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Antimicrobial Resistance Pattern of Clostridium Perfringens Isolated From Broilers

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Abstract

THE global broiler business is heavily impacted financially by *Clostridium perfringens*, the main cause of necrotic enteritis (NE). In this work, *C. perfringens* obtained from NE cases in broiler chickens was examined for prevalence, antimicrobial resistance profiles, and antibiotic resistance genes. Out of 434 collected samples, *C. perfringens* was detected in 166 samples (38.3%). Among these, 137 of 203 intestinal samples (67.5%) and 20 of 141 liver samples (14.2%) from diseased birds (157 of 344; 45.6%) tested positive, along with 9 of 90 (10%) intestinal samples from apparently healthy birds. Identification was confirmed using both conventional and molecular methods. According to antimicrobial susceptibility tests, streptomycin had the highest resistance rates, followed by erythromycin, ampicillin, penicillin G, cefotaxime, tetracycline, ciprofloxacin, amikacin, rifampicin, and amoxicillin/clavulanic acid. Conversely, the lowest resistance was observed against imipenem. Notably, 100% of the isolates of *C. perfringens* exhibited multidrug resistance (MDR). Molecular screening for resistance genes revealed a high prevalence of the macrolide resistance gene *erm*B (141/166; 84.9%), β-lactamase resistance gene *bla*CTX (114/166; 68.7%), tetracycline resistance gene *tet*M (77/166; 46.4%), and fluoroquinolone resistance genes *qnr*A (119/166; 71.7%), *qnr*B (85/166; 51.2%), and *qnr*S (20/166; 12.0%). These results demonstrated that multidrug-resistant *C. perfringens* is highly prevalent in broilers and underscore the need for routine monitoring of antimicrobial susceptibility to inform effective control strategies and mitigate the spread of resistance.

Keywords: Antibiotic resistance, Broilers, *Clostridium perfringens*, Necrotic enteritis.

Introduction

Clostridium perfringens (C. perfringens) is a common gram-positive, spore-producing, anaerobic bacterium that is naturally present in the gastrointestinal tracts of both humans and animals [1]. Furthermore, it exists in the normal animal gut flora and turns harmful when the gut microbiota's equilibrium is upset [2]. Necrotic enteritis (NE), a serious digestive system illness caused by C. perfringens, is a major concern in the chicken business and can result in significant economic losses [3].

A variety of toxin genes mediated by chromosomes and plasmids make up C. perfringens' virulence arsenal. According to the patterns of occurrence of the toxin genes Alpha (α), beta (β), epsilon (ε), and iota (ι) cpe and netB, strains of C. perfringens are divided into seven toxinotypes (A, B, C, D, E, F, and G. While C. perfringens type A strains share the cpa gene encoding alpha-toxin (a phospholipase C), not all toxinotypes share this gene. The cpa gene, which is part of the conserved core genome, is associated with histotoxic diseases like gas gangrene. However, other toxinotypes of C. perfringens are defined by different key toxins, such as beta-toxin in type C, and many toxin genes, including those for intestinal toxins, are found on mobile genetic elements (plasmids) rather than the core chromosome. Toxinotype A is defined by the presence of alpha-toxin (encoded by cpa). Other toxinotypes, like type C, are defined by the presence of beta-toxin (encoded by cpb), which is also a major virulence factor, but is not share by all toxinotypes .

Another issue with C. perfringens infections is antibiotic resistance. The bacterial environment has changed as a result of the prolonged and extensive use of antibiotics in chickens over the past few years, eradicating vulnerable strains and enabling the persistence and dominance of antimicrobial-resistant bacteria. Although many countries currently forbid it, antibiotics have been used as growth enhancers for decades [5]. Since they have less internal transport of the antibiotic, anaerobic bacteria like C. perfringens often have limited susceptibility to aminoglycosides

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[6]. Resistance to streptomycin could be due to the absence of quinones in C. perfringens [7]. Plasmids, transposons, and insertion sequences are the main mechanisms by which C. perfringens develops antibiotic resistance [8].

Concern regarding C. perfringens' resistance to erythromycin and tetracycline has grown in recent years, and this is exacerbated by the bacteria's capacity to generate spores and create biofilm [9]. Importantly, beta-lactamase (bla) and quinolone (qnr) resistance genes were found to be associated with increased resistance to amoxicillin and ciprofloxacin, respectively [10]. The purpose of this investigation was to find antibiotic resistance genes and examine the antimicrobial resistance profiles of Clostridium perfringens isolated from broiler chicken cases of necrotic enteritis.

Material and Methods

Sample collection

From December 2022 to August 2023, 434 samples were taken from various farms in Mansoura, Dakahlia Governorate, Egypt. These samples included 203 intestinal scrapings and 141 liver samples from diseased birds (n=344) exhibiting symptoms of necrotic enteritis, as well as intestinal samples (n=90) from birds that appeared to be healthy. The affected birds displayed symptoms of necrotic enteritis, including emaciation, dehydration, diarrhea (frothy and foamy pings with a clear zone of fluidity), ruffled feathers, acute depression, decreased appetite, and unwillingness to move. For bacteriological analysis, all samples were sent right away to the lab in sterile ice containers.

C. perfringens isolation and identification

Ten milliliters of recently made Robertson cooked meat (RCM) broth (HiMedia, India) were infected with samples. A loopful was streaked onto tryptose sulphite cycloserine agar base supplemented with D-cycloserine (HiMedia, India) and contained 5% egg yolk following an overnight anaerobic incubation at 37°C (using a Gaspak anaerobic jar). Plates were incubated anaerobically for 24 hours at 37 °C. Three to four well-isolated black colonies that showed lecithinase activity and were thought to be *C. perfringens* were streaked on Columbia blood agar (HiMedia, India) that contained 5% defibrinated sheep blood to identify and assess the purity of *C. perfringens*. The colonies were then examined for the typical Double-zone hemolysis associated with *C. perfringens*. Based on microscopic appearance, colonial morphology, dual haemolysis, fermentation of glucose, and lecithinase production, identification of C. perfringens was done [11]. The confirmed isolates were kept at -80°C and cryopreserved in brain-heart infusion broth that contained 24% glycerol.

Molecular identification of Clostridium perfringens and antibiotic resistance genes

The boiling procedure was used to extract the DNA from the sample [12]. Three to five suspected colonies were generally chosen, inoculated in three milliliters of thioglycolate broth, and then incubated under anaerobic condition for 24 h at 37°C using an anaerobic gas pack (BD) in an anaerobic gas jar. The bacterial suspension was centrifuged at 8000g for two minutes using one milliliter, and the supernatant was disposed of. After re-suspending the bacterial pellet in 1 milliliter of nuclease-free water, it was heated to 95 °C for 20 minutes using a Biometra thermal block. After being utilized as a DNA template, the supernatant was kept at -20°C for further molecular analysis. The extracted DNA was screened for *C. perfringens* specific gene (cpa) and antibiotic resistance genes (blaCTX, tetM, ermB, qnrA, qnrB, qnrS) using the uniplex polymerase chain reaction assays. The primer sequences, amplicon size, and PCR condition are shown in Table (1). EmeraldAmp Max PCR Master Mix (Takara, Japan) was used for all PCR reactions. 5 µl of DNA template, 12.5 µl of EmeraldAmp Max PCR Master Mix, 1 µl of each primer with a concentration of 20 pmol, and 5.5 µl of water were used in the final volume of 25 µl. There were particular profiles that were programmed into the Applied Biosystem 2720 heat cycler. The PCR products were electrophoresed on a 1.5% agarose gel (Applichem, Germany). A gel documentation system (Alpha Innotech, Biometra) was then used to photograph the products under ultraviolet light after they had been stained with ethidium bromide (Sigma-Aldrich, U.S.A.). Water devoid of nuclease was employed as a negative control. Positive control was a reference strain of C. perfringens type A (ATCC13124) was kindly gotten from the Animal Health Research Institute's Anaerobic Unit in the Bacteriology Department in Dokki.

In-vitro susceptibility testing of C .perfringens

The disc diffusion method was used to conduct for antibiotic sensitivity on Muller Hinton agar (MHA) (Oxoid, UK) in accordance with the Clinical and Laboratory Standards Institute's guidelines [17]. The choosing of antibiotic discs depended on commonly used antibiotics in human and poultry medicine, containing tetracyclines (tetracycline 30 µg), macrolides (erythromycin 15 µg), quinolones (ciprofloxacin 5 µg), aminoglycosides (streptomycin 10 µg; amikacin 30 µg), and β-lactams (amoxicillin/clavulanic acid 30 µg; penicillin G 10 IU) (Oxoid, UK). Briefly, sterile swabs of cotton were used to streak well-isolated *C. perfringens* colonies plated on Columbia blood agar onto Muller Hinton agar (Oxoid Ltd, Hampshire, England) plates after they had been suspended in a 0.9% sodium chloride solution to a 0.5 MacFarlane standard. Following that, based on guidelines of CLSI (2024)

the inhibition zone was measured and classified as susceptible, resistant, and intermediate. Due to the lack of breakpoints for *C. perfringens*, interpretive criteria for Staphylococcus aureus were also used [18]. Multidrug resistant (MDR) isolates were microorganisms that exhibited resistance to three or more types of antibiotics [19].

Results

Prevalence of C. perfringens

C. perfringens was detected in 166/434 (38.3%) involving; 137/203 (67.5%) intestinal samples and 20/141 (14.2%) liver samples from diseased birds (157/344; 45.6%) and 9/90 (10%) intestinal samples from apparently health birds by the traditional and molecular characteristics technique (Table 2). Alpha toxin genes (cpa) were found in all isolates, confirming their identity as *C. perfringens.*

Antibiotic susceptibility results of C. perfringens

The examined *C. perfringens* isolates showed a higher level of resistance to streptomycin (92.8%), followed by erythromycin (87.4 %), ampicillin (70.5 %), penicillin G (69.3%), cefotaxime (67.8%), tetracycline (59%), ciprofloxacin (58.4%), amikacin (53.7%), rifampicin (33%), and amoxicillin/clavulanic acid (29.5%) (Table 3).

However, the highest susceptible antibiotics were shown in imipenem (90.9%) in comparison with the other tested antibiotics. the *C. perfringens* isolates were resistant three or more antibiotic classes so all, 100% of the isolates of *C. perfringens* exhibited multidrug resistance (MDR).The heat map with hierarchical clustering was applied to group samples based on antibiotic susceptibility helping to phenotypic relationship and differences among samples as illustrated in (Fig.1). A heat map showing the results of antimicrobial susceptibility testing. Blue color indicated resistance. A high resistance was observed against streptomycin, erythromycin, and ampicillin. While red color indicated sensitivity as 90.9% o *C. perfringens* of isolates were completely sensitive to imipenem. Therefore, imipenem, rifampicin were shown to be the most sensitive antibiotics, which appeared to be a potential therapy for *C. perfringens* infection. So, effective antibiotics against *C. perfringens* after using heat map was imipenem in opposite to streptomycin was infective agent against the bacteria.

Identification of β-lactamase, tetracycline, macrolide, and quinolone- encoded genes

Among the examined *C. perfringens* isolates, 141 out of 166 (84.9%) carried the macrolide resistance gene (ermB), 114 (68.7%) carried the beta-lactam resistance gene (blaCTX), 77 (46.4%) carried the tetracycline resistance gene (tetM), and the quinolone resistance genes were detected as follows: qnrA in 119 isolates (71.7%), qnrB in 85 isolates (51.2%), and qnrS in 20 isolates (12.1%)(Fig .2).

Discussion

Clostridium perfringens is a major pathogen in poultry, which causes necrotic enteritis, which is marked by acute depression and an abrupt rise in flock death rates. More significantly, it is linked to subclinical infections that cause long-term harm to the gut mucosa, which lowers weight gain, poor performance, and consequently, significant economic losses [20]. In the current study, all *C. perfringens* isolates harbored the *cpa* gene, which is considered one of the most crucial virulence elements responsible for pathogenicity of necrotic enteritis in chickens [21]. The overall prevalence of *C. perfringens* among the samples examined was 38.3%. This included 67.5% of intestinal samples and 14.2% of liver samples from diseased birds (45.6%), as well as intestinal samples from apparently healthy birds (10%). Notably, the prevalence was higher in diseased birds (45.6%) compared to apparently healthy birds (10%). These findings are consistent with previous studies reporting *C. perfringens* prevalence rates of 38.7% in Egypt and 38.42% in China [22, 23]. [24] Observed a greater isolation rate of 70%. In contrast, lower prevalence rates of 12.6%, and 23.4% have been documented in Egypt, and China, respectively [25, 26]. The observed differences in *C. perfringens* prevalence among studies may be attributed to variations in management practices and/or feed composition. For instance, the inclusion of fishmeal in poultry diets has been shown to alter the gut micro biota profile [27]. Additionally, differences in isolation methods and various predisposing factors such as dietary components, the use of probiotics and prebiotics, and exposure to antimicrobial drugs may also contribute to the variability in prevalence rates reported across different studies [28].

The fact that C. *perfringens* is a normal component of chickens' gut micro flora and may exist in the intestines at low concentrations (less tan10^5 CFU/g) clarifies why it was found in samples from apparently healthy birds in our investigation. However, under certain conditions, its population may increase, predisposing birds to necrotic enteritis [29]. Furthermore, intestinal samples from diseased broiler showed a higher isolation rate of *C. perfringens* (67.49%) compared to hepatic samples (14.18%). This finding is consistent with other studies conducted in Egypt, which reported intestinal and hepatic isolation rates of approximately 56% and 47.4%, respectively [30].

Globally, *C. perfringens* antibiotic resistance is rising, which poses serious risks to public and animal health. The majority of *C. perfringens* isolates in this study were shown to have a total resistance against streptomycin by antimicrobial susceptibility testing.

The heat map results was designed to clarify the results of antimicrobial susceptibility testing. High levels of resistance were also observed to erythromycin, ampicillin, penicillin G, cefotaxime, tetracycline, ciprofloxacin, amikacin, rifampicin, and amoxicillin/clavulanic acid. These results concur with those of earlier Egyptian research that found that chicken isolates of *C. perfringens* were highly resistant to streptomycin and erythromycin, ciprofloxacin, cefotaxime, and rifampicin [31, 32]. Similarly, a study by [10] in Bangladesh reported the highest resistance to streptomycin (92.85%), followed by tetracycline (75%), penicillin G (68.75%), erythromycin (34.82%), amoxicillin/ clavulanic acid (30%), and ciprofloxacin (17.85%). In Romania, [33] documented tetracycline resistance rates of 66.6% and 71.4% among *C. perfringens* isolates from broilers. The great incidence of tetracycline-resistant phenotypes may be explained by the ongoing use of tetracycline as a growth stimulant and the presence of many genes linked to tetracycline resistance that are shared among various *C. perfringens* isolates [34]. Sadly, there are no laws in Egypt controlling the use of antibiotics in chicken farming, whether for medical or growth-promoting reasons. Because of this uncontrolled use, chickens are more likely to develop multidrug-resistant strains of *C. perfringens*, and there is a serious danger that humans could contract antibiotic-resistant bacteria from animals [35]. In order to guarantee food safety and safeguard the public's health, strict regulations on the use of antibiotics in chicken must be developed and strictly enforced. Since antibiotics essential to human medicine are widely used in veterinary operations, the study's findings on antimicrobial resistance raise serious concerns for both public and veterinary health.

C. perfringens can develop antimicrobial resistance through two main mechanisms: mutations in intrinsic genes or the acquisition of resistance genes via horizontal gene transfer [36]. Among the tested isolates, the most frequently detected resistance gene was the macrolide resistance gene *ermB* (84.9%), followed by *bla*CTX (68.7%), *tetM* (46.4%), *qnrA* (71.7%), *qnrB* (51.2%), and *qnrS* (12.1%). These results are in line with other studies, such as [37], which reported that isolates of *C. perfringens* had *erm*B, *tet*M, *qnr*A, and *qnr*B genes with prevalence rates of 72.2%, 47.6%, 74%, and 51.8%, respectively. Likewise, [38] found that 60% of *C. perfringens* isolates from broilers in Egypt carried the *bla* gene, highlighting the potential for horizontal gene transfer of beta-lactamase resistance among *C. perfringens* strains. It's possible that the *erm* gene is stored in the macrolide-resistant C. perfringens, aiding in its conjugal transfer. Additionally, a number of plasmid-borne quinolone resistance gene classes, including *qnrC, qnrB, qnrS, qnrVC* and *qnr*D, can induce fluoroquinolone resistance in *C. perfringens* by reducing susceptibility to fluoroquinolones [39]. Additionally, [40] revealed that fluoroquinolone-resistant C. perfringens isolates from poultry possess qnrB and qnrS genes further supporting the findings of the current study.

There are serious health concerns for humans when β-lactamase and quinolones are used carelessly in chicken feed or for medicinal purposes. These include disruption of the intestinal microflora, the development of antimicrobial resistance, and reduced efficacy of medical treatments. *C. perfringens* is a key public health concern, and its control and prevention in poultry feed and products must be prioritized. Neglecting this issue can result in serious foodborne illnesses. Antimicrobial usage in animals raised for food must therefore be tightly controlled. This includes implementing rationed use, enforcing relevant legislation, and ensuring compliance with appropriate withdrawal periods to effectively combat antimicrobial resistance.

Conclusion

In the current study, *C. perfringens* was found to be more prevalent in diseased broilers compared to apparently healthy birds. However, even apparently healthy broilers should be considered potential reservoirs for the dissemination of this pathogen into the environment. Additionally, a high level of multidrug resistance was observed among C. *perfringens* isolates, with particularly high resistance rates to macrolides and fluoroquinolones. These findings underscore the importance of routine surveillance of antimicrobial resistance patterns in poultry. Given the potential for *C. perfringens* to act as a reservoir for resistance genes that may be transferred to other bacterial species, ongoing monitoring and control strategies are essential.

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Declaration of Conflict of Interest

The authors affirm that they have no competing interests.

Ethical approval

Sample collection procedures were carried out in compliance with the rules set forth by the Faculty of Veterinary Medicine's Animal Research Ethical Committee at Mansoura University in Egypt (code Ph.D/118).

TABLE 1. Oligonucleotide primer sequences and amplified PCR product sizes used in this study

Antimicrobial Class	Target gene	Primers sequences	Annealing	Amplified segment (bp)	Reference
	cpa	F: GCTAATGTTACTGCCGTTGA R: CCTCTGATACATCGTGTAAG	53	324	[13]
β-lactam (cefotaxime)	***bla*$_{CTX}$**	F:SCSATGTGCAGYACCAGTAA R ACCAGAAYVAGCGGBGC-	55	585	[14]
Tetracyclines	***tet*M**	F: GTGGACAAAGGTACAACGAG R :CGGTAAAGTTCGTCACACAC	55	405	[15]
Macrolide	***erm*B**	F: GAAAAGGTACTCAACCAAATA R :AGTAACGGTACTTAAATTGTTTAC	55	636	
	***qnr*A**	F: GGGTATGGATATTATTGATAAA R: CTAATCCGGCAGCACTATTA	55	657	
Quinolones	***qnr*B**	F: GGMATHGAAATTCGCCACTG R: TTTGCYGYYCGCCAGTCGAA	55	263	[16]
	***qnr*S**	F: AGTGATCTCACCTTCACCGC R: CAGGCTGCAATTTTGATACC	55	552	

TABLE 2. Prevalence of *C. perfringens* isolated from broilers.

Birds	Source of sample	No. of examined samples	Positive No. (%)
Diseased birds	**Intestine**	**203**	**137(67.5)**
	Liver	**141**	**20(14.2)**
Total		**344**	**157(45.6)**
Apparently healthy birds	**Intestine**	**90**	**9(10)**
Overall total		**434**	**166(38.3)**

TABLE 3. Antibiotic susceptibility of *C. perfringens* isolates isolated from broilers (n=166).

Antibiotics	Resistant (n) (%)	Intermediate (n) (%)	Susceptible (n) (%)
Streptomycin	154 (92.8)	0 (0.00)	12 (7.2)
Erythromycin	145 (87.4)	21 (12.7)	0 (0.00)
Ampicillin	117 (70.5)	0 (0.00)	49 (29.5)
Pencillin-G	115 (69.3)	44 (26.5)	7 (4.2)
Amoxicillin/Clavulanic acid	49 (29.5)	31 (18.7)	86 (51.8)
Cefotaxime	112 (67.5)	0 (0.00)	54 (32.5)
Tetracycline	98 (59)	68 (40.9)	0 (0.00)
Ciprofloxacin	97 (58.4)	58 (34.9)	11 (6.6)
Amikacin	89 (53.6)	48 (28.9)	29 (17.5)
Rifampicin	55 (33.1)	0 (0.00)	111 (66.9)
Imipenem	15(9)	0 (0.00)	151 (90.9)

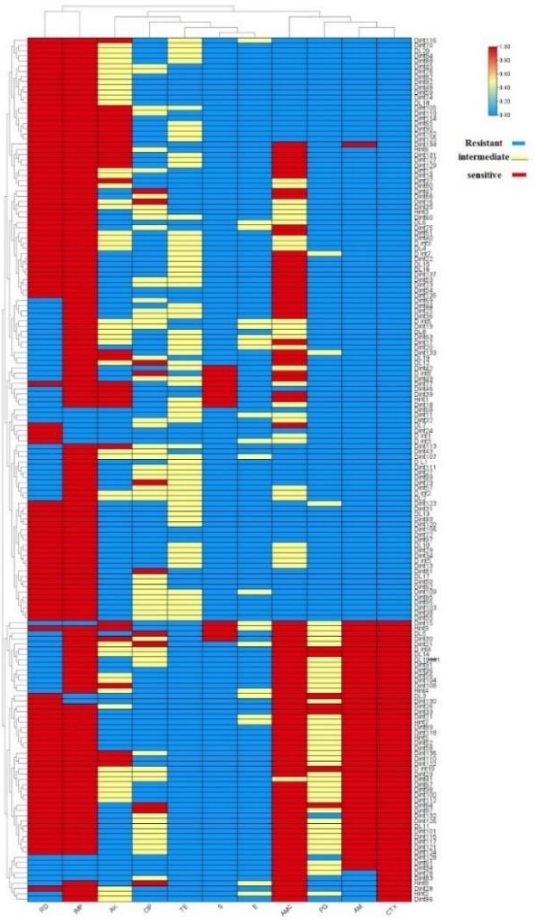

Fig. 1. The heat map with hierarchical clustering analysis representing data on phenotypic detection of 166 *c. perfringenes* strains isolated from diseased and apparently healthy broiler chicken.

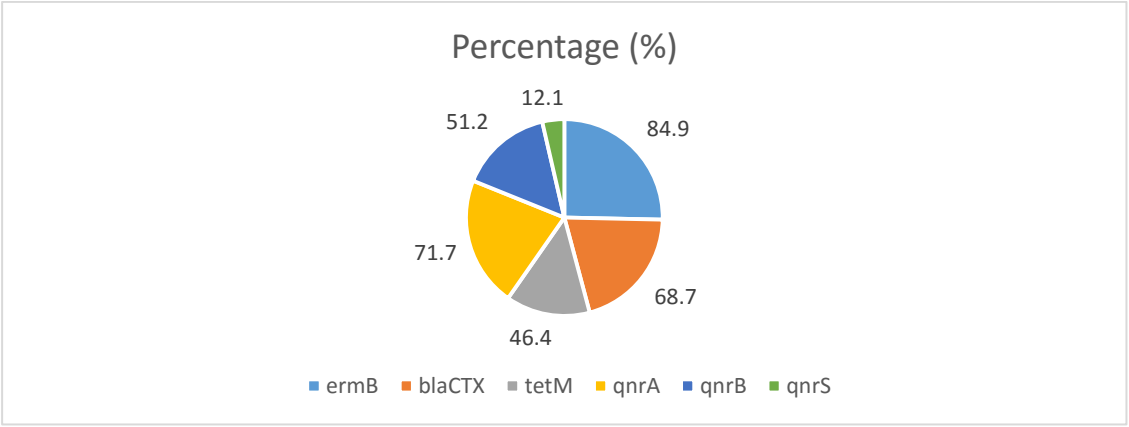

Fig .2. Detection of antibiotic resistant genes in *c. perfringens* strains isolated from broiler chicken.

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نمط مقاومة مضادات الميكروبات لبكتيريا كلوستريديوم بيرفرينجنز المعزولة من دجاج التسمين

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الملخص

تتأثر تجارة دجاج التسمين العالمية ماليًا بشكل كبير ببكتيريا كلوستريديوم بيرفرينجنز، المسبب الرئيسي لالتهاب الأمعاء النخري (NE). في هذا البحث، تم فحص كلوستريديوم بيرفرينجنز المُستقاة من حالات التهاب الأمعاء النخري في دجاج التسمين لمعرفة مدى انتشارها، وأنماط مقاومتها للمضادات الحيوية، وجيناتها. من بين 434 عينة جُمعت، كُشف عن كلوستريديوم بيرفرينجنز في 166 عينة (38.3%). من بين هذه العينات، جاءت نتائج 137 عينة معوية من أصل 203 عينات (67.5%) و20 عينة كبد من أصل 141 عينة (14.2%) من طيور مريضة (157 عينة من أصل 344 عينة؛ 45.6%) إيجابية، بالإضافة إلى 9 عينات معوية من أصل 90 عينة (10%) من طيور سليمة ظاهريًا. تم تأكيد تحديد النوع باستخدام الطرق التقليدية والجزيئية. ووفقًا لاختبارات حساسية مضادات الميكروبات، أظهر الستربتومايسين أعلى معدلات مقاومة، يليه الإريثروميسين، والأمبيسيلين، والبنسلين ج، والسيفوتاكسيم، والتتراسيكلين، والسيبروفلوكساسين، والأميكاسين، والريفامبيسين، والأموكسيسيلين/حمض الكلافولانيك. في المقابل، لوحظت أقل مقاومة ضد الإيميبينيم. والجدير بالذكر أن 100% من عزلات المطثية الحاطمة أظهرت مقاومة متعددة للأدوية. كشف الفحص الجزيئي لجينات المقاومة عن انتشار واسع لجين مقاومة الماكروليدات *erm*B (166/141؛ 84.9%)، وجين مقاومة بيتا لاكتاماز *bla*CTX (166/114؛ 68.7%)، وجين مقاومة التتراسيكلين *tet*M (166/77؛ 46.4%)، وجينات مقاومة الفلوروكينولون *qnrA* (166/119؛ 71.7%)، و*qnr*B (166/85؛ 51.2%)، و*qnrS* (166/20؛ 12.0%). أظهرت هذه النتائج انتشارًا واسعًا لبكتيريا المطثية الحاطمة المقاومة للأدوية المتعددة في دجاج التسمين، مما يؤكد على ضرورة الرصد الدوري لحساسية مضادات الميكروبات لوضع استراتيجيات مكافحة فعّالة والحد من انتشار المقاومة.

الكلمات الدالة: مقاومة المضادات الحيوية، دجاج التسمين، المطثية الحاطمة، التهاب الأمعاء النخري.