NETB, A NEW TOXIN RELATED TO CLOSTRIDIUM PERFRINGENS-INDUCED AVIAN NECROTIC ENTERITIS IN BROILER CHICKENS

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ABSTRACT

Necrotic enteritis is well-defined as poultry disease which occurs as a result of liberation of toxins from Clostridium perfringens type A, C, D, and G pathogenic strains. This study was performed to determine the prevalence rate of C. perfringens in broilers from different farms in Assiut Governorate and to examine the isolates for the existence of alpha, beta, epsilon, and netB gene. A sum of 100 intestinal specimens were compiled from diseased broiler chickens (3-6 W) which had clinical signs and post mortem lesions of Necrotic Enteritis and examined by conventional and molecular methods. C. perfringens was isolated from 52% (52/100) of the bacteriologically examined flocks. Only ten isolates among suspected isolates were examined by using uniplex and multiplex PCR. The results reported 100% positivity for cpa and netB gene in the examined isolates and neither cpb nor etx gene were detected and proved that all isolates were Clostridium perfringens type G.

Keywords: Clostridium perfringens, Broiler, Multiplex Polymerase Chain Reaction, NetB gene.

INTRODUCTION

Necrotic enteritis is a serious disease that affects the poultry industry all over the world (Ali and Islam, 2021). Globally, economic losses to the poultry industry due to NE are estimated to be more than $6 billion per year (Moore, 2016).

NE is the most commonly caused by Clostridium perfringens. It's a rod-shaped, gram-positive, anaerobic spore-forming bacteria that's found throughout the gastrointestinal tract (Ali et al., 2020).

Approximately more than 17 exoproteins which known to be toxic are created by Clostridium perfringens, leading to a new toxigenic classification including seven types (A–G) according to group of six exotoxins which are released from bacteria. (Alpha, beta, epsilon, iota, CPE, and NetB). C. perfringens types A (alpha...
toxin), C (beta, and alpha toxins), and G (Net toxin) are the most common causes of NE (Rood et al., 2018 and McMullin, 2020).

Recently, two novel types of toxins (F and G) were described, Type F generates enterotoxin (cpe gene), which are responsible for food poisoning disease, while type G produces NetB toxin (netB gene) which causes NE in broiler chickens (Anju et al., 2021).

NetB, a pore-forming toxin, is considered to be the major virulence agent (Profeta et al., 2020). It is in charge of the new strain (type G) synthesis which was previously belonged to Clostridium perfringens type A (AKM et al., 2021).

For years, studies have focused only on the function of toxins, Phospholipase C, which induces hydrolysis of the phospholipids and causes destruction of the enterocyte wall and leads to cell death. The function of CPA in the generation of NE was, however, returned when CPA-deleted mutants maintained full virulence in vivo as a result the NetB toxin was discovered (Keyburn et al., 2006 and Paiva and McElroy, 2014).

NetB-positive strains have the capability to enhance lesions of NE, while negative strains unable to induce NE lesions (Keyburn et al., 2008).

The NetB toxin causes pores to creates bilayers in human and animal cell phospholipid membrane, permitting ions (such as Na+, Cl-, Ca2+, and others) to get through and leads to osmotic cell lysis (Datta et al., 2014). So, this study was designed to determine the prevalence of C. perfringens in broiler chickens from different farms in Assiut Governorate.

MATERIALS AND METHODS

Sampling:
From February to August 2021, 100 intestinal specimens were collected aseptically from newly sacrificed 3-6 weeks aged broiler chickens from different 18 poultry farms in Assiut governorate. After a postmortem inspection, the samples were taken from sections of the intestine that had macroscopic lesions which were thought to be NE. As quickly as possible, samples were sent in an ice box to the laboratory of faculty of Veterinary Medicine-Assiut University.

Bacteriological and biochemical examination:
Inoculating intestinal samples into a cooked meat broth medium (Oxoid, UK) and incubation for 48 hours at 37°C in an anaerobic gas pack jar was used to isolate Clostridia. In an anaerobic environment, a loop of broth culture was streaked onto blood agar plates containing 7% sheep blood agar then incubated anaerobically for 48 hours at 37°C. For purification, colonies with double hemolytic zones were chosen and subcultured in Reinforced Clostridial agar. (LAB M 23) (Willis, 1977). Microscopic examination and biochemical tests were used for identification of isolates according to Macfaddin, (2000).

Genotyping of the Toxigenic Clostridium perfringens Isolates:
According to the manufacturer’s recommendations, DNA was extracted from C. perfringens isolates using the QIAamp DNA Mini Kit (QIAGEN). Specific oligonucleotide primer sequences for the α, β and ε genes of C. perfringens and the NetB toxin gene, according to Yoo et al. (1997) and Bailey et al. (2013) respectively were purchased.
from Midland Certified Reagent Company, (Oligos). The details of primers and amplification cycling condition were listed in table (1 and 2).

For DNA amplification 50 µL of the following reaction mixture (For α, β and ε toxins): 6 µL of DNA template, 25 µl Emerald Amp GT PCR master mix (2x premix), 1 µL from each primer (20 pmol µL), and 13 µl of DNase-RNase-free water. Specific preparation of the reaction mixture For NetB toxin was done as the following: 5 µl of DNA template, 12.5 µL EmeraldAmp GT PCR master mix, 1 µL from each NetB primers (20 pmol µL), 5.5 µL of DNase-RNase-free water, to a total volume of 25 µL.

**Table 1:** *Clostridium perfringens* specific genes and primer sequences used for molecular identification and typing

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Primer</th>
<th>Sequence</th>
<th>Amplified product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha</td>
<td>F</td>
<td>GTTGATAGCGCAGGACATGTTAAG</td>
<td>402 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CATGTAGTCTCATCTGTTCCAGCATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta</td>
<td>F</td>
<td>ACTATACAGACAGATCATTCAACC</td>
<td>236 bp</td>
<td>Yoo et al., 1997</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TTAGGAGCAGTATTGAACACTAGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epsilon</td>
<td>F</td>
<td>ACTGCAACTACTACTCATACTGTG</td>
<td>541 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CTGGTGCCCTTAATAGAAAGACTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Net B</td>
<td>F</td>
<td>CGCTTCACATAAAGGTTGGAGGC</td>
<td>316 bp</td>
<td>Bailey et al., 2013</td>
</tr>
<tr>
<td>Toxin</td>
<td>R</td>
<td>TCCAGCACCAGCAGTTTTCCT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2:** Cycling conditions of the different primers during PCR

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Primary denaturation</th>
<th>Secondary denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>No. of cycles</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha, Beta and Epsilon</td>
<td>94°C</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>35</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>5 min.</td>
<td>30 sec.</td>
<td>40 sec.</td>
<td>45 sec.</td>
<td></td>
<td>10 min.</td>
</tr>
<tr>
<td>NetB toxin</td>
<td>94°C</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>35</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>5 min.</td>
<td>30 sec.</td>
<td>40 sec.</td>
<td>40 sec.</td>
<td></td>
<td>10 min.</td>
</tr>
</tbody>
</table>

Finally, using 1.5 percent agarose gel in the presence of a 100-bp DNA ladder provided by QIAGEN 20 µL of the amplified product of the NetB toxin gene and 30 µL of the amplified product of the α, β and ε toxin genes were electrophoresed. To observe under UV light, the agarose gel was treated with ethidium bromide. A gel documentation system was used to photograph the gel, and computer software was used to evaluate the data.

**RESULTS**

**Bacteriological and Biochemical:**
Among 100 intestinal samples 52 were identified. The colony on reinforced clostridial agar were large, regular, round, slightly opaque but shiny colonies, as shown in Fig.1. On sheep blood agar colonies were surrounded by double zone of haemolysis, as shown in Fig.2. Gram's-stained smears showed that the organism was Gram positive large bacilli, and often coccobacillus or short
rod forms with blunt end, as shown in Fig.3.

Biochemically, all isolates produced milk digestion on litmus milk medium as shown in Fig. 4 and lecithinase activity on egg yolk agar as shown in Fig.5.

**Genotypic Detection of C. perfringens Toxins:**
Multiplex PCR was used to genotype toxigenic *C. perfringens* isolates. The 10 toxigenic isolates all contained the alpha gene (402 bp) and none of them had the genes beta (236 bp) or epsilon (541 bp), as shown in Fig. 6. All isolates were proven to be positive for the netB gene, which generates the NetB toxin, using a Uniplex PCR for the identification of this gene, as shown in Fig.7. This explain that all isolates were *C. perfringens* type G (10/10), as justified by the presence of the alpha and Net B genes (table.3)

**Table 3:** New revised classification of *C. perfringens* typing scheme toxin-based (Rood *et al.*, 2018)

<table>
<thead>
<tr>
<th>Toxin produced</th>
<th>α-toxin</th>
<th>β-toxin</th>
<th>ε-toxin</th>
<th>ι-toxin</th>
<th>CPE</th>
<th>Netβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
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<tr>
<td>E</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
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<tr>
<td>F</td>
<td>+</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>G</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Fig.1:** Typical colonies presumed to be *C. perfringens* on reinforced clostridial agar medium

**Fig.2:** *C. perfringens* colonies surrounded by double zone of haemolysis on sheep blood agar medium
Fig. 3: Showing the gram positive bacilli of *C. perfringens*.

Fig. 4: Stormy reaction and clotting of milk of *C. perfringens* on litmus milk media.

Fig. 5: Showing lecithinase activity on egg yolk agar medium.

Fig. 6: Typing of *C. perfringens* toxin genes by Multiplex PCR. Pos: positive control; Neg: negative control; L: DNA ladder (molecular weight marker 100-bp); lane 1-6: cpa positives *C. perfringens* toxigenic isolate.

Fig. 7: Uniplex PCR identification of netB gene. Pos: positive control; Neg: negative control; L: DNA ladder (molecular weight marker 100-bp); lane 1-6: netB positive.
DISCUSSION

Necrotic enteritis (NE) is a serious avian disease, with global losses estimated at $5 to $6 billion (Moore, 2016). The most common encountered signs observed in the examined birds were depression, decreased appetite, lowered growth rates and diarrhoea. These findings were recorded by several authors as Elwinger et al. (1992); Kaldhusdal et al. (2001) and Ibrahim et al. (2017).

The most significant postmortem lesions include swelling in small intestines with an unpleasant-smelling brown fluid, the pseudomembrane formation which is a brownish membrane lining the inner coat of the gut and necrotic hepatitis which characterized by small, white, pinpoint-like foci in the liver (Timbermont et al., 2011; Rizk et al., 2017 and Yadav and Sandeep, 2021).

Different broiler chicken flocks in Assiut Province were examined for Clostridium perfringens and the results recorded that 52 of the 100 suspected samples were positive to Clostridium perfringens. This explains that other infections may have had a role in the development of these lesions.

It is worth mentioning that in the present study that C. perfringens type G was isolated for the first time in Assiut Province (Table 3).

In the current investigation, the prevalence that rate of C. perfringens was 52% (52/100) in the examined flocks. This result is nearly similar to that of Abd El-wahab (2002), Mahmoud et al. (2008) and Shaaban et al. (2017) who recorded that the infection rate of C. perfringens was 54.4% and 54.1% and 55.9% respectively. However, lower prevalence rates of C. perfringens were detected by Abd-El Gwad and El-Kader (2001); Mostafa et al. (2016) and Merati et al. (2017), who detected a prevalence rates of C. perfringens was 44.4%, 16% and 34.44% respectively. Changes in prevalence rates across studies might be due to differences in sample methods, condition of the birds (healthy or diseased), and management practices inside the farm associated with antibiotic usage policies.

NetB has been identified as one of the primary virulence factors in NE outbreaks in birds (Keyburn et al., 2008). Type G of C. perfringens has been associated with cpA and NetB gene for the generation of α and NetB toxin and it is believed to be significant in the pathogenesis of NE in poultry. In the present study, all isolates (10/10) were positive for the CPA and NetB gene and grouped as toxin types G. These results are in line with the findings of AKM et al. (2021) and Khan et al. (2021) who found types G (α-toxin, NetB positive) of C. perfringens from fecal samples of commercial broilers from Pakistan and Japan respectively.

The existence of the netB gene in isolates from various countries was reported by several studies. In a study in Canada out of 41 isolates from broilers with NE 39 (95%) were positive to netB, while just 35% of healthy boilers infected with C. perfringens was identified positive to netB gene (Chalmers et al., 2008). However, in the United States, the prevalence rate of netB gene was 58% in birds with necrotic enteritis, otherwise, the netB gene was found in only 8.75 percent of normal microbiota strains (Martin and Smyth, 2009).
In fact, the netB gene is significantly linked to necrotic enteritis-produced strains, although in some research a small percentage of diseased birds was recorded as negative to netB and a small percentage of healthy birds were reported as positive to netB gene. As NE is a complex disease, the lack of disease in positive netB strains might be contributed to absence of predisposing factors.

Not only netB gene is responsible for NE in birds but also other virulence factor might be produced from *C. perfringens* and induce NE. Despite, in vitro avian models, netB-negative strains do not produce disease. Thus, It’s essential to detect the capacity of both netB-positive strains isolated from healthy birds and netB-negative strains isolated from diseased birds to produce NE to identify if netB is necessary for virulence. Induction of broilers by NetB-positive and NetB-negative strains in a large number produced an excellent association between NetB and the capacity to produce NE. (Keyburn et al., 2010 and Martin and Smyth, 2010).

Based on this finding, the highest prevalence rate of *C. perfringens* was detected in 3-6 weeks old broiler chicks. This might be explained by the risk of NE increases by insufficient levels of maternal antibodies in the circulatory system of birds up to 3 weeks of age. Stress could be one of the major causes produced by intestinal flora changes as a result of alternation of diet from starter to growth which facilities the environment spreading of *C. perfringenes* (Moore, 2016).

**CONCLUSION**

The decreased age (3-6 weeks) is considered as risk factor which cause high occurrence of *C. perfringens* in broiler chicks and *Clostridium perfringens* type G is the most prevalent in Assiut province. The NetB is a key factor in the pathogenesis of NE.

**REFERENCE**


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تجريب نت بي الجدي المرتبط بالكلوستريديوم برفرينجنوز والمسبب لمرض النخر المعوي

رجب فاروق، عمر امين، احمد حسن، رجب سيد ابراهيم

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تُجِربت هذه الدراسة بهدف معرفة مدى انتشار الكلوستريديوم برفرينجنوز في كتائبات التسمين في محافظة اسيوط وكذلك لعزل وتحديد العامل المسبب للتنكر المعوي والهياكل السميحة في المناطق السمية في المزارع. تم جمع منة (١٠٠) عينة من الألبام من الكتاءات المصابة أو أنفة حديثا يتراوح أعمارها من 3 إلى ٥ أسابيع. تم تسجيل الأعراض المرضية الظاهرة على الكتائبات المصابة على شكل انخفاض في حيويتها، وامتناعها عن الأكل والشراب مع وجود اسهالات، ولاحظ أن بعضها مصابا بأعراض اضطرابات حركية. وتُسجيل الصفة التشريحية التي بينت تضخم في جدران الألبام مع وجود مناطق بها نخر شديد، ووجود تشوهات عميقة ومناطق ذات اسطح حادة، كما أن الألبام تحتوي على مخلوقات دموية متساقطة من جدرانها ذات رائحة كريهة. أُسفرت نتائج الاختبارات البكتيرولوجية المستخدمة لعزل الميكروب والتعرف عليه (مروفولوجيا الخلايا والمستعمرات البكتيرية والاختبارات البيوكيميائية) عن عزل اثنين وخمسين (٢٥) عطرة محتملة للميكروب قيد الدراسة وذلك بنسبة ٢٥٪. وللتفتيح الجبيبي تم اجراء تقنية تفاعل البلمرة المتسلسل على بعض العطرات المعزولة عشرة (١٠) والتي أظهر أن جميع العطرات المختبرة إيجابية لبكتريا الكلوستريديوم برفرينجنوز (كلما من الفا والب نت) مما يجدر الإشارة إليه من نتائج الاختبار الأخير (تفاعل البلمرة المتسلسل) ان الكلوستريديوم برفرينجنوز من النوع جي وتم تسجيله لأول مرة في محافظة اسيوط.