكلور (١٠٠%)، سيفوكسيتين (١٠٠%) والسيفادروكسيل (١٠٠%)، ومقاومة عالية جدا للتوبراميسين (٦٦،٧%). في حين أظهرت حساسية كاملة للأزيثروميسين (١٠٠٪) ، السلفا / تريميثوبريم (١٠٠٪) ، النيتروفورانتوين (١٠٠٪) ، الدوكسيسيكلين (١٠٠٪) ، حمض الأموكسيسيلين-كلافولانيك (١٠٠٪) ، فوسفوميسين (١٠٠٪) وأوكسي تتراسيكلين (١٠٠٪). تم تأكيد وجود جينات bla_{TEM} و bla_{SHV} في عزلات السالمونيلا التي أظهرت مقاومة للسيفاكلور و سيفوكسيتين ووجود جين aadA2 في عزلات السالمونيلا المقاومة للتوبراميسين. اما عن جينات الضراوة فتم تأكيد وجود جينات *invA* و *stn* و *sopB* و *hilA* في نوعين السالمونيلا المعزولة بينما تحمل *S. Bovismorbificans* أيضا جين *pefA*.