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Characterization and antimicrobial susceptibility of Salmonella enterica isolated from broiler chicks

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ORIGINAL ARTICLE

Characterization and Antimicrobial Susceptibility of *Salmonella* Enterica Isolated From Broiler Chicks

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Abstract

BACKGROUND: *Salmonellae* are frequently implicated in several harmful processes that affect both humans and animals, including poultry. This study aimed to isolate and identify *Salmonella* serovars from clinically diseased broiler chicks collected from poultry farms and to evaluate the susceptibility of the isolates to antimicrobial agents frequently used in poultry farms.
METHODS: In all, 200 broiler chicks were collected from broiler farms located in the Dakahlia Province and subjected to bacteriological examination to isolate *Salmonella*. *Salmonella* isolates were subjected to serological identification to detect *Salmonella* serovars circulating in broiler farms. In addition, the isolates were tested for susceptibility to antimicrobial agents.
RESULTS: In all, 18 *Salmonella* isolates were detected after confirming the recovered strains biochemically and by polymerase chain reaction targeting the *inv*A gene. Five serotypes were detected in the recovered strains: *Salmonella* Kentucky (n = 8), *Salmonella* Typhimurium (n = 6), *Salmonella* Derby (n = 1), *Salmonella* Infantis (n = 2), and *Salmonella* enteritidis (n = 1). Interestingly, *Salmonella* isolates displayed very high resistance to most antimicrobials. *Salmonella* isolates showed complete resistance to cefotaxime, kanamycin, amikacin, streptomycin, tetracycline, chloramphenicol, penicillin G, oxacillin, ampicillin, amoxicillin-clavulanic acid, nalidixic acid, ciprofloxacin, and fosfomycin. While cefoxitin displayed high sensitivity, all isolates displayed multidrug resistance to 11 or more antimicrobial agents.
CONCLUSIONS: *Salmonella* Kentucky and *Salmonella* Typhimurium were the predominant *Salmonella* serovars circulating on the selected farms. Furthermore, the higher antimicrobial resistance displayed by the recovered isolates necessitates strict strategies against the use of antimicrobials in poultry farms and underscores the importance of finding new substitutes for antibiotics in poultry farms.

Keywords: Antimicrobial susceptibility, Broilers, *inv*A, *Salmonella*, Serotyping

1. Introduction

Salmonella is one of the most significant zoonotic pathogens in foodborne diseases. It has more than 2600 serotypes and can cause gastrointestinal infections in humans and animals, including gastroenteritis, typhoid fever, and paratyphoid fever. It can also cause serious illnesses in younger and older people and can lead to death [1–3]. Humans consume a variety of foods, including meat, eggs, seafood, vegetables, beef, pork, and poultry, particularly broilers and layer chickens [4,5]. Despite multiple control methods, *Salmonella* continues to contaminate poultry meat supply and cause self-limiting gastroenteritis in healthy humans and typically recovers within a week without antibiotics. The infection is severe in immunocompromised individuals, children, and older adults, with a low infectious dose [6]. *Salmonella*-resistant strains can cause severe illnesses, potentially leading to longer hospital stays [7].

Poultry farms have a significant agricultural industry owing to the valuable source of daily protein required by humans [8]. *Salmonella* contamination

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in poultry farms and their products can occur at several stages. In primary production, *Salmonella* contamination can originate from a variety of sources, including contaminated feed and water, asymptomatic birds, wild birds, rats, and flies [9–11]. Second, contact with contaminated cage surfaces during transportation and market display, among other activities, have been identified as potential sources of *Salmonella* infection during the postproduction phase of chicken processing in slaughterhouses [11,12]. Finally, it is impossible to rule out the possibility that food handlers at formal and informal food vendors, as well as in households, directly contaminate poultry items while cooking.

Antibiotics are used as growth promoters in modern food and animal agriculture, particularly in the production of broiler chickens and for therapeutic purposes [13]. Antibiotics were initially designed for diseased animals, but because of contamination transfer, they can be more effective in treating entire flocks through feed or water administration [14]. Antibiotic overuse raises concerns about the transfer of antibiotic-resistant bacteria from farm animals to humans, potentially contaminating food during slaughter or processing [15]. The increasing use of antibiotics has led to the emergence of resistance [16], which has led to a growing public health threat of antimicrobial resistance (AMR) [17]. If appropriate action is not taken, it is predicted that AMR will result in 300 million human deaths, 100 trillion USD financial losses, and an 11% decline in animal productivity by 2050 [18]. Low- and middle-income countries in Asia and Africa account for the vast majority of impacted countries [19]. *Salmonella* causes 410,000 antibiotic-resistant infections annually in the United States, highlighting the long-standing use of antibiotics for disease prevention and growth promotion in poultry production. The overuse of antibiotics has led to a public health crisis, with *Salmonella* developing resistance and potentially rendering antibiotics ineffective in foodborne outbreaks, particularly in improperly handled poultry [20].

Although *Salmonella* spp. have been isolated from poultry, poultry products, and environmental samples in previous studies [21–23], periodic surveillance is still needed for *Salmonella spp.* in Egypt's broiler farms to ensure better poultry production practices that are incorporated into public health. Therefore, this study aimed to determine the prevalence of *Salmonella* in infected broiler farms in Dakahlia Province, Egypt, and to identify the common circulating *Salmonella* serovars in the selected broiler farms. In addition, the isolated serovars were susceptible to the most commonly used antimicrobials.

2. Materials and methods

2.1. Sample collection

This study was conducted from November 2021 to May 2022 on 200 samples from 100 clinically diseased broiler chicks of 20 selected broiler chicken flocks ranging in age from 1 week to 5 weeks. The selected farms are located in Dakahlia, Egypt. Chicks aged 1 week from every broiler farm showed lameness, droopy wings, chalky diarrhea, ruffling feathers, dehydration, decreased body weight, and severe mortality. Five clinically diseased birds were selected randomly from each flock. Pooled samples from the liver, spleen, and kidneys were collected from each bird (n = 100), and intestinal contents (n = 100) were sampled aseptically. With as little delay as possible, the samples were shipped in an ice box to the laboratory for bacteriological analysis.

2.2. Bacteriological examination

Standard operating procedures [24] were followed for isolation and identification of *Salmonella*. Samples were pre-enriched separately with buffered peptone water (BPW, HiMedia, Mumbai, India) (1:9) and incubated at 37 ± 1 °C for this duration. The Rappaport-Vassiliadis Medium (Oxoid, Hampshire, England) was then added to 0.1 mL of the enrichment broth and left at 42 °C for 24 h. The RVS culture was then streaked onto xylose lysine deoxycholate agar using a 10 µl bacteriological loop, and it was then incubated at 37 °C ± 1 for the entire night. The cells were subcultured with xylose lysine deoxycholate (XLD, Oxoid, Hampshire, England). MacConkey's agar medium (HiMedia, Mumbai, India) was used as a selective differential medium for *Salmonella*. Suspected *Salmonella* colonies (pink colonies on XLD with or without black center and pale colonies on MacConkey's agar) were incubated for 24 h at 37 °C ± 1 [25]. To facilitate further processing, suspected *Salmonella* isolates were stored in 25% glycerol and kept at −20 °C.

Suspected *Salmonella* colonies were then subjected to Gram staining for morphological identification and biochemical investigation, in accordance with the recommendations provided by the International Organization for Standardization (ISO 6579:2002).

2.3. Biochemical identification

Pure pink colonies on XLD agar with black center coloration and pale colonies on MacConkey's agar medium were considered suspected colonies of

Salmonella spp. Biochemical identification was performed according to Lamboro [26]. IMViC reactions, which included assays for indole, methyl red, Voges Proskauer, oxidase, and citrate consumption, were performed. In addition, hydrogen peroxide generation and urease hydrolysis were conducted on suspected *Salmonella* isolates [27]. The triple sugar iron (TSI) agar test was conducted according to Waltman [28]. In brief, a pure colony was stab-inoculated into the TSI medium and then aerobically incubated for 24 h at 37 °C.

2.4. DNA extraction

DNA was extracted by the boiling method; briefly, three presumptive *Salmonella* colonies were inoculated into 3 ml of trypticase soy broth and incubated for 18 h at 37 °C. Approximately 1 ml of the previously inoculated broth was centrifuged at 8000×*g* for 2 min. The sediment was washed with DNase/RNase-free water and heated at 95 °C for 15 min, and the supernatants were stored at −20 °C for further molecular characterization to be used as a DNA template.

2.5. Molecular identification of salmonellae

Polymerase chain reaction (PCR) was used to detect *inv*A, a marker gene that serves as a determinant for the detection of *Salmonella* species. PCR was performed using the following sequences: forward, GTGAAATTATCGCCACGTTCGGGCAA and R, TCATCGCACCGTCAAAGGAACCC. PCRs were performed in a total volume of 25 μl using a2720 Thermal Cycler (Applied Biosystem (USA)). The PCR reaction mix consisted of 6 μl of DNA template, 4.5 μL of PCR-grade water, 1 μL of each primer (Metabion, Germany), and 12.5 μL of 2 × PCR master mix (Promega, Madison, USA). PCR cycling conditions for *inv*A amplification were conducted using a previously outlined protocol [29]. The amplicons were separated on a 1.5% agarose gel in TBE buffer and stained with ethidium bromide (Lonza, Rockland, USA). Gel Doc (Cleaver Scientific Ltd., USA) was used to capture images of the gels.

2.6. Serological identification

At the Animal Health Research Institute, Dokki, Cairo, Egypt, serotyping of the confirmed isolates of *Salmonella* was carried out in accordance with the Kauffmann–White–Le Minor technique based on surface antigen identification using polyclonal antisera (Difco Laboratories, Detroit, USA) to determine the somatic (O) and flagellar (H) antigenic epitopes.

2.7. Antimicrobial susceptibility testing

The Kirby-Bauer disk diffusion method was used to investigate the antimicrobial susceptibility of *Salmonella* isolates to various antimicrobial agents, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [30]. Fifteen antimicrobial disks (Oxoid, Basingstoke, UK) representing nine antimicrobial classes were chosen. The disks contained cefoxitin (FOX; 30 μg), ampicillin (AM; 10 μg), amikacin (AK; 30 μg), tetracycline (TE; 30 μg), penicillin (P; 10 lU), fosfomycin (FF; 30 μg), ciprofloxacin (CIP; 5 μg), nalidixic acid (NA; 30 μg), trimethoprim-sulfamethoxazole (SXT; 25 μg), streptomycin (S; 10 μg), kanamycin (K; 30 μg), oxacillin (OX; 1 Mcg), and AMC (30 μg). The results were interpreted according to the interpretation provided by the Clinical Laboratory Standards Institute [30]. Multidrug resistance (MDR) was determined for each isolate if it displayed resistance to at least three antibiotic classes. The multiple antibiotic resistance (MAR) index was calculated by dividing the total number of antimicrobial resistance isolates by the total number of antimicrobials tested, as mentioned previously [31].

2.8. Statistical analysis

To determine the prevalence, descriptive statistics, such as percentages and frequency distribution, were entered into a Microsoft Excel spreadsheet and analyzed using the Statistical Package for the Social Sciences, version 16 (IBM Corp., Armonk).

3. Results

3.1. Prevalence of salmonella in the tested samples

In this study, 200 broiler chicken samples were subjected to bacteriological examination to determine the presence of *Salmonella* serovars. Eighteen *Salmonella* isolates were identified; 11 (11%) isolates were recovered from the intestinal contents, and seven (7%) isolates were successfully identified from the pooled organ samples with an overall prevalence of 18% (18/100). Suspected *Salmonella* isolates produced pink-colored colonies with black centers on XLD plates (Fig. 1A) and pale-colored colonies on MacConkey's agar media (Fig. 1B). Microscopically, the recovered isolates were Gram-negative short rods arranged singly or paired by Gram staining. The suspected isolates were then subjected to biochemical testing, in which *Salmonella* tested negative for indole, urease, and oxidase but revealed positive results with catalase, lysine iron agar, and Simmon's

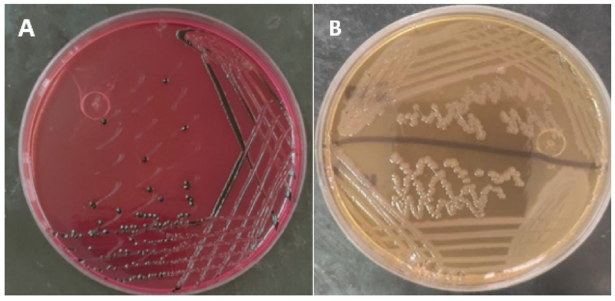

Fig. 1. Culture characteristics of salmonellae. (A) Black colonies of Salmonella on XLD agar and (B) pale colonies of Salmonella *on MacConkey's agar plates.*

citrate tests. On TSI agar, *Salmonella* produced a red alkaline slant and a yellow acidic butt with black color due to H_2S production. Following biochemical confirmation of the isolates, PCR was used to detect *inv*A, which was successfully amplified at 284 bp in all tested isolates (Fig. 2).

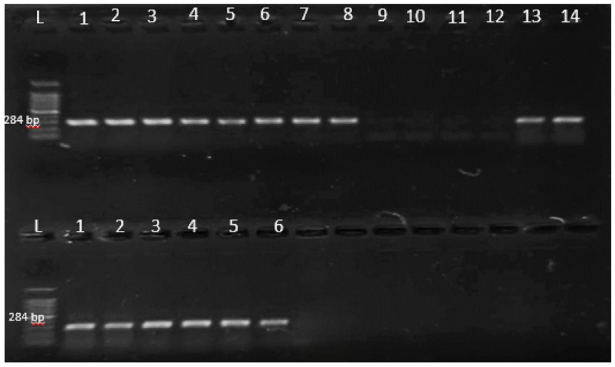

Fig. 2. Agarose gel electophoresis showed amplification of salmonellae *at 284 bp.*

3.2. Serological identification

Based on the Kauffmann–White–Le Minor scheme, five serotypes of *Salmonella*, including *Salmonella* Kentucky (44.4%, n = 8), *Salmonella* Typhimurium (33.3%, n = 6), *Salmonella* Derby (5.6%, n = 1), *Salmonella* Infantis (11.1%, n = 2), and *Salmonella* enteritidis (11.1%, n = 1) were serologically identified (Fig. 3).

3.3. Antimicrobial sensitivity testing

By testing the susceptibility of *Salmonella* isolates to 15 antimicrobial agents, *Salmonella* isolates displayed very high resistance against most of the antimicrobials used. *Salmonella* isolates showed complete resistance (100%) to cefotaxime, kanamycin, amikacin, streptomycin, tetracycline, chloramphenicol, penicillin G, oxacillin, ampicillin, and amoxicillin-clavulanic acid. High resistance was also observed against nalidixic acid (88.88%), ciprofloxacin, and fosfomycin (66.6% each). High sensitivity was observed for cefoxitin (88.8%), as shown in Table 1. Interestingly, all isolates displayed MDR to 11 or more antimicrobial agents (Table 2). The MDR index ranged from 0.7 to 0.9.

4. Discussion

Salmonella species are frequently implicated in a variety of hazardous processes that affect both humans and animals, including poultry [8]. One of the most common foodborne infections in the world is thought to be *Salmonella*. Zoonotic salmonellosis, which is transmitted from animals to humans,

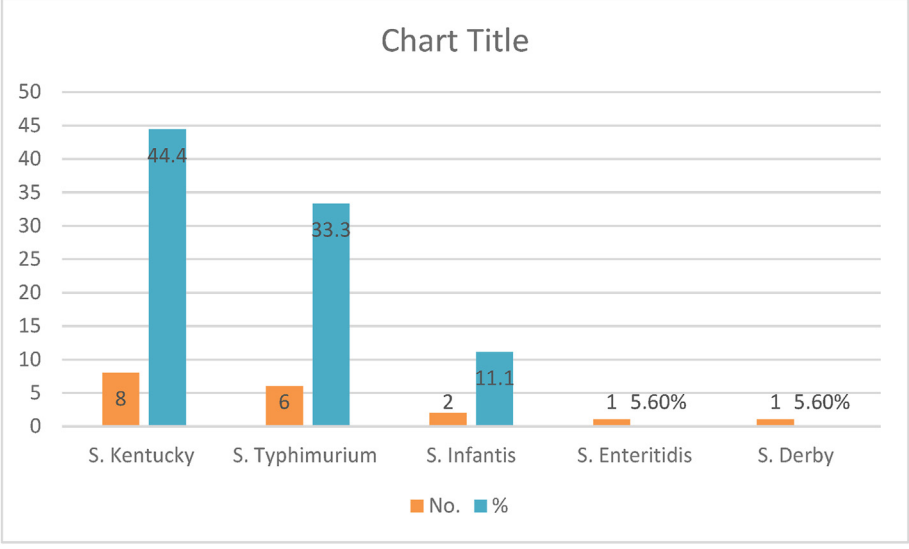

Fig. 3. Distribution of Salmonella *serovars among the recovered isolates.*

Table 1. Antimicrobial susceptibility of salmonellae isolated in this study.

Antimicrobial	Family	Disc code	CPD	*Salmonellae*		
				Resistance	Intermediate	Sensitive
				N (%)	N (%)	N (%)
Pinicillin G	β-lactam	P	10 μg	18 (100)	0	0
Oxacillin		OX	1 mcg	18 (100)	0	0
Ampicillin		AM	10 μg	18 (100)	0	0
Amoxicillin-clavulanic	Penicillin -like, beta lactamase inhibitor	AMC	30 μg	18 (100)	0	0
Cefoxitin	Cephalosporin	FOX	30 μg	2 (11.1)	0	16 (88.8)
Cefotaxime		CTX	30 μg	18 (100)	0	0
Kanamycin	Aminoglycoside	K	30 μg	18 (100)	0	0
Amikacin		AK	30 μg	18 (100)	0	0
Streptomycin		S	10 μg	18 (100)	0	0
Tetracycline	Tetracycline	TE	30 μg	18 (100)	0	0
Trimethoprim-sulfamethoxazole	Sulphonamide	SXT	25 μg	12 (66.6)	0	6 (33.3)
Fosfomycin	Phosphonic acid derivative	FF	30 μg	12 (66.6)	2 (11.1)	4 (22.2)
Ciprofloxacin	Fluoroquinolone	CIP	5 μg	12 (66.6	6 (33.3)	0
Chloramphenicol	Amphenicol	C	30 μg	18 (100)	0	0
Nalidixic acid	Quinolone	NA	30 μg	16 (88.8)	0	2 (11.1)

Table 2. Antimicrobial susceptibility patterns of Salmonella isolates.

Antibiotypes	Antibiotic resistance pattern	MAR index	No of isolates
1	AM - AK- S- K- TE- CTX- OX- AMC- P- C- NA	0.7	2
2	AM- AK- S- K- FF- TE- CIP- CTX- OX- AMC- P- C	0.8	2
3	AM -AK- S- K- FF- TE- SXT- CTX- OX- AMC- P- C- NA	0.8	4
4	AM- AK- S- K- TE- CIP- SXT- CTX- OX- AMC- P- C- NA	0.8	2
5	AM- AK- S- K- TE- CIP- CTX- OX- AMC- P- C- NA	0.8	2
6	AM -AK- S- K- FF- TE- CIP- STX- CTX- OX- AMC- P- C- NA	0.9	4
7	FOX- AM- AK- S- K- FF- TE- CIP- SXT- CTX- OX- AMC- P- C	0.9	2

infects poultry contaminates meat, and is expected to cause a significant number of hospitalizations and deaths each year [32]. Among the bacterial diseases, *Salmonella* is a major hazard in Egypt. According to the current study, 18 (18%) of 100 chicks tested positive for *Salmonella* infection. These results are in close agreement with those of other researchers in Egypt [33–36] who reported lower frequencies of *Salmonella*, ranging from eight to 15.5%. Other studies have reported a higher prevalence of *Salmonella*, ranging from 34 to 73% [37–40]. In another study, *Salmonella* was detected in the liver and intestine of broiler chicks at a rate of 9%, followed by the spleen at a rate of 7.5% of the total distribution in the Egyptian provinces of El-Gharbia, El Behera, Kafr-Elshikh, Alexandria, and Marsa Matrouh [41]. In the Mekong Delta of Vietnam, a study reported a lower prevalence rate of 8.7% (31/357) of intestinal samples taken from ducks that tested positive for *Salmonella* [42], whereas Shang [43] reported a prevalence rate of 7.8%.

In this study, different serovars of *Salmonella* were found in the examined organs and intestinal contents of the examined samples; *S.* Kentucky and *S.* Typhimurium were the most prevalent serovars identified; *S.* infantis, *S.* Enteritidis, and *S.* Derby were recovered at a low frequency. Conversely, six serotypes (9%) of *S.* entericaserovar Enteritidis, 58 (86.6%) of *S.* entericaserovar Typhimurium, and three (4.5%) of non-typable serotypes were recorded in a previous study [44]. In addition, Algammal *et al.* [45] reported 35 isolates of *Salmonella* (20 *S.* Enteritidis and 15 *S.* Typhimurium) obtained from 450 examined samples. Elkenany *et al.* [46] isolated *Salmonella* with a prevalence of 9.3% out of 420 samples, the most often found serotype in the El Sharkia region of Egypt was *S.* Enteritidis (11.4%), followed by *S.* Typhimurium (8.6%).

A severe impact has developed due to antibiotic resistance, especially in the poultry industry. It is estimated that each year, the poultry-borne bacterium *Salmonella* causes more than 600,000 antibiotic-resistant infections. Numerous *Salmonella* strains recovered during food-borne outbreak investigations have exhibited multidrug resistance.

Salmonella isolates in our study demonstrated high resistance (100%) to cefotaxime, kanamycin, amikacin, streptomycin, tetracycline, chloramphenicol, penicillin G, oxacillin, ampicillin, and amoxicillin-clavulanic acid. Moreover, resistance rates

were 88.88% and 66.6% for nalidixic acid, ciprofloxacin, and fosfomycin, respectively. In contrast, the isolates showed 88.8, 33.3, 22.2, and 33.3% susceptibility to cefoxitin, trimethoprim-sulfamethoxazole, fosfomycin, and ciprofloxacin, respectively. Fluoroquinolones, third-generation cephalosporins, and nalidixic acid are the best choices for the treatment of *Salmonella* infections in humans; therefore, the high frequencies of nalidixic acid-resistant *S.* Enteritidis strains in poultry and chicken products are of major public health relevance [47,48]. According to Elsayed *et al.* [49], *S.* Enteritidis strains were remarkably resistant to cefoxitin and nalidixic acid (95.4%), cefotaxime (81.8%), amoxicillin (77.2%), erythromycin (68.1%), chloramphenicol (40.9%), and tetracycline (31.8%). In addition, resistance to beta-lactam and quinolone classes has been shown in numerous investigations conducted globally [50,51].

A different study found that 85.5% and 83.9% of *Salmonella* spp. were resistant to nalidixic acid and ciprofloxacin [52]. Previously, fluoroquinolones, ceftriaxone, and azithromycin were frequently used to control *Salmonella* infections, but resistance to these antibiotics has increased due to AMR in nontyphoid *Salmonella* over the past decade [53,54]. *Salmonella* develops antibiotic resistance by long-term exposure to leftover drugs that are misused or overused, which eventually prevents the effects of drug combinations and leads to the establishment of MDR [55]. Interestingly, the high MDR index in this study confirmed that the samples were delivered from areas with high antibiotic misuse or overuse. According to Sapkota *et al.* [56], serotypes resistant to three or more antimicrobial classes can be classified as MDR. All *Salmonella* isolates isolated in this study revealed MDR against commonly used antimicrobials in poultry farms. These findings are similar to that previously reported Awad *et al.* [57], which detected MDR *Salmonellae* of poultry origin worldwide. These findings necessitate the use of alternative medications to control highly resistant strains, instead of using traditional antibiotics.

5. Conclusions

It is critical to routinely investigate zoonotic bacterial strains circulating in poultry farms, such as *Salmonellae*, to develop effective treatments for managing severe bacterial infections in poultry and the severe economic consequences, as well as to reduce their dissemination across the food chain. In addition, the consequences of the misuse and overuse of antimicrobials on poultry farms lead to the distribution of MDR *Salmonella* strains as shown in the study findings. Therefore, regular monitoring of the production chain and adjusted farming practices, with particularly strict regulations of antibiotic use, must be followed, and alternative medications must be used to decrease the spread of MDR strains to humans.

Ethical approval

The procedures performed in the current study were in accordance with the ethical standards of the Animal Ethics Committee at the Faculty of Veterinary Medicine, Mansoura University (M/112).

Data availability

Data are available within the present study.

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Authors' contributions

Conceptualization, A.A, A.S; methodology, R.H, N.F.M.; validation, A.A., A.S.; investigation, R. H., N. F. M.; original draft preparation, R.H., N.F.M; writing, review, and editing, A.A., A.S; supervision, A.A., A.S.

Conflicts of interest

The authors have no conflicts of interest to declare.

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