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Research Article

Identification and Characterization of *Clostridium perfringens* Isolated from Necrotic Enteritis in Broiler Chickens in Tiaret, Western Algeria

Rachid MERATI [1,2] Soraya TEMIM [2] Ali Alaa Abdel-Fattah MOHAMED [3]

[1] Laboratoire d'Hygiène et Pathologie Animale, Université Ibn Khaldoun, 14000, Tiaret, ALGERIE
[2] Laboratoire de Recherche « Santé et Production Animales », Ecole Nationale Supérieure Vétérinaire, 16000, Alger, ALGERIE
[3] Veterinary Serum and Vaccine Research Institute, 11765, Abassia, Cairo, EGYPT

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Abstract

The present study was carried out to investigate the presence of *Clostridium perfringens (C. perfringens)* in broiler chickens from various locations in Tiaret province, western Algeria, and to characterize the bacterium isolates for the presence of *cpa, cpb, etx, iA* and *netB* gene. A total of 180 samples representing intestinal contents of broiler chickens showing enteric disorder symptoms and lesions suspected to be Necrotic Enteritis (NE) were analyzed by conventional methods and polymerase chain reaction (PCR). *C. perfringens* was isolated at the rate of 34.44% (62/180), and its presence was confirmed by cultural and biochemical characterization. 83.87% (52/62) *C. perfringens* isolates were toxigenic and 16.13% (10/62) were non-toxigenic. Multiplex PCR was performed to toxinotype the 52 toxigenic isolates, and the results showed that all isolates were positive for the gene *cpa* and negative for *cpb, etx* and *iA*. This indicates that all the toxigenic isolates were *C. perfringens* type A (52/52). Uniplex PCR for detection of *NetB* toxin gene was carried out on 22 type A isolates, and these results showed none of the isolates as positive for the gene *netB*. This result indicates that the *C. perfringens* type A was the most predominant etiology of NE without carrying the *netB* gene.

Keywords: *Clostridium perfringens, Necrotic enteritis, Broiler, Toxinotyping, NetB*

Batı Cezayir'in Tiaret Bölgesinde Nekrotik Enteritli Broiler Tavuklardan İzole Edilen *Clostridium perfringens*'in İdentifikasyonu ve Karakterizasyonu

Özet

Bu çalışma Batı Cezayir'in Tiaret Bölgesinin değişik alanlarındaki broiler tavuklarda *Clostridium perfringens (C. perfringens)* mikroorganizmalarının varlığını araştırmak ve *cpa, cpb, etx, iA* ve *netB* genlerinin mevcudiyeti açısından bakteri izolatlarını karakterize etmek amacıyla yürütülmüştür. Enterik bozukluk semptomları ve lezyonları göstererek Nekrotik Enterit (NE) şüpheli olduğu düşünülen toplam 180 broiler tavuğa ait bağırsak içeriği örneği klasik metot ve polimeraz zincir reaksiyonu (PCR) ile incelendi. *C. perfringens* %34.44 (62/180) oranında izole edildi ve mikroorganizmanın kültürel ve biyokimyasal karakterizasyonu teyit edildi. *C. perfringens*'in %83.87 (52/62) izolatı toksijenik ve %16.13'ü (10/62) non-toksijenik olarak belirlendi. 52 toksijenik izolata toksinotiplendirme amacıyla Multipleks PCR uygulandı ve elde edilen bulgular tüm izolatlarda *cpa* geni için pozitif *cpb, etx* ve *iA* genleri için negatif olduğunu gösterdi. Bu durum tüm toksijenik izolatların *C. perfringens* tip A (52/52) olduğuna işaret etti. 22 tip A izolata *NetB* toksin genini belirlemek amacıyla unipleks PCR uygulandı ve sonuçlar izolatların hiç birinin *netB* geni için pozitif olmadığını gösterdi. Bu sonuçlar *netB* geni taşımaksızın *C. perfringens* tip A'nın Nekrotik Enteritin predominant etiyolojik etkeni olduğunu göstermiştir.

Anahtar sözcükler: *Clostridium perfringens, Nekrotik enteritis, Broiler, Toksinotiplendirme, NetB*

INTRODUCTION

Clostridium perfringnens is a Gram positive spore-forming anaerobic bacterium that plays an important role in the etiology of NE disease, which is the cause of the greatest economic losses in the poultry production industry [1,2]. It has been estimated that NE costs the poultry industry 2 billion dollars per year as result of reduction

İletişim (Correspondence)
+213 67 0284000
drmerachi@yahoo.fr

performance, disease treatment and preventive measures [3,4].

C. perfringens is responsible for synthesis and secretion of more than 17 different extracellular toxins, and it has been classified into 5 toxinotypes (A, B, C, D and E) on the basis of their ability to produce the major lethal toxins alpha *(α)*, beta *(β)*, epsilon *(ℰ)* and iota *(ι)* [5-7]. NE disease is caused mainly by type A strains, which produce the alpha toxin, and to a lesser extent, type C strains, which produce both alpha and beta toxin [8,9]. The alpha toxin was long considered the main cause responsible of the induction of the disease, while, a novel pore-forming toxin NetB has been demonstrated in type A strains [2,10,11].

NE can present as an acute clinical disease characterized by severe intestinal necrosis, leading to a sudden 50% increase in flock mortality rates [12,13]. NE can also arise as a sub-clinical infection, associated with chronic damage of the intestinal mucosa, causing problems such as lower performance and reduced weight gain [14,15]. In the past, NE has been controlled in poultry flocks with antimicrobial growth promoters in commercial poultry feed [16,17]. However, since the ban of these supplementations due to policy changes, NE has reemerged as a costly disease in poultry industry [2,16].

In Algeria, poultry meat is the primary source of protein, especially for Tiaret province population where broiler breeding was developing through the last decade. The detection and the characterization of *C. perfringens*, which is known as causative agent of NE in the poultry industry, and one of the most frequently isolated bacterial pathogens in foodborne disease outbreaks in humans [18], remains unknown in this region.

In this study, we aimed to investigate the presence of *C. perfringens,* at various locations in Tiaret province (western Algeria) and to characterize the bacterium isolates for the presence of *cpa*, *cpb*, *etx*, *iA* and *NetB* gene by PCR technology.

MATERIAL and METHODS

Sampling

A total of 180 samples were collected aseptically from freshly sacrificed broiler chickens (2-8 weeks old) reared in 70 poultry farms (average of 2500 broiler chickens by farm) at different locations in Tiaret province, western Algeria, from August 2015 to July 2016. The samples were collected after postmortem examination and the sections of intestine displaying gross lesions suspected to be NE were collected. Samples were taken to the laboratory in an ice box as soon as possible.

Isolation and Identification of Clostridium perfringens

The intestinal content of the collected samples were inoculated into tubes of freshly prepared cooked meat medium (Oxoid,UK) for enrichment and incubated in an anaerobic jar (Oxoid,UK) for 24 h at 37°C in anaerobic atmosphere provided by AnaeroGen atmosphere generation system. 0.1 mL of inoculated fluid media was streaked onto perfringens agar base containing 400 µg/mL of cycloserine (TSC) without egg emulsion (Oxoid, UK) and incubated anaerobically [19]. After 24-48 h incubation at 37°C, typical black colonies presumed to be *C. perfringens* were taken out with the help of the loop, re-streaked onto two plates of 5% defibrinated sheep blood agar and egg yolk agar, and incubated anaerobically for 24 h at 37°C [20]. *C. perfringens* isolates were identified via morphological and biochemical characterization as previously recommended by Koneman *et al.*[21] and Macfaddin [22].

Determination of Toxigenic Clostridium perfringens Isolates

- Mouse Bioassays (Lethality Test)

After an anaerobic incubation for 24 h at 37°C, the cultures of isolated *C. perfringens* strains in cooked meat medium were centrifuged at 3.000 rpm for 15 min, and the cell-free culture supernatants were recovered. 0.3 mL from the clear supernatant fluid was I/V inoculated in the tail vein of each Swiss mouse (25-40 g), and injected mice were observed over a period of three days for nervous symptoms or death [23]. One mouse was injected with broth culture without bacteria as a control.

- Nagler's Reaction (Toxin - Antitoxin Half Plate)

This test was carried out according to the method of Smith and Holdeman [24]. It was performed by spreading *C. perfringens* type A antitoxin serum (National Institute for Biological and Standard Control, UK) on half of the egg yolk agar plate and allowed to dry in the incubator for half an hour. The cultures were then streaked across the plate, beginning at the non-antitoxin coated portion and ending to the side containing the antitoxin. The cultures were incubated anaerobically for 24 h at 37°C.

All isolated toxigenic strains were stored in thyoglycolate medium (Oxoid, UK) with 30% glycerol at -20°C for subsequent toxin genotyping.

The experiment on animals was carried out according to the National Regulations on Animal Welfare.

Genotyping of the Toxigenic Clostridium perfringens Isolates

- DNA extraction and PCR

The DNA was extracted from toxigenic *C. perfringens* isolates using QIAamp DNA Mini Kit (QIAGEN, USA), as indicated per the manufacturer's instructions. Specific oligonucleotide primers sequences corresponding to alpha, beta, epsilon and iota toxin genes of *C. perfringens* and NetB toxin gene, as reported by Yoo *et al.*[25] and

Table 1. *Details of oligonucleotide primers sequences used in this study*

Gene	Primer	Sequence	Product Size (bp)
cpa (α toxin)	F	GTTGATAGCGCAGGACATGTTAAG	402
	R	CATGTAGTCATCTGTTCCAGCATC	
cpb (β toxin)	F	ACTATACAGACAGATCATTCAACC	236
	R	TTAGGAGCAGTTAGAACTACAGAC	
etx (ε toxin)	F	ACTGCAACTACTACTCATACTGTG	541
	R	CTGGTGCCTTAATAGAAAGACTCC	
iA (ι toxin)	F	GCGATGAAAAGCCTACACCACTAC	317
	R	GGTATATCCTCCACGCATATAGTC	
NetB (NetB toxin)	F	GCTGGTGCTGGAATAAATGC	560
	R	TCGCCATTGAGTAGTTTCCC	

Keyburn *et al.*[10], respectively were procured from Midland Certified Reagent Company, (Oligos, USA).The details of primers are given in *(Table 1)*.

The PCR reaction mix for alpha, beta, epsilon and iota toxins was prepared as follows: 8 µL of extracted DNA template from bacterial cultures, 25 µl EmeraldAmp GT PCR master mix (TAKARA, USA), 1 µL of each alpha, beta, epsilon and iota forward and reverse primers (20 pmol μL^{-1}), and 9 µl of PCR-grade water, to a total volume of 50 µL. For NetB toxin, the reaction mixture was prepared as follows: 6 µl of extracted DNA template from bacterial cultures, 12.5 µL EmeraldAmp GT PCR master mix (TAKARA, USA), 1 µL NetB forward and reverse primers (20 pmol μL^{-1}), 4.5 µL of PCR grade-water, to a total volume of 25 µL.

The PCR amplification for detection the toxins (α, β, ε and ι) was programmed in TRIO thermal cycler (Biometric, Germany) as follows: initial denaturation step at 94°C for 5 min, followed by 35 cycles of amplification. Each cycle comprised denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 45 sec. There was then a final extension for 10 min at 72°C. For detection of *netB* gene PCR, an initial denaturation step at 94°C for 5 min was followed by 35 cycles. Each cycle comprised denaturation at 94°C for 30 sec, annealing 58°C for 45 sec, and extension at 72°C for 45 sec. There was a final extension for 10 min at 72°C. Finally, 30 µL of the amplified product for alpha, beta, epsilon and iota toxin genes and 20 µL of the amplified products for NetB toxin gene were electrophoresed in 1.5% agarose gel and stained with ethidium bromide. Standard DNA fragments (Gel Pilot100-bp DNA molecular weight marker; QIAGEN, USA) were used as molecular weight markers to indicate the sizes of the amplification products. Amplified bands were visualized and photographed by a gel documentation system (Alpha Innotech, Germany), and the data was analyzed through computer software (Automatic Image Capture Software, ProteinSimple formerly Cell Biosciences, USA).

The positive DNA samples for alpha, beta, epsilon, iota and NetB toxins were obtained from reference laboratory for veterinary quality control on poultry production. (Animal Health Research Institute of Cairo, Egypt). Distilled water was used as negative control.

RESULTS

Necropsy Findings

At necropsy, all the 180 broiler chickens, from which the intestinal contents were collected, showed gross lesions suspected to be NE. Lesions were seen in the middle of the small intestine that had friable wall and distended with gases. Intestinal mucosa was covered by a tan to yellow necrotic membrane with or without hemorrhagic foci *(Fig. 1)*.

Bacteriological and Biochemical Examination

Out of the 180 intestinal samples examined, 62 *C. perfringens* isolates (34.44%) were detected. The isolates produced 1 to 2 mm typical black colonies on TSC agar medium, as shown in *Fig. 2*. Also produced were smooth, round, glistening colonies surrounded by double zone of haemolysis on sheep blood agar medium, as shown in *Fig. 3*. Biochemical characterization revealed that all the isolates were positive for lecithinase activity on egg yolk agar medium, sugar fermentation, milk digestion, while indole production, catalase and oxidase tests were negative.

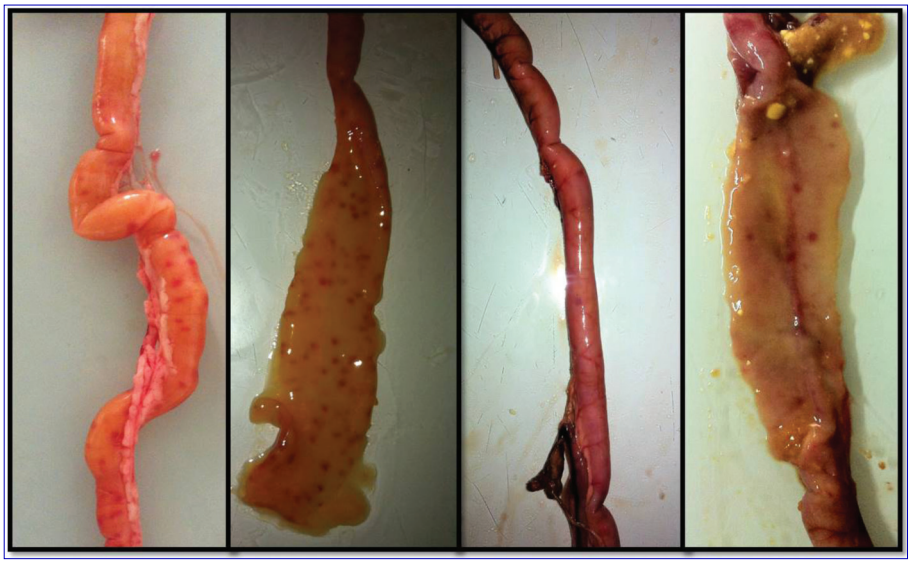


Fig 1. Parts of chicken intestine from field cases with lesions suspected to be NE

Fig 2. Typical black colonies presumed to be *C. perfringens* on TSC agar medium

Fig 3. *C. perfringens* colonies surrounded by double zone of haemolysis on sheep blood agar medium

Toxigenic Activities of Clostridium perfringens Isolates

52 isolates out of 62 *C. perfringens* isolates (83.87%) were toxigenic, as indicated by the death of the inoculated mice in mouse bioassays, and positive reaction on Nagler's tests expressed by zone of opacity surrounding the toxigenic *C. perfringens* colonies on the half of the plate without antitoxin while no change was observed on the other half containing the antitoxin *(Fig. 4)*. The remaining 10 *C. perfringens* isolates (16.13%) were non-toxigenic.

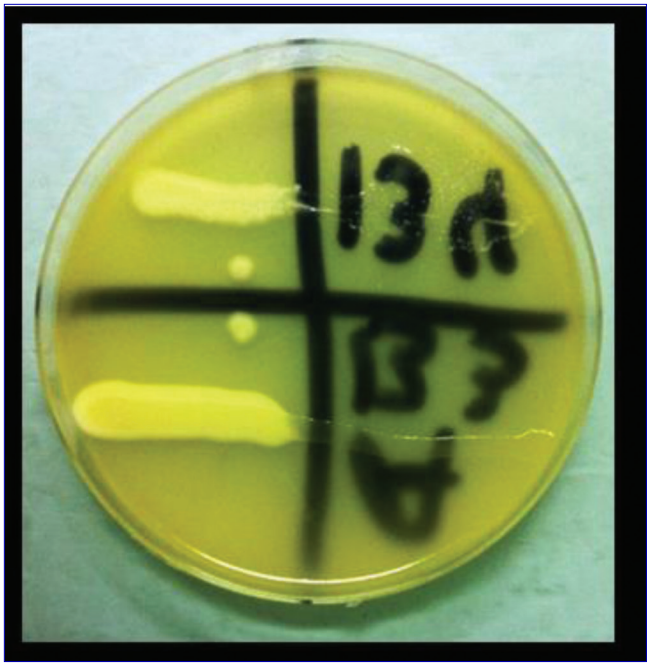


Fig 4. Nagler's test by half antitoxin plate

Multiplex PCR for the Genotyping of Toxigenic C. perfringens Isolates

The genotyping of the 52 toxigenic isolates revealed that all the isolates carried the gene *cpa* (402 bp), coding for the alpha toxin as illustrated in *Fig. 5*, and none of the isolates carried the genes *cpb* (236 bp), *etx* (541 bp) and *iA* (317 bp) coding for beta, epsilon and iota toxins, respectively. This indicates that all the toxigenic isolates were *C. perfringens* type A 100% (52/52).

Uniplex PCR for the Detection of netB Gene

A selection of 22 toxigenic isolates were confirmed to be *C. perfringens* type A and analyzed by uniplex PCR to detect the presence of the *netB* (560 bp) gene. None of the isolates were found positive for this gene that expresses for the NetB toxin, as illustrated in *Fig. 6*.

DISCUSSION

C. perfringens has been demonstrated in several regions in the world. It is one of the most common causes of severe gastro-intestinal infection and necrotic enteritis in poultry [1,13,26]. However, no studies on the detection and molecular characterization of *C. perfringens* inducing NE in broiler chickens were carried out in Algeria.

The presence of *C. perfringens* was investigated in different broiler chicken flocks located at Tiaret province. *C. perfringens* was isolated in 62 from 180 samples analyzed

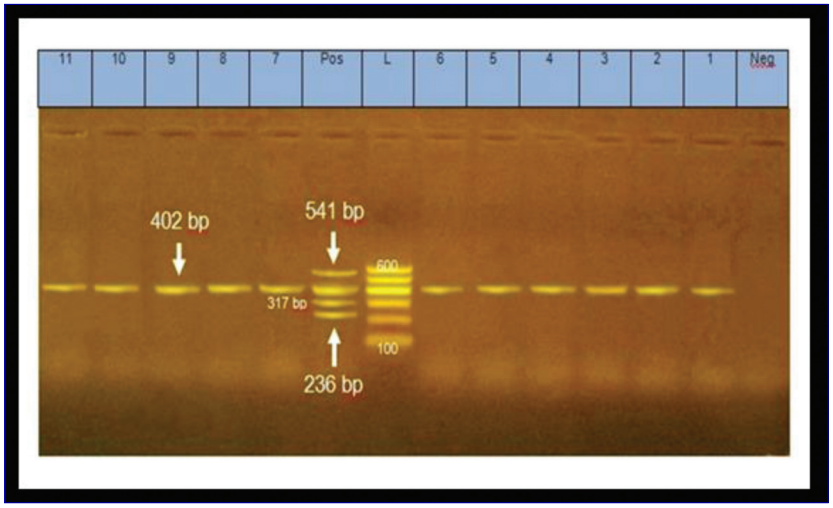
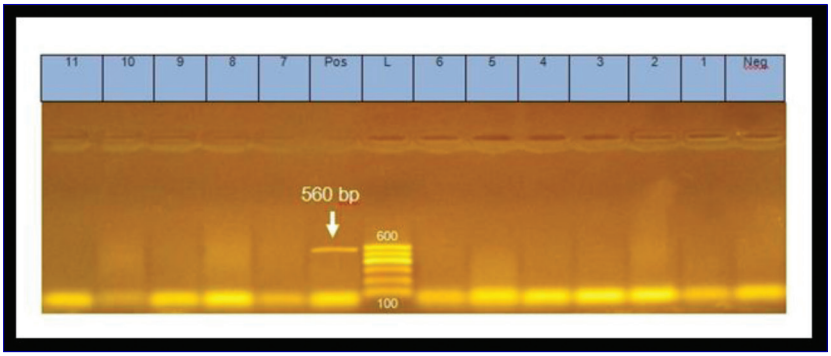


Fig 5. Typing of *C. perfringens* toxin genes by Multiplex PCR. Pos: positive control; Neg: negative control; L: DNA ladder (molecular weight marker 100-bp); lane 1-11: cpa positives *C. perfringens* toxigenic isolates

Fig 6. Uniplex PCR identification of *netB* gene. Pos: positive control; Neg: negative control; L: DNA ladder (molecular weight marker 100-bp); lane 1-11: netB negatives *C. perfringens* type A isolates



at the rate of 34.44%, this finding indicates that not all the intestinal lesions observed in the field were due to *C. perfringens* infection. Other pathogens may be incriminated in the etiology of these lesions. It is also believed that the sampling was carried out on farms receiving curative antibiotics which 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.*[27] isolated *C. perfringens* at the rate of 18.39%, and Schocken-Iturrino *et al.*[28] analyzed 560 intestinal contents and reported that *C. perfringens* was found in 94 samples at the rate of 16.78%. Manfreda *et al.*[29] have detected *C. perfringens* in 87 from 149 samples analyzed (58.40%), while the lowest frequency of isolated *C. perfringens* was reported by Kalender and Ertas [30], who found that only 5% of intestinal contents were positive for *C. perfringens*. This variation may be due to the different methodologies used for the isolation, selection of samples (number and nature of samples, from healthy and/or diseased birds) and poultry farm management (the use or not of antibiotics as growth promoters in feed).

C. perfringens is considered a commensal organism of normal chicken intestinal flora [31], for this reason, we should differentiate between toxigenic and non-toxigenic isolates. Our results revealed that, out of the 62 isolates that were previously identified morphologically and biochemically as *C. perfringens*, 52 isolates (83.87%) were toxigenic. The high rate of toxigenic *C. perfringens* isolates recorded in our study confirm the role of this bacterium in the occurrence of the NE disease due to the high production of toxin that is responsible of the destruction of intestinal mucosa.

Multiplex PCR is a rapid and effective method for typing of *C. perfringens* toxins. The typing of 52 toxigenic isolates revealed that all the isolates were *C. perfringens* type A, in agreement with previous investigations carried out by Keyburn *et al.*[32], Crespo *et al.*[33], Svobodova *et al.*[27], Drigo *et al.*[34] and Trinh *et al.*[35]. However, there were no *C.perfringens* type C and D that were isolated from broiler chickens which demonstrated NE, disagreeing with the results of Shane *et al.*[36] and Heier *et al.*[37], who isolated *C.perfringens* type D and type C from broiler chickens suffering from NE.

Several studies have reported the role of other toxins in the induction of NE. The most important of these is Necrotic Enteritis toxin B (NetB), a pore forming toxin capable of causing lesions typical of NE [10]. Since the discovery of this new virulence factor, the presence of *netB* gene in *C. perfringens* isolates was investigated in different regions of the world. According to our study none of the selected toxigenic *C. perfringens* type A isolates were positive for NetB toxin. These results agree with Datta *et*

al.[38], who investigated the presence of NetB toxin in 26 isolates of *C. perfringens* type A and reported that none of the isolates were positive for this toxin. Similar results were reported by Thomas *et al*.[39], who recorded tested isolates for the presence of *netB* gene were negative. In contrast to our finding, several studies demonstrated the existence of *netB* gene in *C. perfringens* isolates. Johansson *et al*.[40] investigated the prevalence of this toxin and reported that more than 90% of all isolates from cases of NE carried *netB* gene. In addition, the presence of this gene was examined in 36 isolates of *C. perfringens* and was detected in 19 isolates at the rate of 52.8% from diseased flocks by Talooe *et al*.[41]. The lack of *netB* gene in our study may be explained by the insufficient number of samples analysed or the inexistence of this gene in our region.

Our study indicated the presence of *C. perfringens* in broiler chicken flocks in Tiaret province, and type A was the most predominant etiology in the occurrence of NE, an important bacterial disease of poultry. Hence, considerable attention should be paid in the prevention of this disease as *C. perfringens* type A is considered one of the most important causes of foodborne disease in human.

All the selected toxigenic *C. perfringens* type A isolates were negatives for the *netB* gene. This finding represents the first report on the detection of *netB* gene in Algerian field isolates of *C. perfringens*. We suggest further study to find other toxins that are the cause of NE, as in the case of NetB negative isolates. Future investigations should be carried out on an important number of *C. perfringens* isolates and on other regions in Algeria.

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