



RESEARCH ARTICLE

Clostridium perfringens type A Causing Necrotic Enteritis Outbreaks among Chickens in Egypt

Sarah S. Helal*¹, Noura M. Khalaf², Alaa A. El menisy² and Mohammed A. Lebdah¹

¹Avian and Rabbit Medicine Department, Faculty of Veterinary Medicine, Zagazig University, 44511, Zagazig, Sharkia Governorate Egypt.

²Veterinary Serum and Vaccine Research Institute 11381, Abbassia, Cairo, Egypt

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Abstract

Undoubtedly, necrotic enteritis is identified as one of the most threatening diseases which face poultry industry in Egypt and need radical solution to avoid huge economic losses. The present work was designed for typing *Clostridium perfringens* isolated from diseased chickens suspected to have necrotic enteritis from different outbreaks in six Governorates in Egypt (Gharbia, Dakhalia, Sharkia, Ismailia, North Sinai and Kafr El Sheikh) during the period from August to December 2018. Intestine and liver samples were obtained from eighty six diseased broiler chickens representing 60 flocks. *C. perfringens* was isolated and toxigenicity of the recovered isolates was determined by Nagler's and dermonecrotic reactions. Furthermore, multiplex polymerase chain reaction (PCR) targeting alpha, beta, epsilon and iota toxins' genes was performed for result confirmation and typing of the toxigenic isolates. Sixty-six *C. perfringens* isolates (38.37%) were recovered from 172 intestine and liver samples and twenty isolates (30.3%) were toxigenic and typed as *C. perfringens* type A producing alpha toxin only. These findings established the fact that alpha toxin is the only main toxin of *C. perfringens* type A which is basically responsible for its pathogenicity and virulence. In addition, most of the positively toxigenic isolates were isolated from hepatic lesions (15 isolates) rather than intestinal lesion (5 isolates). In conclusion, alpha toxin is a major toxin for NE development in chickens. Genotyping of *Clostridium perfringens* by multiplex PCR is a useful adjunct to diagnosis of necrotic enteritis in chickens.

Keywords: Necrotic enteritis, chickens, *Clostridium perfringens*, Alpha toxin, Multiplex PCR..

Introduction

Disease prevention is considered as the main target in the poultry industry as outbreaks of several poultry diseases can drastically decrease growth and increase mortality rate especially outbreaks which occur as a result of enteric diseases [1]. Necrotic enteritis (NE) is one of the most serious and critical enteric diseases characterized by a high mortality rate and severe economic loss. The disease is named as enterotoxaemia after being reported for the first time in England and then, it occurred in many countries [2].

The main etiological agent of NE is *Clostridium perfringens* which is a gram positive, anaerobic and spore-forming bacterium. The sources for disease transmission are contaminated feed, drinking water, housing

particles and sometimes insects [3-9]. According to the classical classification, *Clostridium perfringens* has five toxigenic subtypes according to the secretion of major toxins "Alpha, Beta, Epsilon, Iota" [10]. Recently, a novel toxin has been named necrotic enteritis B-like toxin (NetB) and is found to be associated with NE in broilers. It is one of the pore-forming toxins which forms plasma membrane pores with an estimated pore diameter of 1.6 to 1.8 nm [11]. In addition, TpeL, a family member of large clostridial cytotoxins which was detected in some *C. perfringens* type A strains isolated from NE cases [12]. The recent discovery of these new toxins has shown the necessary for an updated classification [13].

The foremost strain isolated in NE outbreaks is *C. perfringens* type A which mainly secretes alpha toxin as a major toxin and type C may be

*Corresponding author e-mail: (dr.sarah.saied@gmail.com), Avian and Rabbit Medicine Department, Faculty of Veterinary Medicine, Zagazig University, 44511, Zagazig, Sharkia Governorate, Egypt.

rarely involved [1, 14-20]. Alpha Toxin is a major virulence factor which produced by all five types of *C. perfringens*. In addition, it has phospholipase, sphingomyelinase, and hemolytic activities [3, 21, 22]. The mode of action of alpha toxin mainly depends on hydrolysis of phospholipids and promoting membrane disorganization [23, 24]. Hydrolysis of phospholipids results in the formation of diacylglycerol, activation of protein kinase C, and stimulation of the arachidonic acid cascade. As a result of this, the synthesis of inflammatory mediators is induced such as leukotrienes, thromboxane, platelet-agglutinating factor and prostacyclin [25, 26]. These mediators cause blood vessel contraction, platelet aggregation and myocardial dysfunction, leading to acute death [6]. Globally, the economic loss as a result of avian NE is estimated to cost the United States \$2 billion per year, mainly because of medical treatment and imperfect growth performance [27, 28]. Therefore, a critical need to develop managerial strategies became necessary to control and prevent disease occurrence [7]. NE has become an emerging threat in broiler chicken flocks either in clinical or subclinical form mainly after the restrictions of the in-feed antibiotics application and modern high-density housing conditions [6,7].

Various predisposing factors for NE can control the disease occurrence and fate including environmental stress, high stocking density, concurrent infection with infectious bursal disease and/or coccidiosis [7, 29], high protein and fat levels in diet [30], and change in mucus production and gut transit time [31]. Other dietary factors including lectins, trypsin inhibitors, tannins and mycotoxins in the diet are also critical predisposing agents for NE [32].

The present work was designed for typing *C. perfringens* isolated from diseased chickens, suspected to have necrotic enteritis, from different outbreaks in six Governorates in Egypt.

Materials and Methods

All animal care and experimental procedures were reviewed and approved by Zagazig University Institutional Animal Care and Use Committee (ZU-IACUC/ 2/F/102/ 2019).

Chickens

Chicken (Broilers and Sasso) flocks from six Governorates (Gharbia (5 flocks), Dakhalia (38 flocks), Sharkia (12 flocks), Ismailia (1 flock), North Sinai (3 flocks) and Kafr Sheikh (1 flock), with age ranged from 2-9 weeks were investigated. 1-4 chicken were collected from each flock. The birds were clinically examined for observation of clinical signs and gross lesions related to NE.

Samples

Intestinal and liver samples (86 for each) were collected from either euthanized or freshly dead chickens showing signs of diarrhea, low growth rate and imperfect feed conversion rate and gross lesions suspected to be NE. Samples were placed in plastic bags and transferred in an ice box to the laboratory in the department of anaerobic bacteria at Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, Egypt for further anaerobic bacteriological examination. Samples were collected according to Cruickshank *et al.* [33].

Bacteriological examination

Isolation of *Clostridium* species

About one gram of each sample was inoculated in freshly prepared cooked meat media (Oxoid). The media was previously heated in boiling water bath for 10 min, for driving off any dissolved oxygen and then rapidly cooled in a cold bath just prior to their inoculation with the samples. Tubes were incubated anaerobically at 37°C for 24 h for enrichment. Then, a loopful from each suspension was streaked on sheep blood agar plates and incubated anaerobically at 37°C for 24 h [34].

Purification of suspected colonies was carried out on 10% sheep blood agar until pure culture was obtained. This media was prepared according to Carter and Cole [35] with or without neomycin for the proliferation of *Clostridium* isolates and detection of its hemolytic characteristics. Colonies of *C. perfringens* that were characterized by double zone of hemolysis (inner complete zone and outer incomplete zone of hemolysis) were sub cultured on two plates of 10% sheep blood agar and two plates of egg yolk agar which were previously prepared according to

Cruickshank *et al.* [33]. One plate from each inoculated solid media was incubated aerobically and the other plate was incubated anaerobically.

The colonies that grew only in anaerobic condition and lecithinase producer were picked up separately subjected to catalase test. Colonies that were catalase negative, lecithinase producer and showed the target haemolysis were kept on cooked meat broth or thioglycolate broth (Oxoid) for further identification.

Identification of Clostridium isolates

Suspected *Clostridium* colonies were examined morphologically according to Vaikosen and Muller [36]. Smears from suspected *Clostridium* colonies were stained with Gram's stain and examined microscopically for detection of morphological character of *Clostridium* organisms [33].

Suspected purified *Clostridium* isolates were identified according to the schemes of Koneman *et al.* [37] and MacFaddin [38] using catalase and lecithinase test.

Determination of toxigenic strains of Clostridium isolates

Nagler's reaction and toxin-antitoxin test

This test was applied according to Willis and Hobbs [39].

C. perfringens type A antitoxic sera was distributed on half of the egg yolk plate and allowed to dry in the incubator for several minutes. The suspected colonies were streaked heavily across the plate starting from the free half of the plate and ended to the side soaked with antiserum. The plates were incubated anaerobically for 48 hours at 37°C. In case of positive results: Nagler's reaction was positive in the free half of the plate and the opalescence was formed, but it was inhibited on another half of the plate which was soaked with antiserum.

Mice lethality test

The test was applied for detection of the toxigenicity of *C. perfringens* isolates according to Holdman *et al* [40].

Twenty white Swiss mice (Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, Egypt) were injected in the

tail vein with 0.3 ml of 5 hrs. supernatant of cooked meat inoculated broth containing 1ml of 60% glucose via an insulin syringe of gauge (No.20). The injected mice were kept under observation, In case of toxigenic strains, mice died shortly after toxin injection.

Typing of C. perfringens isolates by dermonecrotic test

The test is applied in albino Guinea pigs (Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, Egypt) , according to Oakley and Warrack [41].

Toxin production media was prepared according to Roberts *et al* [42]. Two parts of 50 ml of the medium to which glucose was added at 1% were prepared to ensure anaerobic state and inoculated with 10 ml of 24 h - incubated cooked meat cultures of toxigenic isolates of *C. perfringens*. One part incubated at 37°C for 5-6 h for detection of *C. perfringens* types A, B and C and adjust pH every one hour at 7.5 and the other part incubated at 37°C for 48 h for detection of type D and adjust pH at 7.5 twice daily, trypsin was added to the final concentration of 0.3% to activate protoxin and incubated at 37°C for one hour.

The first part of the culture was siphoned after 6 h of incubation under complete aseptic condition and centrifuged at 3000 rpm for 20 min.

The clear supernatant fluid was divided into four portions: the first (0.1 ml) was neutralized with 0.1 ml of the type A antiserum, the second was neutralized with 0.1 ml of the type B antiserum. The third (0.1 ml) was neutralized with 0.1 ml of the type C antiserum and the fourth 0.1 ml was added to 0.1 ml of saline as control. The supernatant was incubated for one hour at room temperature. The other part of the supernatant was neutralized by type D antiserum and one for control in the same toxin-antitoxin ratio 1:1.

The hair on the back and two sides of albino Guinea pigs was shaved carefully and marked longitudinally onto both sides.

On the right side, 0.1 ml of 6 h or trypsinized 48 h incubated supernatant fluid from each *C. perfringens* culture was inoculated intradermally and 0.2 ml from the

neutralized one was injected at the left side by the same manner and arrangement.

The inoculated Guinea pigs were kept under observation for 24 h for any dermal reaction. The results were interpreted by the color degree of the dermonecrotic reaction and its neutralization according to Stern and Batty [43].

Typing of *C. perfringens* isolates using multiplex PCR

C. perfringens isolates were typed using multiplex PCR targeting alpha, beta, epsilon and iota toxins genes (Table 1) as described by Yoo *et al* [44].

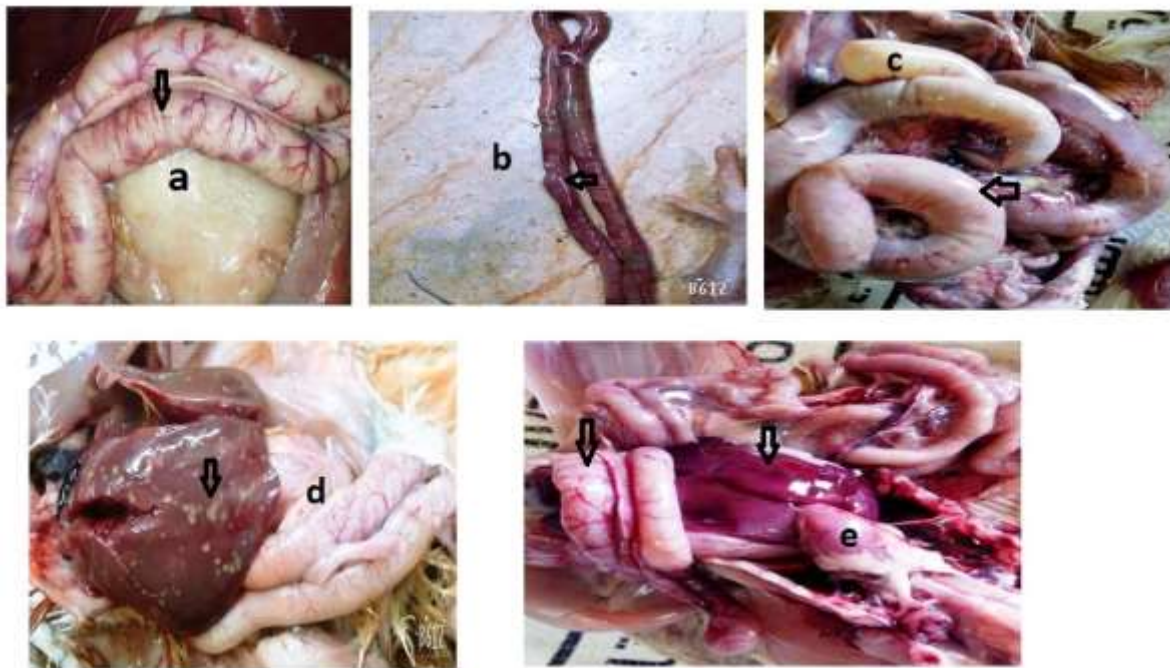


Figure 1: Post mortum lesions of freshly dead and euthanized birds suspected to have necrotic enteritis. (a) Hemorrhagic ulcers at serosa of small intestine, (b) Severely congested intestine, (c) Ballooning of the intestine due to gas accumulation, (d) Necrotic foci unevenly distributed on the liver surface and (e) Severe enteritis and hepatitis as a result of toxemia.

Phenotypic characters of *C. perfringens* isolates

Sixty-six *C. perfringens* isolates (38.37%) were recovered from 172 intestine and liver samples. On blood agar plates, grayish whitish colored colonies recovered anaerobically surrounded by double zone of hemolysis (Narrow and complete inner zone of hemolysis, wide and incomplete outer zone of hemolysis). Gram stained smear from suspected colonies showed gram +ve rod shape bacilli. Suspected colonies were sub cultured to be biochemically identified by catalase test and the result was negative (No gas bubbles formation).

Clear opalescence area due to lecithinase activity of alpha toxin was shown on egg yolk agar media for twenty *C. perfringens* isolates (30.3%).

Nagler's reaction showed clear opalescence zone in the antitoxin free side while it was inhibited in the half of the plate where the antitoxin was added (no clear opalescence zone due to toxin-antitoxin neutralization).

Mice lethality test

All twenty mice were died within few hours shortly after being injected by toxigenic *C. perfringens* strains.

Dermonecrotic reaction

Albino Guinea pigs which were inoculated with the supernatant of broth culture showed severe dermal reaction due to toxigenic strains (Figure 2). The appearance of an irregular area of yellowish to greenish necrosis and the lesion tends to spread downward refers to alpha toxin.

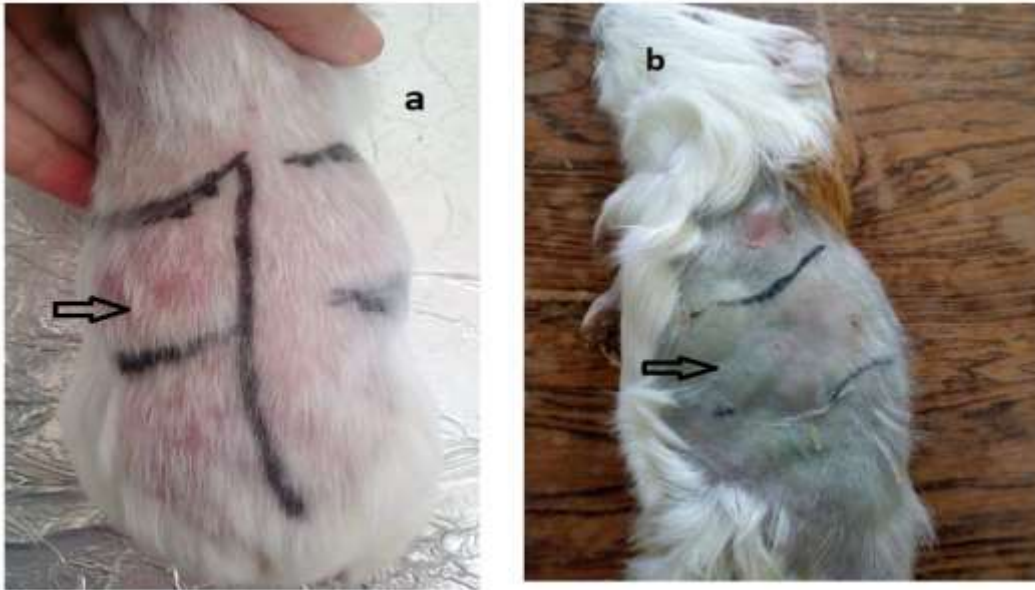


Figure 2: Dermonecrotic reaction in albino Guinea pig (a) The skin before the toxin injection, (b) An irregular area of yellowish to greenish necrosis and the lesion tends to spread downward (as a result of intra dermal injection of alpha toxin).

Typing of *C. perfringens* strains by multiplex PCR

The results showed that 20 representative *C. perfringens* isolates were positive for alpha

toxin and produced amplicons at 402 bp, so were considered type A (Figure 3).

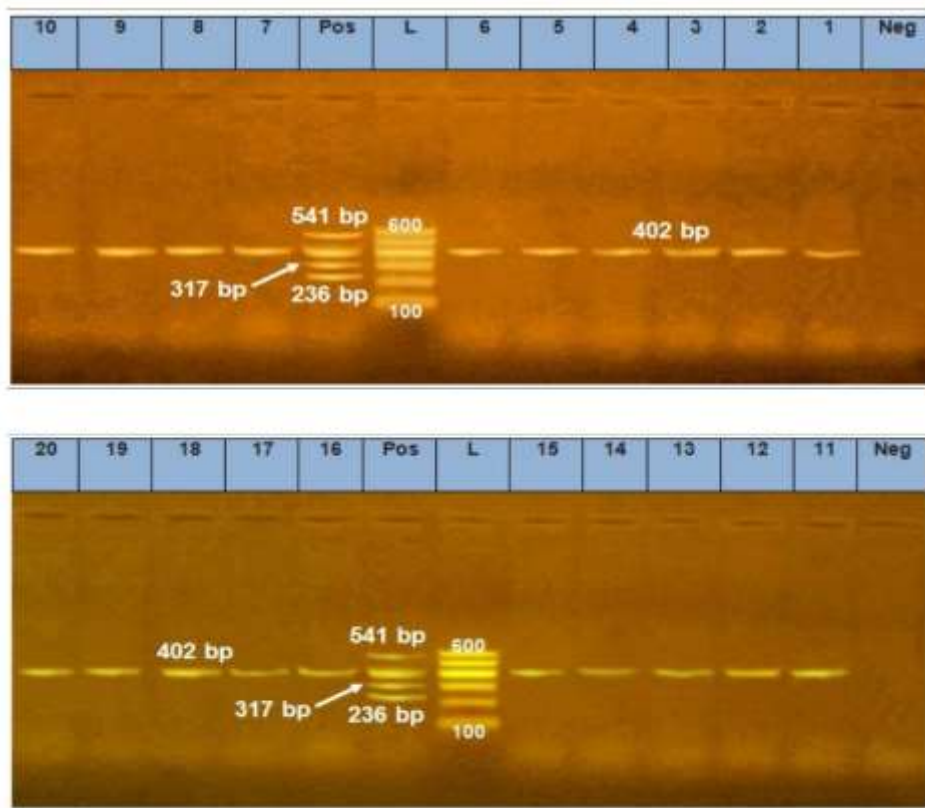


Figure 3: Typing of *C. perfringens* isolates using multiplex PCR. Lane (L): 100bp DNA Ladder, Lane Pos: Spiked samples with alpha, beta, epsilon and iota toxins served as a positive control, Lane Neg: negative control, Lane: 1-10 and 11-20 : positive *C. perfringens* isolates type A to alpha toxin at 402bp.

Discussion

Examination of diseased chickens suspected to have NE revealed general signs of illness and diarrhea which was typically recorded and previously mentioned during outbreaks of Clostridial infection [45-47].

Examination of freshly dead and euthanized chickens revealed various intestinal lesions as: severe congestion of the mucous membrane of the intestine, ballooning of small intestine, friable intestinal wall, and intestinal mucosa covered with necrotic tissue with presence or absence of hemorrhage and in some cases the necrotic area coalescence to each other forming pseudomembrane. Liver appeared severely congested with or without necrosis. The same gross lesions associated with necrotic enteritis were declared in other studies [45, 48].

The age of freshly dead birds suspected to have NE, ranged from 2 to 9 week old. The same susceptible age was recorded by Cooper and Songer [49] and that was previously interpreted as the maternal derived immunity begins to disappear [45].

C. perfringens was isolated in 66 from 172 chicken samples at the rate of 38.37%. This finding indicates that not all the intestinal lesions observed in the field were due to *C. perfringens* infection and other pathogens may be incriminated as etiological agents. Samples taken for isolation were obtained from chicken farms receiving curative antibiotics which subsequently lead to the destruction of the intestinal microbial population, thus explaining the low isolation rate of *C. perfringens* observed in our study. Several studies have reported different isolation rates; Svobodova *et al.* [50] isolated *C. perfringens* at the rate of 18.39%, Schocken-Iturrino *et al.* [51] investigated the presence of *C. perfringens* in 560 intestinal samples and reported that it was found in 94 samples at the rate of 16.78% and Manfreda *et al.* [52] who detected *C. perfringens* in 87 out of 149 samples (58.38%). This variation may be due to the different isolation methods, selection of samples (number and nature of samples, from healthy and/or diseased birds) and poultry farm management (the presence or

absence of predisposing factors and different ranges of antibiotic administration).

The results showed that 20 isolated strains were toxigenic and all were typed as *Clostridium perfringens* type A after being confirmed by multiplex PCR, in agreement with previous studies carried out by Keyburn *et al.* [9], Crespo *et al.* [53] and Svobodova *et al.* [50].

The PCR results revealed that alpha toxin was the only toxin which was secreted by the obtained isolates. Those results came in contact with the fact that alpha toxin is the only virulence factor which is produced by all biotypes of *C. perfringens* [22]. Besides *C. perfringens* type A strains only produce the chromosomal encoded alpha toxin as a major toxin [54].

The results confirmed that the main virulence factor to induce necrotic enteritis in broiler chicken is alpha toxin production. This confirmation runs against a study reported by Keyburn *et al.* [9], who declared that alpha toxin may not be an essential factor in NE development in broiler chickens after using an alpha-toxin deficient *C. perfringens* strain (JIR4107). In contrast, the basic role of alpha toxin in necrotic enteritis (NE) occurrence was supported by the study of Coursodon *et al.* [55], who reported that birds experimentally infected with the alpha-toxin deficient *C. perfringens* strain (JIR4107) developed NE and that diseased birds had high level of the accumulated alpha toxin in the intestine.

The results also reported that the positively - toxigenic and easily purified strains were not only isolated from intestinal samples but also from liver samples. The isolation of *C. perfringens* from liver samples was previously recorded by Løvland and Kaldhusdal [56] who suggested that the detection of liver lesions in broilers may be associated with NE occurrence. In addition, Lovland, and Kaldhusdal [57] also reported that the *C. perfringens* associated hepatitis within NE cases was highly indicative for *C. perfringens* infection. Besides, the isolation of toxigenic *C. perfringens* type A strains from liver lesions was previously recorded by EI-Jakee *et al.* [58] who isolated 90 (75%) *C. perfringens* from

120 intestine and liver samples of diseased chicken and the incidence of toxigenic *C. perfringens* type A isolates among chickens was 53.8%.

Conclusion

Genotyping of *C. perfringens* by multiplex PCR is a useful adjunct to diagnosis of necrotic enteritis in chickens. Moreover, the obtained findings confirmed that alpha toxin is a major toxin for NE development in chickens.

Conflict of interest

None of the authors have any conflict of interest to declare.

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الملخص العربي

كلوستريديوم بيرفرينجنز من النوع أ المسببة لتفشي مرض التهاب الأمعاء التكرزي بين الدجاج في مصر
سارة سعيد هلال^{١*}، نورة محمد خلف^٢، علاء عبد الفتاح المنيسي^٢ و محمد عبد العزيز لبد^١
^١ قسم طب الطيور والأرانب، كلية الطب البيطري، جامعة الزقازيق، ٤٤٥١١، الزقازيق، محافظة الشرقية، مصر.
^٢ معهد بحوث الأمصال واللقاحات، ١١٣٨١ العباسية، القاهرة، مصر

لا شك أن التهاب الأمعاء التكرزي هو واحد من أكثر الأمراض التي تهدد صناعة الدواجن في مصر وتحتاج إلى حل جذري لتجنب الخسائر الاقتصادية الهائلة. صممت هذه الدراسة الحالية من أجل تصنيف الكلوستريديوم بيرفرينجنز و التي سبق عزلها من دجاج مريض يشتبه اصابته بالتهاب الأمعاء التكرزي وذلك في ست محافظات في مصر (الغربية، الدقهلية، الشرقية، الاسماعيلية، شمال سيناء وكفر الشيخ) في الفترة ما بين أغسطس الى ديسمبر ٢٠١٨. تم الحصول على عينات من الأمعاء والكبد من ستة وثمانين دجاجة تسمين مريضة ممثلة لستين قطيع. تم عزل كلوستريديوم بيرفرينجنز و تم التحقق منسمية العزلات بشكل رئيسي من خلال Nagler's reaction ورد الفعل الجلدي Dermonecrotic reaction. علاوة على ذلك، تم إجراء تفاعل سلسلة البلمرة المتعددة (PCR) الذي يستهدف جينات توكسين ألفا وبيتا وإسيلون و أيوتا لتأكيد النتائج ومعرفة نوع العزلات السمية. ستة وستين عزلة من الكلوستريديوم بيرفرينجنز (٣٨.٣٧٪) والتي تم الحصول عليها من ١٧٢ عينة من الأمعاء والكبد، كان منها ٢٠ عزلة سمية (٣٠.٣٪) تم تصنيفها ككلوستريديوم بيرفرينجنز من النوع أ المنتج للألفا توكسين فقط. أثبتت هذه النتائج أن توكسين ألفا هو التوكسين الرئيسي الوحيد الذي يفرز من النوع أ كلوستريديوم بيرفرينجنز وهو المسؤول بشكل أساسي عن المرض وضراره. بالإضافة إلى ذلك، تم عزل معظم العزلات السامة من الآفات الكبدية (١٥ عزلة) بدلاً من الآفات المعوية (٥ عزلات). ختاماً، يعتبر توكسين ألفا هو التوكسين الرئيسي في حدوث مرض التهاب الأمعاء التكرزي في الدجاج. التصنيف الجيني لميكروب الكلوستريديوم بيرفرينجنز عن طريق إجراء تفاعل سلسلة البلمرة المتعددة هو مساعد مفيد لتشخيص مرض التهاب الأمعاء التكرزي في الدجاج.