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Role of Clostridial Species as Causative Agents of Cellulitis in Turkey

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Abstract
The present study was conducted to evaluate the role of clostridial species as the etiology of cellulitis in turkey. Fifty recently dead turkeys were obtained from different localities (Ismailia, Giza, and Qalyubia Governorates) in Egypt with a previous history of short onset of clinical signs including reluctant of walking, recumbence and pain of thigh muscles under palpation. Eighty percent (40 turkey samples) gave positive results when isolated under anaerobic conditions. Cultivation of different liver tissues and heart blood revealed that 100% (40 out of 40 turkeys submitted to bacterial isolation) were confirmed as *C. septicum*, including 87.5% in single isolation, however, mixed infection with *C. perfringens* type A was detected in only 12.5%. These isolates were confirmed by culture characters; biochemical tests; toxin neutralization test; pathogenicity test, and Polymerase Chain Reaction (PCR). The study concluded that the predominant causative agent of cellulitis in turkeys was *C. septicum*.

Keywords: Cellulitis, Turkeys, *Clostridium septicum*, PCR

Introduction

Cellulitis in poultry is described as an inflammation of the skin and subcutaneous tissue with an accumulation of focal yellow or yellowish brown exudates in the subcutis of the breast and tail areas [1,2]. The disease is poorly understood and there are no many reports available regarding this condition. The lesions associated with cellulitis are more common at approximately 13 to 16 weeks of age and persist until the birds are marketed [1].

Recent observations from the field outbreaks of cellulitis suggested early onset of cellulitis in poults even at 7 weeks of age [3]. *Clostridium perfringens* and *C. septicum* have been suspected in playing a role in causing cellulitis, dermatitis and mortality in turkey breeder hens [4]. However, *C. septicum* have been reported to be more pathogenic than *C. perfringens*.

Clostridial dermatitis (cellulitis/gangrenous dermatitis) has become an issue of concern in recent years; in 2010 the United States Animal Health Association (USAHA) ranked clostridial dermatitis among the top three disease issues in turkeys [5]. Although *C. septicum*, *C. sordellii*, *C. colinum*, *C. perfringens* and *Staphylococcus aureus* can cause cellulitis, *Escherichia coli* and *Streptococcus* species have occasionally been isolated from birds suffered from cellulitis [6]. *C. septicum* plays an important role as an etiologic agent in traumatic gas gangrene and clostridial myonecrosis in animals and humans [7].

Four major toxins are produced by *C. septicum*, they are responsible for tissue damage and toxemia [8]. These toxins include: the lethal necrotizing and hemolytic toxin (alpha-toxin); DNase (beta-toxin); hyaluronidase (gamma-toxin); and the thiol-activated toxin or septicolysin (delta toxin) [9]. Other enzymes such as protease and neuraminidase are also produced.

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In Egypt, data about the distribution and sources of *C. septicum* in poultry production facilities is limited. Therefore, the objective of this study was to isolate and characterize clostridial spp. as causative agents of turkey cellulitis depending on clinical signs, as well as histopathological examination of infected organs and microbial isolation and identification. In addition, molecular identification, pathogenicity and toxin neutralization were carried out.

Material and Methods

Turkeys and sampling

Fifty recently dead turkeys arrived to the Anaerobic Bacterial Vaccine Research Department, at the Veterinary Serum and Vaccine Research Institute (VSVRI) from different localities (Ismailia, Giza, and Qalyubia Governorates) in Egypt between January, 2014 and February, 2015, were included in the study. The representative birds were aged between 12-20 weeks, and they were obtained from farms with a previous history of lameness followed by sudden death of affected birds and no concurrent diseases were detected.

The recorded mortality rate was about 1-2% per week. The birds were subjected to post-mortem examination. Specimens were taken from liver and heart blood using aseptic techniques for anaerobic bacteriological and histopathological investigations.

Table 1: Primer sequences and expected product sizes of the multiplex PCR for *Clostridium perfringens*

Toxin	Gene	Primer sequence (5' - 3`)	Expected product size bp
	cpa		
Alpha (α)	Forward	5`GCTAATGTTACTGCCGTTGA 3`	324
	Reverse	5`CCTCTGATACATCGTGTAAG 3`	
	cpb		
Beta (β)	Forward	5`GCGAATATGCTGAATCATCTA 3`	196
	Reverse	5`GCAGGAACATTAGTATATCTTC 3`	
	etx		
Epsilon (ε)	Forward	5`GCGGTGATATCCATCTATTC 3`	655
	Reverse	5`CCACTTACTTGTCCTACTAAC 3`	
	cpi		
Iota (ί)	Forward	5`ACTACTCTCAGACAAGACAG 3`	446
	Reverse	5`CTTTCCTTCTATTACTATACG 3`	

Bacteriological isolation and identification

Samples were inoculated into cooked meat medium supplied with 1% glucose and 0.05% thioglycollate. Subsequently, the samples were incubated under anaerobic conditions at 37ºC for 24 hours. They were then subcultured onto sheep blood agar plates and incubated anaerobically at 37ºC for 24 hours [10]. Suspected colonies were purified by inoculation again into cooked meat medium and brain heart infusion (BHI) broth (Oxoid, Ogdensburg, NY, USA) then incubated under anaerobic conditions and streaked on blood agar. The colonies were examined for their morphology, Gram staining, cultural characteristics, and biochemical tests using API 20A [11, 12].

In vitro estimation of toxin production

Hemolytic activity of the suspected cultures was determined by a microtiter assay [13,14]. Briefly, 3 ml of sheep blood was centrifuged and the sedimented RBCs were washed 3 times with normal saline and 1% RBCs suspension in PBS was made. The culture supernatants were serially diluted in two-fold across the micro titer plate in 100 µl of PBS and than 100 µl of 1% sheep RBCs suspension was added into all the wells and the micro titer plate was incubated at 37ºC for one hour. The reciprocal of highest dilution of suspected culture supernatant producing visible hemolysis was considered as the hemolytic toxin titer.

In vivo lethality assay

The isolated strains of *Clostridium* species were subjected to the lethality test in mice for evaluation of toxin production and for assessment of the Minimal Lethal Dose (MLD) [15]. Toxin neutralization test in mice was done for the determination of different clostridial toxins by using specific standard antitoxin against alpha toxin for *C. septicum* [16]. Nagler reaction was carried out to differentiate *C. perfringens* by neutralization of lecithinase C by standard antitoxins against alpha; beta, epsilon, and iota toxins on egg yolk agar [17].

Identification of clostridial isolates by Polymerase Chain Reaction (PCR)

PCR was carried out for the detection of hemolysin (alpha toxin) gene of *C. septicum* [18]. From the sequence of *C. septicum* hemolysin gene, two oligonucleotides were used to amplify a DNA fragment of 270 bp [19]. The sequences of the synthesized primers were F-5`-AATTCAGTGTGCGGCAGTAG-3` and R-5` CCTGCCCCAACTTCTCTTTT-3`. PCR amplification was performed in 100 µl reaction volume containing 10 µg of the template DNA, 20 pmol of each primer, 2.5 mM of the four deoxynucleotides, and 2.5 units of *Taq* polymerase, and subjected to 35 cycles of amplification in a PCR thermal cycler. PCR for the identification of *C. perfringens* toxin associated genes using specific primers for the amplification of alpha, beta, epsilon, and iota toxins encoding genes was carried out [20, 21]. The sequences of the primer sets and the expected product size are illustrated in Table (1). Amplification products were resolved in 1.2% (w/v) agarose gels along with 100 bp molecular weight ladder. The agarose gel was prepared in 1 x TBE (89 mM Tris-Borate; 2 mM EDTA; and pH 8.3) stained with 5 µM ethidium bromide. The gels were run in 1 x TBE, 5 µM ethidium bromide for at least 45 min at 100 V and then visualized under Ultra Violet light of ultraviolet transilluminator (Transilluminator, UVP, USA).

Pathogenicity test of clostridial isolates in turkey

The test was done to assess the biological effect and reproducibility of the disease using two of the clostridial isolates. Nine 8-weeks old commercial turkey poults obtained from a farm with no history of cellulitis were assigned into three groups of three birds in each. Group (I) was inoculated intramuscularly with 2 ml of 24 hour old whole culture of *C. septicum* isolate ($5x10^7$ CFU/ml). Group (II) was inoculated intramuscularly with *C. perfringens* type A isolate with the similar dose, and Group (III) was used as a control group inoculated with 2 ml cooked meat medium only.

Results

Post-mortem findings of naturally infected turkeys

Post-mortem examination of field dead turkeys revealed signs of severe inflammation under feathers at breast and thigh muscles in which, under palpation, there was crepitating and beginning signs of gangrene with the presence of serosanguineous exudate over the thigh region. Liver showed enlargement with severe inflammation and large area of necrosis (Figure 1- A,B).

Histopathological examination

Histopathological examination of liver from field dead turkeys revealed marked alteration in the hepatic blood vessels (Figure 2-A, B). There was a large fibrin thrombotic mass filling the lumen of the hepatic blood vessels and also severe hepatocellular degenerative and necrotic changes.

Histopathological examination of liver from the experimentally infected birds with isolate (I) showed marked dilatation and congestion of the hepatic sinusoids and fibrin thrombi in the hepatic vessels and multiple fibrin thrombi, with brownish granules of bile pigments indicting cholestasis (Figure 3-A,B,C). The necrotic cells either appeared with pyknotic nuclei or without any nuclear structure or sometimes appeared completely destructed.

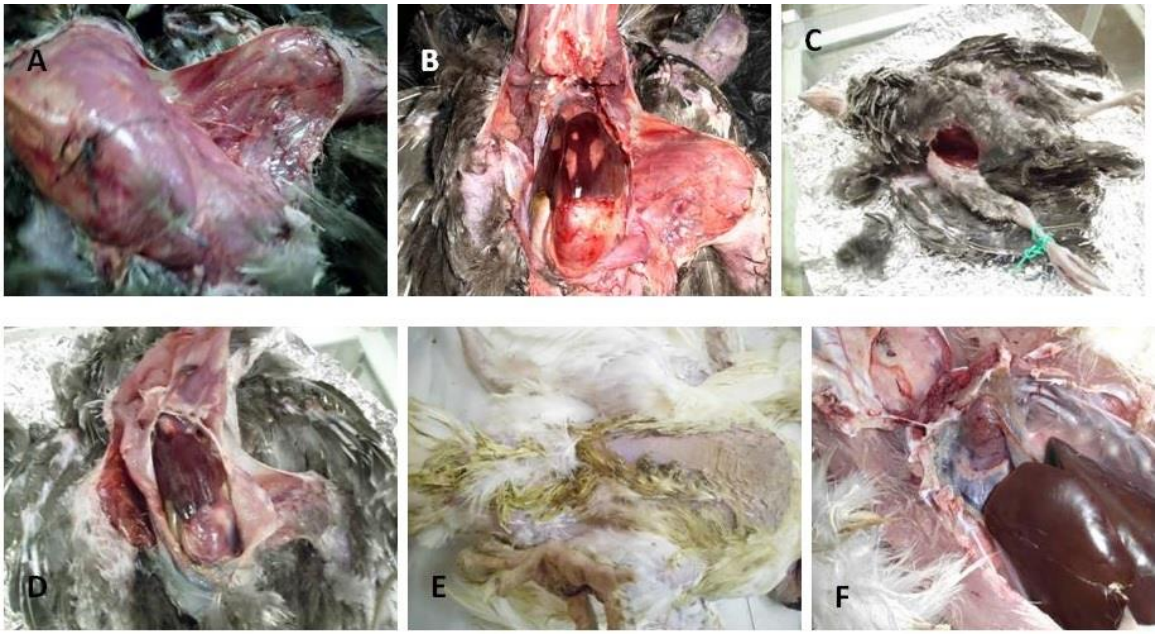

Figure 1:Field dead turkeys: (A: severe inflammation of breast and thigh muscles with crepitating and accumulation of gases, B: enlarged liver with focal necrosis and inflammation). Experimentally infected turkeys with *C. septicum* isolate: (C: inflammation of thigh muscle, D: congestion and enlargement of liver with serofibrinous perihepatitis). Control turkeys: (E: normal breast and thigh muscles, F: normal liver with no evidence of congestion).

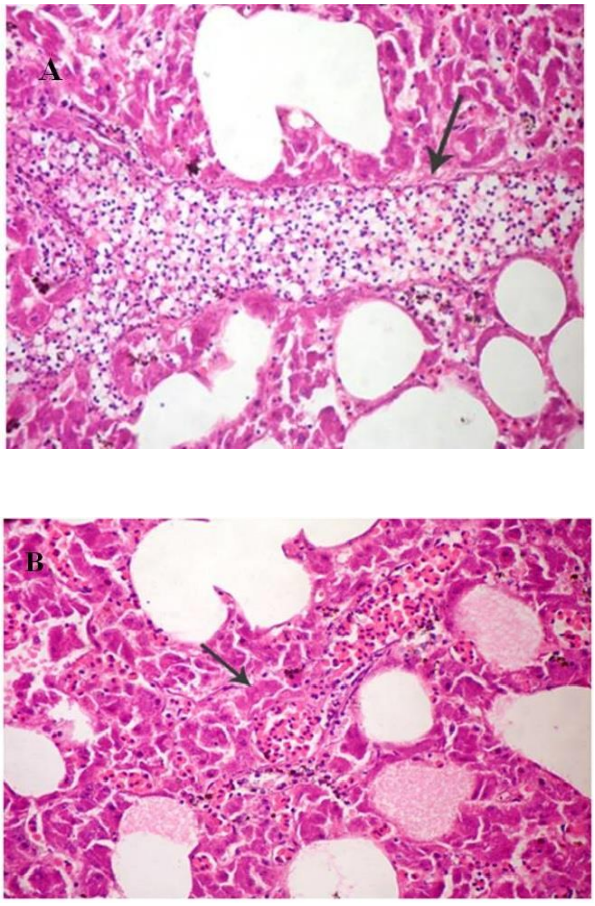

Figure 2: Histopathological examination of liver tissues from field dead turkeys: A: large fibrin thrombotic mass filling the lumen of a hepatic blood vessel, B: Congestion of the hepatic vessels, severe hepatocellular degeneration and necrotic changes (arrow).

Microbiological examination

The results of bacteriological investigation of field dead turkeys revealed that, 40 out of 50 samples (80%) were positive for *Clostridium* spp. when grown under anaerobic conditions. The isolates showed swarming growth with a narrow zone of β-hemolysis on sheep blood agar. These isolates were identified as *C. septicum* (40 out of 40) of which, 87.5% (35 out of 40) were in pure form, while 12.5% (5 out of 40) had mixed infection with *C. perfringens* type A by using morphological characters and biochemical tests. The isolates were Gram positive, very long bacilli, by examination of a liver impression smear, they appeared as very long filamentous long chain. They were positive for DNase, esculin and gelatin hydrolysis, neuraminidase, neutral red reduction, substrate utilized and or acid production weak from glucose, lactose, maltose, mannose, trehalose, and fructose. Negative results were detected with casein hydrolysis, indole production, lecithinase, lipase, urease, catalase and oxidase.

Morphological characters of *C. perfringens* on sheep blood agar were large, smooth, regular convex colonies with a double zone of β-haemolysis and the inner one showed complete zone of hemolysis and the outer zone showed incomplete zone of hemolysis. They appeared as Gram positive short thick non-motile bacilli. Biochemical tests revealed that the isolates were positive for lecithinase; Nagler reaction; ferment carbohydrates (glucose, sucrose, maltose, lactose) and gelatin hydrolysis, while negative for oxidase, catalse, indole, and lipase.

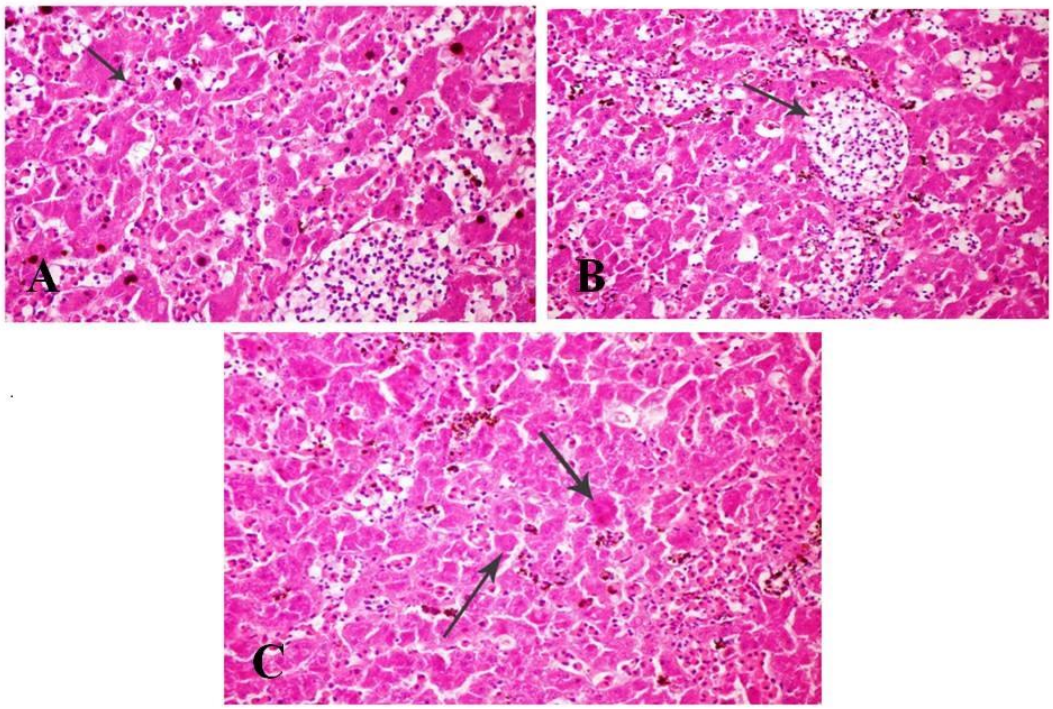

Figure 3: Histopathological examination of liver tissues from experimentally infected turkeys with *C. septicum* isolate: A: marked dilatation and congestion of the hepatic sinusoids (arrow) and fibrin thrombi in the hepatic vessels (arrow head). B: liver showing multiple fibrin thrombi in the hepatic vessels, notice the brownish granules of bile pigments. C: wide spread hepatocellular inflammation with marked vascular congestion and appearance of brownish bile pigments granules.

Hemolytic assay

Results of hemolytic assay for *C. septicum* isolates were 32 hemolytic Activity (HA) after its cultivation on toxin production medium at 37ºC for 24 hours.

Lethality assay

Five representative *C. septicum* and *C. perfringens* isolates, were subjected to the lethality assay for evaluation of toxin production and assessment of the MLD. The results revealed 100MLD/ml for *C. septicum* isolates however, *C. perfringens* type A was 80MLD/ml.

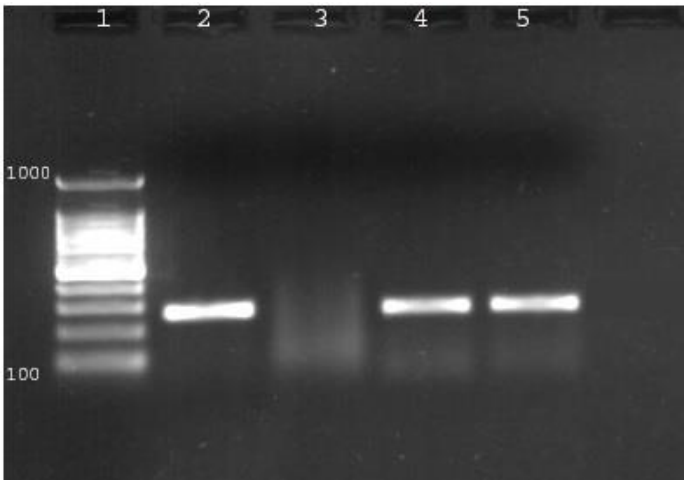

Figure 4: PCR product running on agarose gel (1%) for identification of *C. septicum* alpha toxin at 270 bp [lane 1: 100 bp molecular weight marker; lane 2: reference strain of *C. septicum*; lane 3: *C. perfringens* type A isolate; lanes 4 and 5: *C. septicum* isolates.

Toxin Neutralization Test

For further identification of the toxins produced by closterdial isolates, toxin neutralization test results revealed that, *C. septicum* isolates (n=5) were neutralized by standard alpha toxin of *C. septicum* antiserum. On the other hand, *C. perfringens* type A isolates (n=5) were neutralized by standard alpha toxin of *C. perfringens* antiserum only but they were not neutralized by standard beta; epsilon, and iota antisera of *C. perfringens*. Also, Nagler reaction using specific antiserum against alpha toxin for *C. perfringens* type A on egg yolk agar revealed inhibition of lecithinase activity.

Polymerase Chain Reaction

Out of the suspected five toxin producing *C. septicum* isolates, two were chosen randomly for confirmation by PCR using specific primers for hemolysin alpha-toxin gene. The two isolates were confirmed as *C. septicum* by the amplification of hemolysin alpha toxin associated gene of 270 bp product size (Figure 4). Moreover, one representative suspected *C. perfringens* was subjected to PCR using specific primers for alpha (*cpa*); beta (*cpb)*; epsilon *(etx*), and iota (*cpi*) toxin encoding genes. The results revealed that the isolate was only positive for *cpa* toxin gene at 324 bp indicating that it was *C. perfringens* type A (data not shown).

Pathogenicity test

C. septicum and *C. perfringens* type A isolates confirmed by PCR were used for the pathogenicity test. Experimentally infected turkeys with *C. septicum* isolate in Group (I) showed 100% mortality, 24 hours post-inoculation. Post-mortem examination revealed congestion and severe inflammation of the thigh muscle at the site of inoculation, as well as, congestion and enlargement of the liver (Figure 1-C, D). Histopathological examination of the naturally infected birds showed congestion and inflammation of hepatocytes (Figure 3-A,B,C). The experimentally infected turkeys with *C. perfringens* type A isolate (Group II) failed to show mortalities or lesions. The control Group

(III), birds appeared normal with no clinical signs after 24 hours post-inoculation. Post-mortem examination revealed that the breast and thigh muscles and liver appeared normal with no evidence of any pathological changes (Figure 1-E,F).

Discussion

Recently, the impact of cellulitis in turkeys has caused a significant concern in many farms due to great economic losses resulted from high mortalities which ranged between 1-2% per week especially between 12-20 weeks old [3,4]. The results of the current study revealed that *C. septicum* was isolated from 40 out of 50 (80%) birds; 35 isolates of *C. septicum* was found as single infection, while, 5 isolates were mixed with *C. perfringens* type A. These results were in agreement with that of Thachil *et al.* [22] who reported that *C. septicum* was considered the primary agent causing cellulitis in turkeys based on the isolation results and experimental induction of gross cellulitis lesions. According to a report of Animal and Plant Health Inspection Service (2012) of US Department of Agriculture, an overall 42.3% percent of turkey-grower farms had problem with clostridial dermatitis. The disease was reported to be more severe at 16-17 weeks of age group of turkey [23]. Also, Clark *et al.* [4] described the clinical signs of Clostridial Dermatitis in Turkeys (CDT) due to *C. septicum*, *C. perfringens*, *C. sordelli*, and *S. aureus*. The signs ranged from sudden death to inappetence, depression, leg weakness, recumbency and ataxia.

The disease is characterized by severe inflammation of the skin around the thighs, abdomen, keel, tail region, back, and wings. The lesions can extend into the underlying muscles, and accumulation of gases result in crepitating under palpation. Incidence of mortality from this condition can be severe and acute (i.e., rapid onset of high mortality). Overcrowding, aggressive birds, poor-wet litter, decreased down time, a contaminated environment including feed and water, poor hygienic conditions, and contaminated vaccines and vaccine equipment, etc., can predispose birds for CDT [4].

Our observation in naturally affected turkeys with cellulitis were comparable with the aforementioned results which pointed out to closterdial species to be incriminated as etiological agents.

Polymerase chain reaction was performed to confirm the biochemical results. Hemolysin associated gene was amplified from the examined two isolates of *C. septicum*, but *C. perfringens* isolate failed to amplify 270 bp of the same gene. These results came parallel with Takeuchi *et al*. [18] and Imagawa *et al*. [19] who used specific primers for the detection of *C. septicum* nucleotide sequence for hemolysin gene. They recorded that the sequence was unique because no significant sequence homology has been found between this gene and the GenBank nucleotide sequence database including various hemolytic toxins of *C. perfringens*; C. *chauvoei*; *C. novyi*; *C. hemolyticum*, and *Streptococcus pyogenes.* Another suspected isolate obtained from turkey cellulitis was confirmed as *C. perfringens* type A by the amplification of alpha toxin associated genes at 324 bp. These results are in accordance with Erol *et al*. [25] who isolated and identified *C. perfringens* from turkey meat and referred that all isolates belonged to type A by multiplex PCR. Also, *C. perfringens* was isolated from chickens, turkeys, quail, and psittacines [26].

Pathogenicity test for the assessment of the reproducibility of the disease picture was performed using PCR confirmed *C. septicum* isolate. The post-mortem and histopathological examination revealed findings similar to those produced by natural infection. These results are in agreement with Norton *et al*. [24] who reproduced avian cellulitis in broiler and developed characteristic fibrino caseous plaques after 18 hours post infection. Lesions appeared as serosanguinous exudate with hypreamia extended in breast muscles.

Group (II) inoculated with *C. perfringens* type A isolate showed neither clinical signs nor mortality. This result could be attributed the presence of 2 domains (N-terminal catalytic domain, and C-terminal binding domain) in alpha toxin produced by *C.*

perfringens type A. The C-terminal has specific binding receptors to phospholipids membrane of the intestinal tract leading to subsequent toxin-dependent hydrolysis of phosphatidylcholine resulting in necrotic enteritis, cholangiohepatitis as well as gizzard erosion [27]. However, in the current study, *C. perfringens* type A was injected intramuscular and resulted on no clinical signs and mortalities.

Conclusion

The obtained findings provide evidence that *C. septicum* was the most predominant and potential cause of cellulitis in turkeys and we believe that *C. perfringens* type A share to a certain extent in the production of the disease. Further studies are needed to prepare a vaccine from *C. septicum* isolates to control the disease in turkey farms, also to clarify if there is any role of *C. perfringens* type A in producing these cases.

Conflict of interest

The authors declare no conflict of interest.

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الملخص العربي

دور ميكروبات الكلوستريديا كعوامل مسببة لمرض الالتهاب الخلوي في الرومي

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قسم بحوث اللقاحات البكتيرية اللاهوائية-معهد بحوث الأمصال واللقاحات البيطرية- العباسية-القاهرة

في هذه الدراسة تم الحصول علي ٥٠ رومي نافق حديثا من اماكن مختلفة في جمهورية مصر العربية وكانت تعاني من صعوبة في المشي ورقود والآم في عضلات الفخذ، وكان معدل الوفيات يتراوح بين ١-٢ % اسبوعيا. اتضح من محاولات العزل من الرومي أن ٨٠ % من العينات أعطت نتائج ايجابية في العزل للبكتريا اللاهوائية كانت ١٠٠ % من المعزولات كلوستريديم سبتكم منها ٣٥ معزولة بنسبة ٨٧.٥ % بصورة منفردة بينما خمس معزولات بنسبة ١٢.٥ % كلوستريديم سبتكم و كلوستريديم بيرفرينجينز نوع أ. تم تأكيد هذه المعزولات باستخدام خصائص الزرع ،والاختبارات البيوكيميائية ، اختبار التعادل السمي ، اختبار الضراوة واختبار البلمرة المتسلسل ، ويستنتج من هذه الدراسة ان السبب الرئيسي لمرض التهاب الخلوي في الرومي ميكروب الكلوستريديم سبتكم.