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Bacterial Screening of Sporadic Cases of Captive African lions (Panthera leo) at the Egyptian National Circus

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ABSTRACT

Although African lions (Panthera leo) are listed as vulnerable species, it is a famous practice in certain nations to display them in the circus. To date, the gastrointestinal bacterial flora of lions is poorly understood. This study aimed to clarify the zoonotic potential isolation of the bacteria posed by lions of the Egyptian national circus along with PCR screening for their virulence and antibiotic resistance genes. Nine captive African lions were examined bacteriologically. *Clostridia spp.* was the most prevalent bacteria (55.5%) in lions, then *Staphylococcus spp.* (33.3%), followed by *E. coli* and *Edwardsiella spp.* (22.2% for each) and *Serratia spp.* (11.1%). The isolated bacteria showed multiple drug resistance patterns of multiple antibiotics (ampicillin, cefepime, cefoxitin, piperacillin, amoxicillin, chloramphenicol, ceftazidime and tetracycline). Two isolates of *C. perfringens* were positive for Alpha toxin and *bla* resistant genes using PCR. Also, all *S. aureus* isolates were confirmed by the presence of 16s rRNA gene and exhibited the virulence (*spa*, and *coa)* and resistance (*bla*Z) genes. Additionally, the two isolated *E. coli* strains were positive for virulence (*pho*A, *eae*A) and resistance (*Aada1*) gene but, Aada2 was completely absent. In conclusion, this study is actually unprecedented and provides a start for the upcoming research to investigate the current health status of captive wild animals exhibited at the circuses.

INTRODUCTION

Displaying wild animals in circuses continues to be a famous practice today in certain nations although; it first originated in Egypt in the remnants of Memphis and Thebes and later to the monster tamers of old Rome and Greece. However, animal circuses are a debatable practice that can reduce their welfare and expose them to multiple mental and health states. Therefore, regular investigations of the animal health status of the circus animals would contribute to keeping them safe in their life (Mota-Rojas *et al.*, 2022).

According to Groom *et al.* (2014), African lions (Panthera leo) are considered the culturally adored and iconic species of wildlife in Africa, which balance the animal populations through predation, which is crucial for biodiversity conservation. However, lions are listed as vulnerable on the red list of the International Union for the Conservation of Nature (IUCN) as a result of the massive global decline of their numbers which recorded approximately 43% over the course of the past 21 years (Bauer *et al.,* 2016).

Big carnivores are very important to the integrity and stability of most ecosystems (Murray *et al.,* 1999). Meanwhile, exposure to infectious diseases might negatively impact their populations especially wild felids (Akanbi *et al.,* 2021). Moreover, several bacterial infections were documented in large felids, the causative bacteria were isolated mainly from the mouth, nostril, or rectum of both diseased and healthy wild or domestic animals and under certain conditions, such as stress could recur, develop into pathogenic form causing the disease in host animals (Sabapara *et al.,* 2010).

Gastrointestinal illnesses could affect the captive animals more frequently due to poor management, inappropriate diet, or infectious bacterial agents such as *Salmonella*, *Shigella*, *E. coli* and *Clostridium* spp. (Uddin *et al.,* 2017). Also, the rates of *Salmonella* shedding in captive felids had been decreased when they were fed a good safe uncontaminated diet free from *Salmonella* infection minimizing the zoonotic health risk for animal care workers (Lewis *et al.,* 2002) reported.

The typical bacterial flora of large felids' gastrointestinal is poorly understood. Although, Jia *et al.* (2017) revealed the intestinal bacterial diversity of adult and young African lions in the same breeding environment, showed that it was lower in young lions and has a significant difference in various stages of African lions. Samu *et al.* (2021) conducted a study to determine the enteric fecal bacterial pathogen cultures of some zoo animals, and their diagnostic value as a part of the routine protocols of preventive medicine at zoos.

According to our knowledge, this study was carried out for the first time in Egypt to comprehend the zoonotic potential isolation of the bacteria and the health risks posed by lions; the inhabitant of the national circus along with the PCR screening for their virulence and antibiotic resistance genes of the isolated bacteria.

MATERIALS AND METHODS

1. Ethical Approval:

All the procedures of the study were adapted according to the ethical and humane principles of the Ethics and Animal Experimentation Committee of Suez Canal University (Approval No.2022031). Nine captive apparently healthy African lions (Panthera leo) of different ages and sex (3 males and 6 females) were selected and examined as shown in Table (2). They were belonging to the Egyptian national circus in which they were living in individual cages and feeding every other day.

2. Sampling:

The fecal samples were collected under sterilized conditions using clean sterile bacteriological swabs then they were transported in an icebox within 2-3 hours without any delay to be examined bacteriologically at the bacteriological laboratory at Animal Health Research Institute (AHRI), Ismailia branch, ARC, Egypt.

3. Laboratory Scheme For Bacterial Isolation In Lion Cases:

All fecal swabs were enriched in buffered peptone water broth for the isolation of aerobic bacteria and cooked meat medium (CMM) broth for the isolation of anaerobic bacteria. The isolation of *Staphylococcus* spp. was carried out according to (ISO/IEC 6888-1:1999-AMD/2003) and for isolation of *E. coli*, it was done according to Mac Faddin (2000). However, for the isolation of anaerobic bacteria (*Clostridium species*),

the procedures were performed according to Smith and Holdeman (1968). Moreover, all the recovered bacterial isolates were phenotypically, microscopically and biochemically identified (Quinn *et al.*, 2002). The serological identification for the recovered *E. coli* isolates was done according to the manual of the Reference Lab for Veterinary Quality Control on Poultry Production, Animal Health Research Institute (AHRI), Dokki, Giza, Egypt (Ewing, 1986). Furthermore, all the recovered identified bacterial isolates were preserved in tryptone broth at 1% after adding glycerol. Then these bacterial strains were kept at −20°C for further PCR analysis.

4. Antimicrobial Susceptibility Testing:

The susceptibility of the purified yielded bacterial isolates were tested against the most commonly used antibiotics (Ampicillin, Gentamycin, Tobramycin, Cefepime, Cefoxitin, Levofloxacin, Piperacillin, Trimethoprim/Sulfa, Amoxicillin, Ciprofloxacin, Amikacin, Chloramphenicol, Ceftazidime, Minocycline, Tetracycline) in this investigation study according to the Standard Kirby-Bauer disc diffusion method and then the results were interpreted according to CLSI (2020).

5. DNA Extraction:

DNA extraction from fecal samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. About 200µl of the sample suspension was incubated with 10µl of proteinase K and 200µl of lysis buffer at 56°C/10 min. After incubation, 200µl of 100% ethanol was added to the lysate. The samples were washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100µl of elution buffer provided in the kit.

6. PCR Investigation Of Virulence and Antibiotic Resistance Genes Of Isolated Bacteria:

The genomic DNA of bacterial isolates was extracted following the manufacturer's instructions for QIAamp DNA Mini Kit (Qiagen, Germany). Oligonucleotide primers (Metabion, Germany) were listed in Table (1), and utilized in a 25µl reaction volume with; 12.5µl of Master Mix (EmeraldAmp Max PCR Takara, Japan), 1µl of each primer of (20 pmol), 5.5µl of Dnase free water, and 5µl of DNA template were added. The reaction was performed in an Applied biosystem 2720 thermal cycler. All reactions included a negative and Positive control of reference strains provided from AHRI, Dokki, Giza, Egypt.

Finally, PCR products were separated for the analysis step using gel electrophoresis. The gel was prepared using 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. Then, for gel analysis, 20µl of the products were loaded in each gel slot and a 100 bp DNA ladder (Fermentas, Germany) also was used to determine the DNA fragment sizes. After that, the gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table 1: Primers sequences, target genes and amplicon sizes.

Target agent	Target gene	Primers sequences	Amplified segment (bp)	Reference
Clostridium perfringens	Alpha toxin	GTTGATAGCGCAGGACATGTTAAG	402	**(Yoo et al., 1997)**
		CATGTAGTCATCTGTTCCAGCATC		
	bla	ATGAAAGAAGTTCAAAAATATTTAGAG	780	**(Catalán et al., 2010)**
		TTAGTGCCAATTGTTCATGATGG		
Staphylococcus aureus	*coa*	ATA GAG ATG CTG GTA CAG G	350/430/570/630	**(Iyer and Kumosani, 2011)**
		GCT TCC GAT TGT TCG ATG C		
	spa	TCA ACA AAG AAC AAC AAA ATG C	226	**(Wada et al., 2010)**
		GCT TTC GGT GCT TGA GAT TC		
	16S rRNA	CCTATAAGACTGGGATAACTTCGGG	791	**(Mason et al., 2001)**
		CTTTGAGTTTCAACCTTGCGGTCG		
	blaZ	TACAACTGTAATATCGGAGGG	833	**(Bagcigil et al., 2012)**
		CATTACACTCTTGGCGGTTTC		
E. coli	*phoA*	CGATTCTGGAAATGGCAAAAG	720	**(Hu et al., 2011)**
		CGTGATCAGCGGTGACTATGAC		
	eaeA	ATG CTT AGT GCT GGT TTA GG	248	**(Bisi-Johnson et al., 2011)**
		GCC TTC ATC ATT TCG CTT TC		
	Aada1	TATCAGAGGTAGTTGGCGTCAT	484	**(Randall et al., 2004)**
		GTTCCATAGCGTTAAGGTTTCATT		
	Aada2	TGTTGGTTACTGTGGCCGTA	622	**(Walker et al., 2001)**
		GATCTCGCCTTTCACAAAGC		

RESULTS

Incidence of Different Bacteria in The Examined Lion Cases:

The bacterial investigation of fecal samples of the 9 African lions declared the presence of 13 bacterial species which were referred to as different 5 bacterial genera (5 of *C. perfringens*, 3 of *S. aureus*, 2 of *E. coli*, 2 of *Edwardsiella* spp. and 1 of *Serratia* spp.). As shown in Table (2), all cases harbored at least one bacterial species, 6 samples were positive to a single bacterium. Two samples were positive for two bacteria. One sample was positive for three bacterial species. In Table (3), the percentage of different yielded bacteria was calculated in relation to the total number of isolates (n=13) and to the total number of samples (n=9). About 55.5% of lion cases of both sexes at the age of 8-11 years old exhibited *Clostridia spp.*, meanwhile lesser rate (33.3%) of infection with *Staphylococcus spp.*, followed by *E. coli* and *Edwardsiella spp.* (22.2%, for each) in two female lions but one only male case was positive for *Serratia spp.* (11.1%) The five *C. perfringens* positive samples were confirmed only and typed as type A with conventional PCR technique since they failed to be isolated by the traditional culturing method. For *E. coli* serotyping, the results reported two different serovars; Polyvalent 2 O55 K99 and Polyvalent 3 O25 K11.

Table 2: Distribution of different bacterial species isolated from African lions.

No.	Sex	Age	*Clostridia*	*Staphylococcus*	*E. coli*	*Edwardsville*	*Serratia*
1	Female	11 years			+		
2	Female	7 years				+	
3	Female	7 years		+		+	
4	Female	8 years	+				
5	Female	11 years	+				
6	Female	11 years		+	+		
7	Male	8 years	+				
8	Male	9 years	+				
9	Female	10 years	+	+			+

Table 3: Incidence of different isolated bacterial species in African lions**.**

Isolated bacteria	No. of isolated bacteria	% To No. of isolates (n=13)	% To No. of swabs (n=9)
C. perfringens	5	38.5	55.5
S. aureus	3	23.1	33.3
E. coli	2	15.4	22.2
Edwardsiella spp.	2	15.4	22.2
Serratia spp.	1	7.7	11.1

Results of Antimicrobial Sensitivity Testing:

The yielded 3 isolates of *S. aureus spp.* in this study demonstrated different resistance patterns of multiple antibiotics (ampicillin, cefepime, cefoxitin, piperacillin, amoxicillin, chloramphenicol, ceftazidime, and tetracycline). Meanwhile, they were 100% sensitive to gentamycin, tobramycin, levofloxacin, trimethoprim/ sulfa, ciprofloxacin, amikacin, and minocycline. Concerning the 2 recovered *E. coli* strains, they were 100% sensitive to all tested antibiotics except minocycline and tetracycline antibiotics; they were resistant.

Virulence and Antibiotic Resistance Genes of Recovered *Clostridium perfringens, Staphylococcus aureus* and *E. coli*:

Direct PCR investigation for the 9 fecal swabs of the African lion cases detected 5 positive *C. perfringens* isolates in them. These 5 *C. perfringens* isolates were subsequently typed as 2/5 as of Alpha toxin using a specific toxin primer. Also, the *bla* resistant gene was clearly shown in two of *C. perfringens* strains Fig (1).

Regarding *S. aureus spp.*, it was confirmed by the presence of the 16s rRNA gene in all yielded strains. Moreover, all *S. aureus* isolates exhibited virulence (*spa,* and *coa)* and resistance (*bla*Z) genes Fig (2). In addition, the 2 isolated *E. coli* strains were positive for virulence (*pho*A, *eae*A) and resistance (*Aada1*) gene although, Aada2 was completely absent Fig (3).

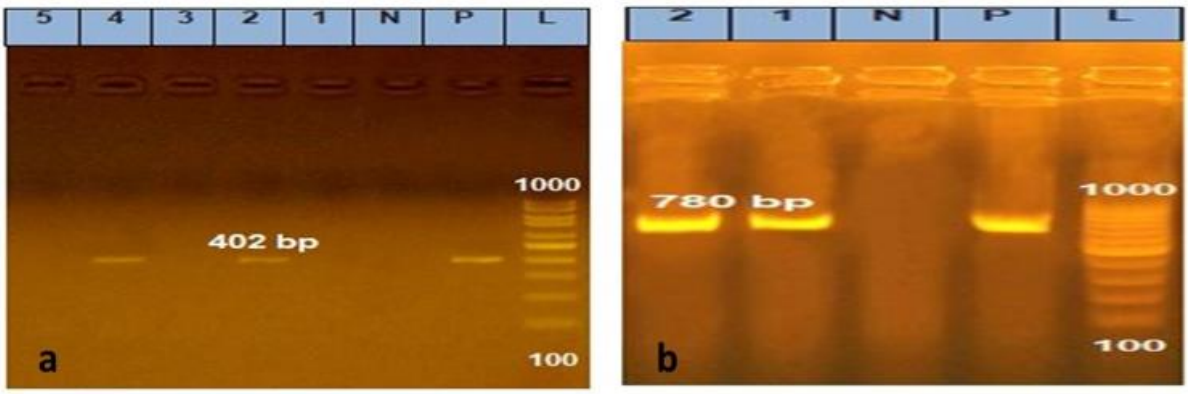

Fig. 1: (a, b) PCR Electrophoretic gel products for *C. perfringens* isolates (lanes 1-5), lanes 2,4 positive for *alpha toxin* at 402 bp and (1,2) positive bands of *bla* gene at 780 bp**.** Lane L: 100 bp DNA ladder. Lane P: positive control. Lane N: negative control.

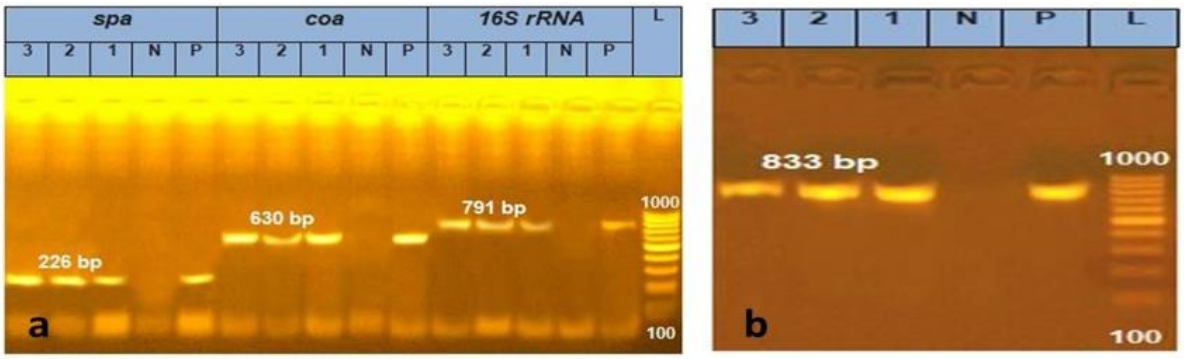

Fig.2: (a, b) PCR Electrophoretic gel products for *S. aureus* isolates (lanes 1-3), **a:** (1-3) positive for 16srRNA identification gene at 791 bp, (1-3) positive for virulence (*coa* and *spa)* genes at 630 bp and 226 bp, respectively. **b:** (1-3) positive for *bla*Z resistant gene at 833 bp. Lane L: 100 bp DNA ladder. Lane P: positive control. Lane N: negative control.

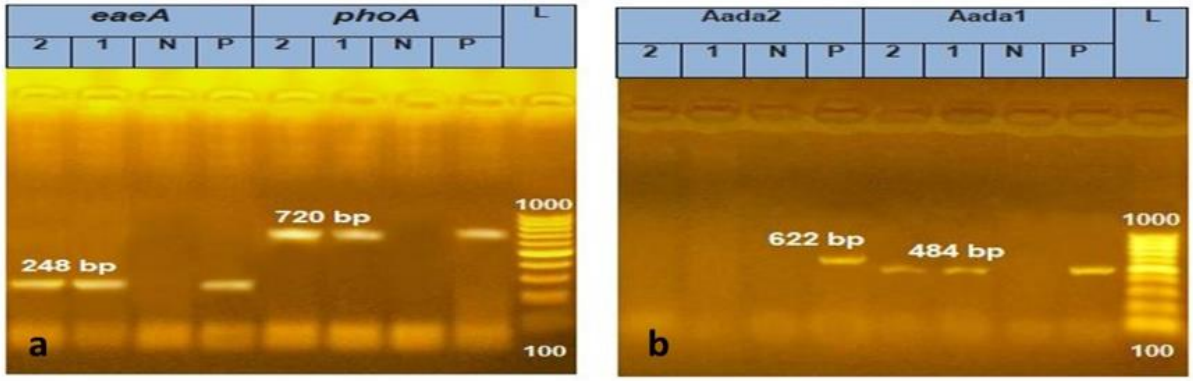

Fig. 3: (a, b) PCR Electrophoretic gel product of *E. coli* isolates lanes (1-2). **a:** (1-2) positive bands for virulence (*pho*A, *eae*A) genes at 720 bp and 248 bp, respectively. **b:** (1-2) positive bands for resistance genes ($Aada_1$) at 484 bp and negative for ($Aada_2$) at 622 bp, respectively.

DISCUSSION

African lions in entertainment and other commercial purposes have been popular for a long time (Green *et al.,* 2020). Lions could be live as actors in traveling circuses or might be held as a captive in zoos, and sanctuaries (Brando, 2016). Captive animals are generally more often under high-stress situations than wildlife (Marková *et al.,* 2019). Lions in circuses could have direct contact with people; such close contact between them and humans could permit multiple chances for spreading the most serious zoonotic human health hazards (Green *et al.,* 2020) and (Saad *et al.,* 2021). Many studies mentioned that *Clostridium perfringens* had been associated with disease in many animal species however; the shedding of *C. perfringens* in wild animals had been still limited and rare in wild animals (Hamzah, 2017). It is commensally inhabitant in animal GIT, soil and feedstuffs (Silva *et al.,* 2014). These organisms could revert to the pathogenic form under special circumstances like stress or immunocompromising conditions. The major virulence factors of *C. perfringens* are their toxins which are associated mainly with diarrhea and enterotoxaemia in animals (Hamzah, 2017).

In this work, *C. perfringens* was mostly recovered in (38.5%) of all examined cases of African lions that inhabited the Egyptian national circus. In a similar way, the prevalence of *C. perfringens* organisms in some captive wildlife species (including 20 leopards and 9 tigers) in India was 30% and 33.3% respectively, and this might be owed to the same captive conditions (Milton *et al.,* 2017). On the other hand, other studies in

large felids failed to isolate *Clostridia* spp (Howard *et al.,* 1993).

Moreover, *E. coli* organisms are crucial agents that are accused primarily for many cases of bacteremia, and coli-septicemia in lions, tigers and leopards, with gastroenteritis (Sabapara *et al.,* 2010). They also could cause urinary tract infections or pyelonephritis in captive jaguars (Wronski *et al.,* 2020). *E. coli* was isolated from aborted fetii and associated with abortion in lions and tigers (Singh *et al.,* 2022). Also, it was responsible for the death of 12 days old in lion cubs (Azam *et al.,* 2023).

Regarding *E. coli* spp. in this study, about (22.2%) of the examined fecal samples were positive for *E. coli* spp. Similarly, many retrospective studies discussed the main cause of high morbidity and mortalities in nondomestic felids was *E. coli* infections especially in the geriatric stage (Wronski *et al.,* 2020). Additionally, *E. coli*, *Mannheimia haemolytica* and *Salmonella* Spp.; were accounted for 41% of mortality cases in the examined wild captive animals in Nigerian zoological gardens (Akanbi *et al.,* 2021). Many diseases cases of lions and tigers had been found to be infected with multiple kinds of bacterial species comprising *E. coli* and *Klebsiella* (Singh *et al.,* 2022). Also, more than 63 different pathogenic organisms have existed in both captive and wild lions and some of them were of public health concern (Green *et al.,* 2020). In addition, many authors declared that *E. coli* was frequently isolated bacteria from the rectal swabs of cheetah and domestic cats, in zoo lions, tigers and leopards and in African lions and Cheetah at Kuwait zoo, respectively (Howard *et al.,* 1993; Mahmoud, 2015; Sabapara, 2002). Similarly, *E.coli* was recovered from fecal samples of tigers and lions at (55.55% and 72.72%), respectively (Uddin *et al.,* 2017). The variation could be attributed to the small size of samples in the circus. Also, Akanbi *et al.* (2021) reported that *E. coli* was responsible for the death of some ungulates rather than felids in 3 different zoological gardens.

Over the past several years, *S. aureus* is regarded as the zoonotic bacterial colonizer in humans and a variety of wildlife species (Heaton *et al.,* 2020). In the current investigation, *S. aureus* was detected in 33.3% of examined lion cases in (2 females and 1 male) with a (23.1%) prevalence rate. Many reviews stated the presence of *S. aureus* in captive animals either in zoos or research facilities, also in animals in sanctuaries and parks. In addition, in the United Kingdom, *S. aureus* along with other mecA-positive staphylococci was identified in foxes (Carson *et al.,* 2012). The staphylococcal infection was documented in many species of wild life as in black rhinoceros (Diceros bicornis) in Kenya, red deer in Poland (Gnat *et al.,* 2015) and in fecal samples from slaughtered reindeer in Finland and Norway (Laaksonen *et al.,* 2017). Also, it was isolated from lions at a zoo in Denmark in 2012 (Heaton *et al.,* 2020)

Furthermore, other bacteria were also studied. For example, *Edwardsiella* and *Serratia* spp. were recovered for the first time from African lions in 15.4% and 7.7%, respectively however, *Edwardsiella* failed to be isolated from the tested zoo collection (Samu *et al.,* 2021).

Wildlife could play a vital role in the spreading of the epidemiology of virulence and resistance genes in both animal and human environments. The inherited elements or bacteria that are naturally present in the environment, soil, or water can be stored and widely transmitted in wildlife and could be amplified with improper human activity (Heaton *et al.,* 2020). A combination of potent virulence genes like papC, sfa, hlyA, and cnf1 of *E. coli* strains was detected frequently (Tramuta *et al.,* 2011). This is corresponding to our results in this study that clarified the presence of more different virulence (*pho*A, *eae*A) and (*spa,* and *coa)* genes of the isolated strains of *E. coli* strains

and *S. aureus* spp isolates as shown in (Figs. 2 & 3).

Recently, the antimicrobial drug resistance (AMR) phenomenon has been globally detected and widely spreading in wild animals making a threat to human health safety since it has been hard to monitor bacterial infection in wild animals and the best time for administration of antibiotics in their treatment (Singh *et al.*, 2022). The MDR of *E. coli* and *S. aureus* spp. to different antimicrobials had been reported in this study. In the same trend, MDR with the carbapenems-producing activity of *E. coli* strains and *K. pneumoniae* strains with ESBL activity were recorded in the tracheal swab and spleen of lions; both bacterial species were highly susceptible to colistin (Singh *et al.*, 2022). Moreover, the isolated *E. coli* strain in felids exhibited great resistance to penicillin, cephalothin, and amoxicillin, and intermediate susceptibility to ampicillin (Wronski *et al.*, 2020). Also, in the United States, 98% of most strains of *E. coli* from the urinary tract of domestic felids were found resistant to at least one antibiotic, and 62% of strains were resistant to cephalothin and ampicillin, respectively (Liu *et al.*, 2015). Similar findings to the antibiotic resistance profile of some *E. coli* strains from domestic and wild felids, as well as for humans were also recorded (Carvallo *et al.*, 2010).

It was also stated in previous reports that several populations of *S. aureus* in wildlife could serve as important reservoirs transmitting the infection to the nearby domestic livestock, poultry and directly or indirectly to humans. Such a reservoir of *S. aureus* might lead to the creation of novel resistant strains due to the exchange of antibiotic-resistance genes of *S. aureus* among humans and various animal species. The resistance to one or more antibiotics especially methicillin-resistant *S. aureus* (MRSA) was reported (Heaton *et al.*, 2020). Correspondingly, this study indicated the MDR traits of isolated *E. coli* and *S. aureus* strains in African lions which were regarded as zoonotic pathogens and thus might threaten other circus animals and the workers too. The spreading of these MDR bacteria could explain the negative response to antibiotic treatment in wildlife like lions and jaguars that was adopted by veterinarians in the zoo or circuses (Wronski *et al.*, 2020).

Conclusion:

In fact, there is a dearth of information concerning the diversity of bacterial species that could be a commensal inhabitant in the gastrointestinal or the respiratory tract of captive lions which in turn might be converted to pathogenic ones causing serious disease. This study could provide some potentially valuable information about some isolated bacteria in African lions inhabiting the Egyptian Circus for the first time with the detection of their genetic virulence and MDR resistance traits. Moreover, there is a special need for scientists and researchers to review and update the current preventive and management policies to identify and control the spreading of these bacteria in wildlife. The strict hygienic measures for humans who deal with lions should include hand sanitizing and stepping points to disinfect shoes since the presented data indicated a potential zoonotic risk to wild animals and public health.

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ARABIC SUMMARY

الفحص البكتيري لحالات متفرقة من الأسود الأفريقية الأسيرة (*Panthera leo*) في السيرك القومي المصري

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على الرغم من أن الأسود الافريقية (*Panthera leo*) مدرجة كأنواع معرضة للخطر إلا أن عرضها في السيرك يعتبر ممارسة مشهورة في بعض البلاد. حتى الآن البكتريا المعدية المعوية للأسود غير مفهومة بشكل جيد. ولذلك هدفت هذه الدراسة إلى توضيح البكتريا المعزولة من أسود السيرك القومي المصري إلى جانب فحص تفاعل البلمرة المتسلسل لشدة ضراوتها وجيناتها المقاومة للمضادات الحيوية. تم فحص تسعة أسود أفريقية أسيرة بكتريولوجيا وكانت الكلوستريديا هي البكتيريا الأكثر انتشارا في الأسود (55.5٪), ثم المكورات العنقودية (33.3٪) ، تليها الإشيريشيا القولونية والإدواردسييلا (22.2٪ لكل منهما) ثم السيراتيا (11.1%). وقد أظهرت البكتيريا المعزولة أنماطا متعددة المقاومة للمضادات الحيوية مثل (الأمبيسلين ، السيفيبيم ، سيفوكستين ، بيبيراسيلين ، أموكسيسيلين ، الكلورامفينيكول ، السيفتازيديم و التتراسيكلين) . وأوضح تفاعل البلمرة المتسلسل أن هناك عزلتان من C. perfringens ايجابيتان لتوكسين الألفا وعزلتان ايجابيتان لجينات المقاومة *bla*. أيضا تم تأكيد جميع عزلات المكورات العنقودية من خلال 16s rRNAوجينات الضراوة (*spa* & *coa*) وجين المقاومة *bla*Z. بالإضافة إلى ذلك، كانت عزلتا الايشيريشيا القولونية ايجابيتان لجينات الضراوة (phoA &eaeA) وجين المقاومة (Aada1) بينما جين (Aada2) كان سلبيا. وأخيرا تعتبر هذه الدراسة غير مسبوقة وتوفر البداية للأبحاث القادمة لفحص الحالة الصحية للحيوانات البرية الأسيرة في السيرك.