INTESTINAL HISTOPATHOLOGICAL STUDY, ISOLATION AND MOLECULAR DETECTION OF *CLOSTERDIUM PERFERNGES*, FROM LAYER CHICKEN IN BASRAH GOVERNORATE

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ABSTRACT

This study was designed for the isolation and molecular identification of *Clostridium perfringens*. From a total of 200 samples, *C. perfringens* was isolated from 30 samples (25%) of laying hens and confirmed using a PCR assay by amplifying a segment of a gene (*16S rDNA* gene) from *C. perfringens*. The result of histopathology study showed severe necrotic area surrounded by inflammatory cells, erosions of the intestinal epithelium, ulceration of the epithelium, villous necrosis finding surrounded by inflammatory cells, vascular congestion, goblet cell hyperplasia. For the type of toxin, the bacterial isolates were analyzed by PCR using specific primers in order to determine the presence of cpa genes (*cpb*, *iA*, *cpe*, *cpb2*). Among the 30 *C. perfringens* isolates, 10 isolates were identified (33.33%) with C. She is type A (carrying the alpha toxin gene). Of these 10 isolates, 5 isolates (50%) were identified as type A simplex and 5 (50%) were identified as heterozygous but no None of the isolates carry both *cpb2* and *cpe* genes. As the dominant species, 20 isolates (66.66%) were identified as C. The research concluded that *C. perfringens* was one of the most important isolates from laying hens, and the toxin type A was identified as heterozygous (carrying *cpb2*), and type C is the most prevalent toxin in Basrah, and the detected toxins cause pathological changes in the intestine.

Keywords: Necrotic enteritis, toxins.

دراسة نسجية مرضية وعزل جزيئي وكشف عن مطثيات البيرفرنجس المعزولة من الدجاج البياض في محافظة البصرة

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المستخلص

صممت هذه الدراسة لعزل والتعرف الجزيئي لبكتيريا المطثية الحاطمة. من إجمالي 200 عينة، تم عزل البكتيريا المطثية الحاطمة من 30 عينة (25٪) من الدجاج البياض وتم تأكيدها باستخدام اختبار PCR عن طريق تضخيم قطعة من الجين (*16S rDNA* gene) من بكتيريا المطثية الحاطمة. أظهرت نتائج دراسة التشريح المرضي وجود منطقة نخرية شديدة محاطة بالخلايا الالتهابية، وتآكل ظهارة الأمعاء، وتقرح الظهارة، ونخر زغبي محاط بالخلايا الالتهابية، واحتقان الأوعية الدموية، وتضخم الخلايا الكأسية. بالنسبة لنوع السم، تم تحليل العزلات البكتيرية بواسطة تقنية PCR باستخدام بادئات محددة لتحديد وجود جينات (*cpb*, *iA*, *cpe*, *cpb2*)، من بين 30 عزلة من عزلات المطثية الحاطمة، تم التعرف على 10 عزلات (33.33%) مصابة بالنوع C. وهي من النوع A (تحمل جين توكسين ألفا). من بين هذه العزلات العشر، تم تحديد 5 عزلات (50٪) على أنها من النوع A البسيط و5 (50٪) على أنها متغايرة الزيكوت ولكن لم تحمل أي من العزلات جينات *cpb2* و*cpe*. باعتبارها الأنواع السائدة، تم تحديد 20 عزلة (66.66٪) على أنها من النوع C. وخلص البحث أن بكتيريا المطثية الحاطمة كانت واحدة من أهم العزلات من الدجاج البياض، وتم تحديد السم A على أنه متغاير الزيجوت (يحمل cpb2)، ويعتبر النوع C أكثر السموم انتشارا في البصرة، وتسبب السموم المكتشفة تغيرات مرضية في الأمعاء.

الكلمات المفتاحية: التهاب الأمعاء النخري، سموم.

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INTRODUCTION

Necrotic enteritis is a disease that affects poultry and spreads all over the world. This disease is characterized by several signs, including severe diarrhea and necrotic inflammation in the intestine, as well as diffuse necrotic foci in the liver, kidneys and cecum (35,37). This disease causes huge economic losses, sometimes exceeding two billion US dollars per year, due to treatment costs, bird losses, and the cost of disease prevention measures (38). *Clostridium perfringens*, one It is one of the most common types of bacteria that cause diseases in the environment. The bacteria are anaerobic, Gram-positive, spore-forming bacteria (1,3). These bacteria are the fastest growing foodborne pathogens, they are found in soil, air, water, and food as well as in the intestinal tracts of humans and animals (36). This microorganism is probably one of the most prevalent pathogenic bacteria and is undoubtedly the most important cause of Clostridium in humans and animals (15,16,32). On the other hand, pets are known to be the source of human food poisoning. Accordingly, this risk must be reduced or eliminated, and strategies must be developed to diagnose and prevent infected animals from entering the food chain (4,33). One of the things that can be caused by *C. perfringens* is poor production performance in chickens. The function of the intestine is to achieve optimal food conversion. There are some factors that reduce trophic shift such as necrotic intestinal lesion and abnormal Clostridium predominance in the intestinal microflora with Clostridium toxin all reducing productivity (20,22,27). The clinical form of the disease is associated with a huge economic burden, while the clinical form of the disease significantly reduces the growth performance of chickens by causing severe damage to the gut epithelial layer (21). Molecular characterization and toxicological profiling are a rapid tool for the detection of *C. perfringens* from suspected cases of necrotizing enteritis. There is a strong association between type A isolates carrying the *cpe* gene and C. chromosomal. Some C, D and E isolates also carry functional *cpe* genes on large plasmids (14,30). Aims of study this study was designed to isolate and confirm *C. perfringens* by polymerase chain reaction (PCR) and typify enterotoxins from laying hens in Basra province. To achieve these goals, we need to take the following steps to achieve these goals: To confirmed *C. perfringens* bacteria using a PCR assay by amplifying a segment of a gene (*16S rDNA* gene). The type of toxin was analyzed by bacterial isolates by polymerase chain reaction, using specific primers in order to determine the absence or presence of genes (*cpa, cpb, iA, cpe,* and *cpb2,*).

MATERIALS AND METHODS

Samples collection a total of 200 samples of layer chicken were collected from different poultry farms chickens suffer severe depression, diarrhea, unwillingness to move, feather reproduction, and sometimes sudden death and increased mortality. We use the rinsing technique to restore surface bacteria as follows: The intestine sample tacked for grossly study. Samples were also taken for the purpose of histological cutting, by placing them in formalin at a concentration of 1%, and then making a histological section, as well as layer intestine was placed in a sterile container, 300 mL of phosphate buffer was added for molecular study. The container was then shaken over the sample for 15 seconds, then the rinse suspension was transferred to the laboratory on ice and bacterial analysis began within 1 to 4 hours.

Bacterial isolation All laboratory work was performed in the Public Health Laboratory for postgraduate students in the Public Health Department of the College of Veterinary Medicine at the University of Basra. The samples were taken from the intestine, and it was diagnosed macroscopically at the beginning, then a sample of the intestine was placed in the transport medium (peptone water). In the laboratory, a medium consisting of nutrient agar was prepared under suction conditions. The sample was cultivated on medium by means of a ring, a petri dish was placed in the jar, and a pruning candle in the jar, and closed tightly (13). After that, it will be explained later. Place the jar in the incubator at 37°C for a period of 48 hours. After the end of the incubation period, the jar is taken out, and the bacteria are grown under anaerobic conditions.

Procedure of Candle Jar a candle-extinguishing jar or bottle with a large mouth for petri dishes. Several plates were placed in the jar after they were inoculated. After placing the dishes in the jar, a small candle was placed near the bottom of the jar. The upper part has been replaced and tightened. I will cause the lit candle to increase the amount of carbon dioxide in the jar, and eventually the oxygen content will decrease, and then the candle will stop burning. The jar is then placed in the incubator at 37 °C. The jar was removed, the dishes opened, and then the cultures were removed and the cultures performed for a reading. After that, the indole, catalase, urease tests were performed on it, then it was stained with a gram stain to identify the bacteria in preparation for molecular work and its diagnosis by PCR technique. After that, colonies were taken in order to perform the molecular work. The nuclear material was extracted from the bacteria for the purpose of technology (11,23).

DNA extraction and PCR DNA extraction was performed in duplicate for all samples using the Genomic DNA Extraction Kit (G-spin Total). Where bacterial DNA was extracted according to the bacterial extraction kit from the manufacturer.

To confirm the presence of bacterial DNA, a Nanodrop technique was used to determine the presence and purity of the nuclear material. periodically mixed the solution by gently tapping the tube, DNA was stored at 2-8°C. The purified products were then detected by electrophoresis on 1% agarose (5). Go Taq Green Master Blend used from Promega and Primary Oligonucleotide used from Alpha DNA/Canada. The primers that were used are shown in Table 1. Sample DNA was amplified in a total reaction volume (25 ml) (28) containing genomic DNA (3 ml), primers (1 µl) for each primer, master mix (12.5 µl) and complete volume by adding 7.5 µl of ddH_2O. The polymerase chain reaction (PCR) test was performed as follows: [Initial denaturation step at a temperature (95°C for 5 minutes), which is followed by 35 cycles for each of: a denaturation step (95°C for 45 seconds), followed by annealing step which was (50°C for 45 seconds), then use a thermal cycler to perform the extension step (72°C for 45 seconds)]. After that, a final elongation is done at (72°C for 6 minutes) (Techne-UK).

PCR products detection the amplified PCR product was detected on agarose gels (1%) prepared with agarose in Tris-borate-EDTA buffer (1X), stained with a fluorescent stain, and images were recognized by electrophoresis. The band's size was determined by comparing it to a standard (100 bp) DNA ladder. After that, toxin A samples were sent to Macrogene for sequencing in Korea, the sequences were edited and aligned and compare it with what is in NCBI to find out the source of the strain in Iraq, then draw the phylogenetic tree of this gene.

Table 1. ***16S rDNA, cpa, cpb, cpb2, iA*** **and** ***cpe*** **primers used in current study.**

Target gene	Primers sequences (5`-3`)	Annealing temperatures	Product length (bp)	References
16S rDNA	AAAGATGGCATCATCATTCAAC TACCGTCATTATCTTCCCCAAA	53 ˚C	279	Wu, et al (39)
cpa	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	55 ˚C	324	Meer and Songer (29)
cpb	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	55 ˚C	196	Meer and Songer (29)
cpb2	AGATTTTAAATATGATCCTAACC CAATACCCTTCACCAAATACTC	55 ˚C	567	Bueschel (10)
iA	ACTACTCTCAGACAAGACAG CTTTCCTTCTATTACTATACG	55 ˚C	446	Meer and Songer (29)
cpe	GGAGATGGTTGGATATTAGG GGACCAGCAGTTGTAGATA	55 ˚C	233	Meer and Songer (29)

RESULTS AND DISCUSSION

Morphology and phenotypic characterization: The samples were diagnosed in from different farms of Basra Governorate. Chickens in these fields suffer from signs that give a preliminary diagnosis of necrotic enteritis. Two possible types of disease were diagnosed, there are two types of necrotizing enteritis (NE), namely the acute clinical form

and the second type is the clinical form. Although it can be seen at any age, the main disease that affects young chickens is the acute clinical form, which presents with unwillingness to move, severe depression, diarrhea, enlarged feathers, increased mortality and sudden death. The subclinical form does not produce external signs but has a significant impact on performance. In the current study, all samples were detected through the gross study, and Figures 1, 2 and 3 represented gross changes in the infected intestine of chicken layer, and we have shown in Figure 1 balloons with gas, bloody content in the intestinal lumen, mixed with necrotic detritus and gas bubbles in Figure 2, while Figure 3 represents ulceration of intestinal mucosa Figure 4 represented a culture of *C. perfringens* in an anaerobic condition. *C. perfringens* is a spore-forming bacillus that commonly inhabits soil, poultry droppings, and harbors as enteropathogens in chickens and other animal (6,18,19,34). Different types of toxins cause a wide range of gastrointestinal and systemic diseases in various animals, including chickens. It is also important that it participates in food poisoning (diseases transmitted through food of animal origin), which has developed in the current period with the consumption of various raw and canned foods, especially chicken meat and meat products (40). *C. perfringens* type A, is a bacterial pathogen that causes necrotic enteritis in broiler chickens and thus leads to visible and invisible economic losses. During the results of the current study, the prevalence of *C. perfringens* was 15% of 200 diseased layer chickens. previous studies by Kalender and Ertas (24) who reported a lower prevalence of enterocolitis 8 and 5% of enteric broiler chickens, respectively. This difference may be due to the different methodologies used for isolation, classification of microorganisms as well as the management of poultry farms used such as the use of growth promoters (12,31). These bacteria are used as a source for PCR technology to confirm the diagnosis and then a limited type of toxin. Table 2 shows the results of the growth of bacteria by the anaerobic method as well as the results of the PCR technique.

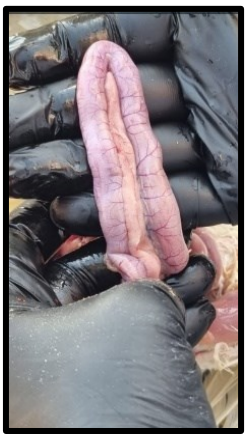

Figure 1. Gross lesion of layer chicken intestine infected with *C. perfringens* showing balloons with gas.

Figure 2. Gross lesion of layer chicken intestine infected with *C. perfringens* showing bloody content in the intestinal lumen, mixed with necrotic detritus and gas bubbles.

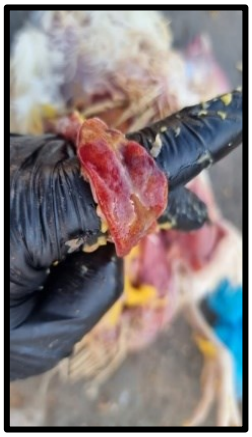

Figure 3. Gross lesion of layer chicken intestine infected with *C. perfringens* showing ulceration of intestinal mucosa.

Figure 4. Nutrient agar showing culture of *Clostridium spp.* in anerobic condition.

Table 2. Conventional bacteriological analysis positive results and PCR

umber of animals	Tested No.	Conventional bacteriological and PCR analysis No. (%) Plating Characterization	Indole, catalase, ureases tests and gram stain	PCR
200	200	50 (15%)	50 (25%)	30 (15%)

The results of histopathological examination: The results of histopathological examination showed in Figures (5-13) represented various changes in tissue of

intestine. These Figures show sever necrotic area and surrounded by inflammatory cells in Figure 5, erosion of the epithelium of the intestine in Figure 6, as well as showed sloughing of villi and surrounding by fibrinoid exudate in the Figure 7. Figure 8 represented coagulative necrotic of the villi surrounded by inflammatory cells, congestions of blood vessels and area of hemorrhage represented in Figure 9, while Figure 10 shows area of hemorrhage, congestions of blood vessels and distraction of the epithelium. Figures 11 and 12 shows congestions of blood vessels and area of hemorrhage respectively, while Figure 13 shows hyperplasia of goblet cells.

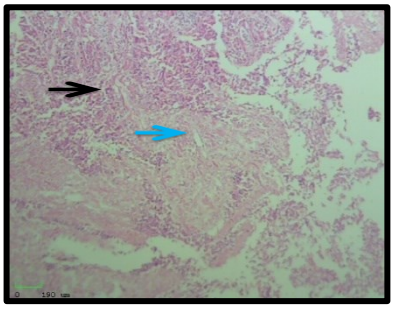

Figure 5. Histopathological section of layer chicken intestine infected with *C. perfringens* shows intensive necrotic at tip of villi ( ) with inflammatory cells infiltration mild ( ). H&E 100X

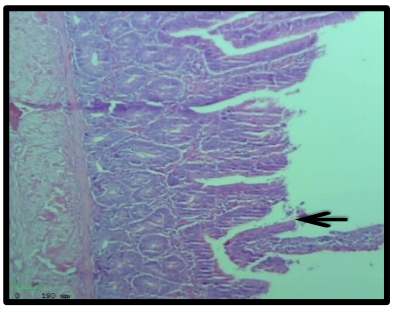

Figure 6. Histopathological section of layer chicken intestine infected with *C. perfringens* shows erosion of the epithelium of the intestine ( ). H&E 100X

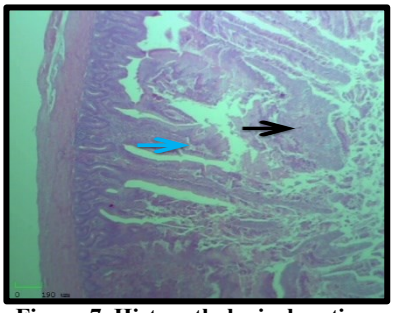

Figure 7. Histopathological section of layer chicken intestine infected with *Clostridium perfringens* shows sloughing of villi ( ) surrounded by fibrinoid exudate ( ). H&E 10X

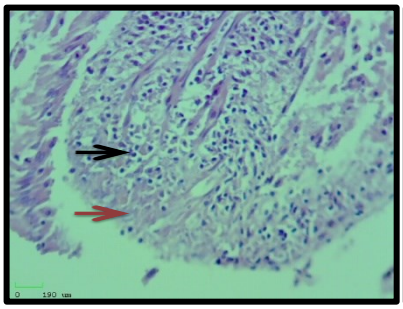

Figure 8. Histopathological section of layer chicken intestine infected with *C. perfringens* shows coagulative necrotic of the villi ( ) surrounded by inflammatory cells ( ). H&E 10X

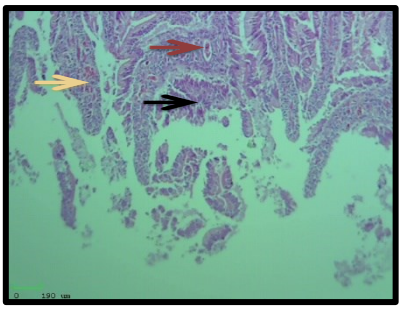

Figure 9. Histopathological section of layer chicken intestine infected with *C. perfringens* shows congestions of blood vessels ( ) distraction of the epithelium ( ) and area of hemorrhage ( ). H&E 10X

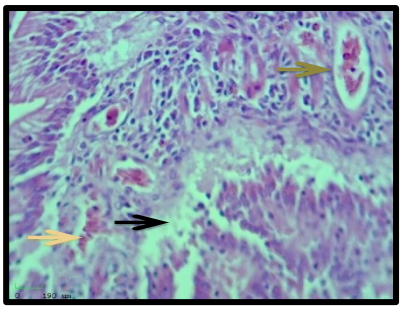

Figure 10. Histopathological section of layer chicken intestine infected with *C. perfringens* shows congestions of blood vessels ( ) distraction of the epithelium ( ) and area of hemorrhage ( ). H&E 10X

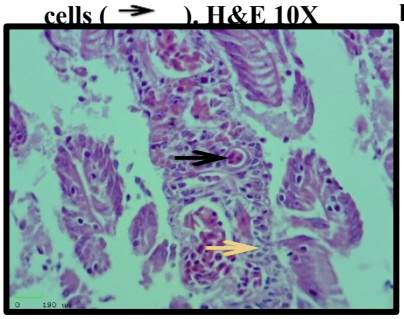

Figure 11. Histopathological section of layer chicken intestine infected with *C. perfringens* shows congestions of blood vessels ( ) with epithelium sloughing ( ). H&E 100X

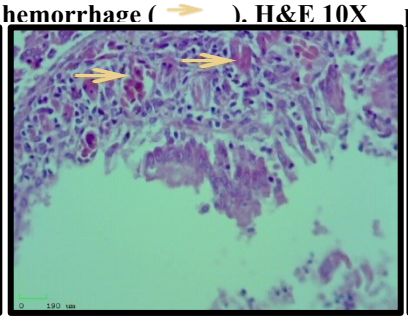

Figure 12. Histopathological section of layer chicken intestine infected with *C. perfringens* shows area of hemorrhage ( ). H&E 10X

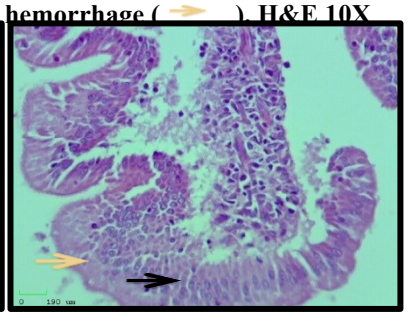

Figure 13. Histopathological section of layer chicken intestine infected with *C. perfringens* shows irregular hypertrophy of enterocytes ( ) with mild cellular infiltration in proprial tissuee mucus ( ). H&E 400X

This results agreement with Al-Sheikly and Truscott (7) in the appearance of necrotic areas reaching the crypts and massive necrosis of the villi, cellular debris in the intestinal lumen at this stage and fibrin. The histopathological lesions visible to us are also similar to those recorded by researchers Brynestad and Granum (9). Explain correspondence with what was stated in Filip Van Immerseel (22). **PCR** *C. perfringens* was isolated from 50 samples out of 200 samples by bacteriological diagnosis, while only 30 samples (15%) showed a positive result confirmed using a PCR test to amplify a specific segment of the *16S rDNA* gene identified from *C. perfringens*. As for the determination of the type of toxin, this was done by analyzing the bacterial isolates by means of the multiplex polymerase chain reaction technique, by using alopecia primers for the purpose of determining the presence or absence of the genes under study (*cpa*, *cpb*, *iA*, *cpe* and *cpb2*). Then the PCR results corresponding to the negative controls were shown in Figure 14 showing the result for *16S rDNA*. Of the 30 *C. perfringens* isolates, 10 isolates (33.33%) were identified as type A (carrying an alpha-toxin gene). Of these 10 isolates, 5 isolates (50%) were identified as type A simplex (carrying neither *cpe* nor *cpb2* gene) and 5 isolates (50%) were identified as heterozygous types (carrying *cpb2* gene) but no any of the isolates. She carries both the *cpb2* and *cpe* genes. As the dominant species, 20 isolates (66.66%) were identified as C-type (Figure 15). Thus, identification of the toxicogenic strain in a sample, by using PCR technique, can be done before toxin producing. The PCR technique is a strong for finding and diagnosing the minimum number of microorganisms. A multiplex PCR technique was developed, which can detect all major toxin genes of *C. perfringens*. Then, it was confirmed that all isolates taken from the intestines of laying hens in Basra governorate are *C. perfringens* type A by detecting the *cpa* gene using multiplex PCR technology. Not surprisingly, all *C. perfringens* isolates in this study were positive according to the multiplex PCR results, and all were identified as type A.

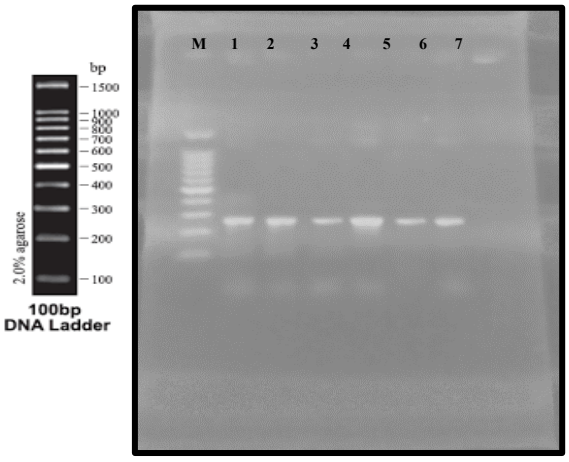

Figure 14. The PCR amplification of *16S rDNA* gene products of *Clostridium.* Lane 1 M: Marker (DNA ladder, 100 bp). Lanes: 2, 3, 4, 5 and 6, PCR amplification of *16S rDNA* gene products of *C. perfringens* isolates (279-bp). Lane 7 negative control.

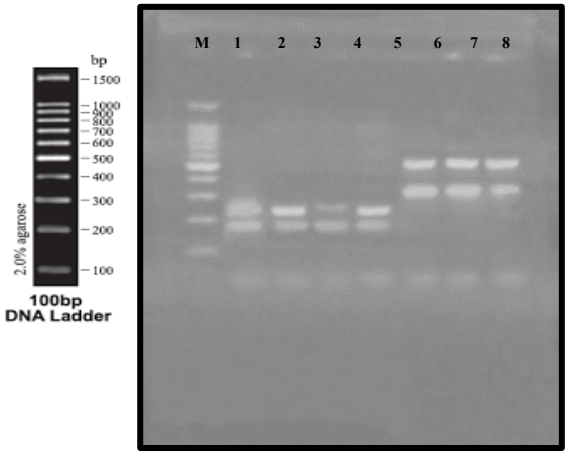

Figure 15. PCR amplification for toxins detection. Lane 1 M: Marker (DNA ladder, 100 bp). Lanes Lane 1,2,3 and 4: *C. perfringens* type C isolates with *cpb* and *cpe* genes. Lanes 5,6 and 7: *C. perfringens* type A with *cpb2* and *cpa* genes. Lane 8 negative control.

This can explain this by the fact that the cpa toxin gene is a common gene expression for all *C. perfringens* species, and is also common among *C. perfringens* species worldwide, and predominant in almost all research on poultry is *C. perfringens* type A (17). In Norway in the 1990s, *C. perfringens* has been recorded as the most common cause of food poisoning (9). The prevalence rate in other countries, such as Japan, the United States and the United Kingdom, is also high (9). In Wales and England, *C. perfringens* was the second most

frequently reported organism that was associated with foodborne enteric disease outbreaks in the United States in the 1990s (22). The infection vehicles are usually poultry products and meat. A survey by Lin and Labbe showed that these foods were the most heavily contaminated with isolates of *C. perfringens* isolates (26). The results of this study confirm that alpha-toxin is primarily responsible for the occurrence of necrotizing enteritis in chickens. Basically, PCR is used to detect virulence genes of *C. perfringens* due to its high sensitivity and specificity (7,8). All toxin species of *C. perfringens* are capable of producing an alpha toxin that has lecithinase activity and causes tissue necrosis especially in the small intestine. Mainly responsible for necrotizing enteritis in chickens is alpha-toxin (25). Necrotic enteritis is a serious global poultry disease caused by *C. perfringens* that results in production losses through increased feed consumption and mortality rates and reduced chicken welfare (2). Necrotizing enteritis is characterized by a sudden increase in mortality without any significant clinical symptoms. Meanwhile, the subclinical form becomes more prevalent and is characterized by decreased nutrient digestibility, absorption, feed conversion rate and intestinal mucosal damage. During the current study, all strains were identified as type A and type C. These results agreed with Afshari *et al.* (3). It can be explained why species B, D and E were not found in the samples in the first place. Where Songer and Meyer reported that 92.70% of the isolates in their study were type A. while type B was 0.10%; Type C gave 4.50%; It was followed by type D, with a rate of 2.10%, and finally type E, with a percentage of 0.60% (29). It can be concluded that the most widespread type in this region, in Basra Governorate, is type C, and here the importance is great because this type causes major and fatal diseases in humans. Accordingly, more investigations must be done and samples taken in larger sizes and in different and larger geographical areas in Iraq. The results of sequencing of the genetic material in the genetic tree showed that the diagnosed isolate of toxin type A (*cpb2* gene) was 100% similar to the isolates in Iran and America. GU581180 / Iran / 100%, AY884040 / USA / 100%, AY884035 / USA / 100%, MN503254 / Italy and Turkey 99.81%, changes MK285060 / Australia 99.81% and CP025503 / Australia 99.81%. Figure 16 represented the phylogenetic tree of toxin type A (*cpb2* gene) was 100% similar to the isolates in Iran and America. The phylogenetic tree of toxin type A (*cpb2* gene) was 100% similar to the isolates in Iran and America. The research recommends the following: Search for other genes and investigate the presence of other strains of toxins. Search for other causes of *Clostridium* other type of *C. perfringens* bacteria, and sample collection and identification of Clostridium bacteria from broiler chicken.

Conclusions: The research concluded that *C. perfringens* was one of the most important isolates from laying hens, and the toxin type A was identified as heterozygous (carrying *cpb2*), and type C is the most prevalent toxin in Basrah, and the detected toxins cause pathological in the intestine.

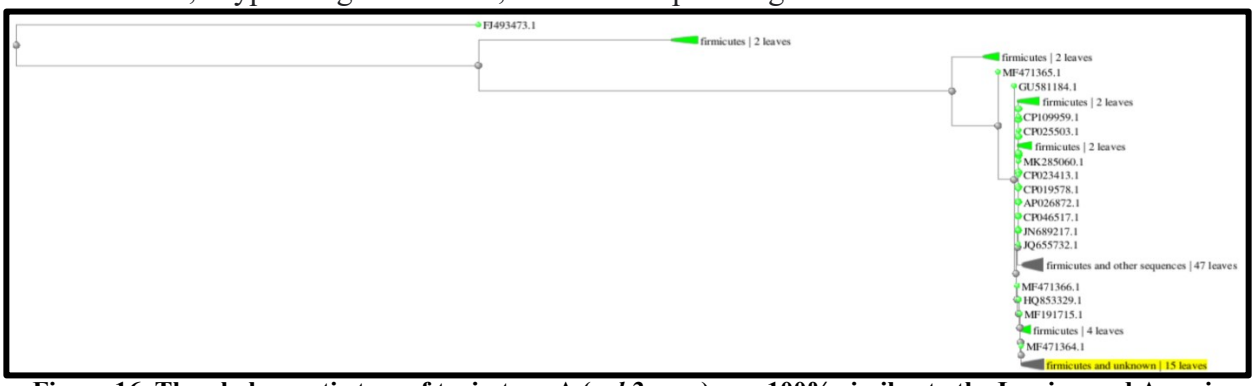

Figure 16. The phylogenetic tree of toxin type A (*cpb2* gene) was 100% similar to the Iranian and American isolates.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare

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