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MOLECULAR CHARACTERIZATION OF *CLOSTRIDIUM PERFRINGENS* ISOLATED FROM TURKEYS

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ABSTRACT

A total of 82 *C. perfringens* isolates (41%) were recovered from 200 samples collected from 100 turkeys (20 apparently healthy, 40 clinically diseased and 40 freshly dead), 4-6 weeks old. The clinical signs of diseased birds were sudden mortality, depression, ruffled feathers, diarrhea, dehydration, emaciation and decrease in feed consumption with increase in water consumption. While, postmortem findings were consistent with necrotic enteritis (NE), the small intestine was severely affected. Small and demarcated lesions were found in the duodenal loop, hepatitis and cholecystitis were also observed. Unopened intestines were gas-filled and enlarged with thin wall. The intestinal contents were dark due to necrotic material. The opened intestines showed necrotic lesions of varying severity of the mucosa. Isolation and biochemical identification of *C.perfringens* were done. Five *C.Perfringens* field isolates were analyzed by PCR assay to determine the presence of some toxin genes. In all tested isolates, the *Cpa* gene (alpha toxin) was detected (100%) confirming the isolates were *C.perfringens.* While, *Cpb* (beta toxin) gene was detected in two samples (40%). But the *Etx* gene (epsilon toxin) was not detected in any isolate (0%). *NetB* gene was detected in two isolates (40%). Antimicrobial susceptibility of 40 *C.perfringens* field isolates recovered from turkeys revealed that *C.perfringens* isolates were sensitive to Ampicillin (85%), Amoxicillin (85%), Penicillin (82.5%), Florfenicol (72.5%), Enrofloxacin (67.5%), Vancomycin (65%), Bacitracin (60%), Oxytetracyclin (32.5%), Lincomycin (22.5%) and Clindamycin (15%).These five *C. perfringens* isolates were also screened by PCR which detected the presence of tetracycline resistance gene *tet*(K) in three isolates (60%) and Lincomycin resistance gene *lin*(B) in three isolates (60%).

Key words: *C. perfringens,* multiplex PCR, toxin and resistance genes.

INTRODUCTION

Clostridium perfringens is an important bacterial pathogen, especially in poultry, where it can lead to both subclinical and clinical disease. Necrotic enteritis is caused by toxins produced by *C. perfringens*, which is often found in the intestinal tract of healthy birds, and when it grows in the intestinal tract, it can produce toxins. The disease may occur in the form of outbreaks in poultry and especially in broiler and turkeys flocks, causing acute clinical disease characterized by necrotic enteritis (Engstrom *et al.,* 2003).

Clostridium perfringens is a Gram-positive, spore-forming and anaerobic bacterium responsible for a wide range of diseases in humans and animals (Manteca *et al.,* 2002). The pathogenicity of *C. perfringens* is closely related to the production of major lethal toxins (alpha, beta, epsilon, and iota toxins) and other toxins, including enterotoxin (Hatheway, C. L., 1990). *Clostridium perfringens* is commonly classified to toxigenotypes based on the types of toxins they produce. The main toxins produced by strains of *C. perfringens* are alpha, beta, epsilon, and iota toxins (Songer and Meer, 1996).Necrotic enteritis is caused predominantly by *C. perfringens* type A, and to a lesser extent by type C (Cooper and Songer, 2009). Alpha-toxin has long been believed to be the critical virulence factor in NE (Al-Sheikhly and Truscott, 1977), but Cooper *et al.* (2010) showed that alpha toxin may not be an essential causative factor of NE. More recently, a novel toxin, NE toxin B (*NetB*), has been discovered and strongly associated with the pathogenesis of NE (Keyburn *et al.,* 2010). Some authors consider *NetB* the most important bacterial virulence factor for development of NE, although both *NetB*-positive and *NetB*-negative strains have been found associated with NE (Timbermont *et al.,* 2011).

The conventional method of *C.perfringens* typing is based on the detection and typing of the toxins with toxin neutralization test in mice. This procedure consumes a lot of antisera and experimental animals.

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Moreover, it is time consuming. In recent years, molecular techniques such as polymerase chain reaction (PCR) are increasingly used to type *C. perfringens* (Baums *et al.*, 2004). The Present study aimed to determine the prevalence of necrotic enteritis in turkeys, detect some toxin genes and antimicrobial resistance genes in *C.perfringens* isolated from turkeys (field isolates) using PCR assay and study antimicrobial susceptibility to choice the effective antibiotics against *C.perfringens.*

MATERIALS AND METHODS

Samples:
A total of 200 samples (100 liver and 100 intestine) were collected from 100 turkeys, 4-6weeks age (20 apparently healthy, 40 clinically diseased and 40 freshly dead) were obtained from different turkey farms in Dakahlia province. The samples were collected aseptically in sterile separate labeled bags in an ice box then were transferred without delay to be examined bacteriologically for isolation and identification of the causative microbe.

Clinical and Postmortem examination:
All turkeys were examined clinically, then sacrificed and immersed in a disinfectant before being autopsied. Gross pathological changes were recorded, summarized and presented with results for both freshly dead and clinically diseased turkeys.

Isolation and identification:
The samples were inoculated into tubes of freshly prepared boiled then rapidly cooled cooked meat medium (CMM) (Oxoid) and incubated anaerobically for 24 hours at 37°C in a Gaspak anaerobic jar *(Willis, A.T. ,1977).* A loopful of inoculated fluid medium was streaked onto neomycin sulphate (200ug/ml) sheep blood agar plates then re-incubated anaerobically for 24 h at 37°C (Cruickshank *et al.*, 1975). The lecithinase activity of suspected *C. perfringens* colonies were tested on egg yolk agar medium. Typical colonies (lecithinase producer and showed double zone of hemolysis on blood agar medium) were picked up, sub-cultured and purified for further biochemical identification tests (Koneman *et al.,* 1983).

Molecular characterization of *C. perfringens* by PCR:
Five *C.perfringens* isolates (field isolates) were subjected to PCR test in PCR unit, Animal Health Research Institute (AHRI), Egypt.

DNA extraction from *C. perfringens* isolates:
Extraction was done by using Patho Gene-Spin TM, DNA/RNA Extraction kit iNtRON cat. No. 17154 Korea according to the instructions of the manufacturer.

Oligonucleotide Primer:
The PCR primers used in this study are listed in table (1).
Oligonucleotide primer used in PCR reactions were synthesized by Sigma Company, (Germany). PCR reaction was performed in Gradient Thermal cycler (S 1000 Thermal cycler Bio-RAD USA). The reaction mixture (total volume of 50 µl) was 25 µl M. Mix (Cosmo PCR red Master Mix (2X) Willowfort W1020300x), England), 2 µl target DNA, 1 µl of each primers (containing 10 p mole/ µl) and the mixture was completed by water nuclease free to 50 µl.

Analysis of the PCR Products:
Run 5-8µl of the PCR product in parallel with a 100bp ladder molecular weight marker (100bp DNA Ladder: Thermo Scientific Gene Ruler, Cat. No. SM0243 or SM0321 USA) on a 1.5 % agarose gel (Agarose, Sigma, USA) in TBE (Tris Boric EDTA) 1X buffer. Run for 90 min at about 110V on a mini horizontal electrophoresis unit (BIO-RAD, USA). The gel was stained in ethidium bromide for 20-30min. The gel was visualized under UV transilluminator (Spectroylyne Model TR-312 A) under UV light and photographed by Canon digital camera.

Table 1: Target genes, PCR Primers and Length of amplification products of *C. perfringens.*

Target gene	Primer sequence (5'-3')	Reference	Length of amplification products (bp)
Cpa	F: GCTAATGTTACTGCCGTTGA R: CCTCTGATACATCGTGTAAG	***Ahsani et al., 2010***	324
Cpb	F:GCGAATATGCTGAATCATCTA R:GCAGGAACATTAGTATATCTTC		196
Etx	F: GCGGTGATATCCATCTATTC R: CCACTTACTTGTCCTACTAAC		655
NetB	F:GCTGGTGCTGGAATAAATGC R:TCGCCATTGAGTAGTTTCCC	***Anthony et al., 2010***	383
Lin(B)	F: CCTACCTATTGTTTGTGGAA R: ATAACGTTACTCTCCTATTC	***Bozdogan et al., 1999***	906
Tet(K)	F: TTATGGTGGTTGTAGCTAGAAA R: AAAGGGTTAGAAACTCTTGAAA	***Masco et al., 2006***	382

Table 2: PCR cycling conditions for target genes.

Target gene	Stage	Temp.(°C)	Time	No. of cycles
Cpa *Cpb* *Etx*	Initial denaturation	94	2 min.	1
	Denaturation	94	15 sec.	35
	Annealing	55	30 sec.	
	extension	68	1 min.	
NetB	Denaturation	94	30 sec	30
	Annealing	55	30 sec	
	extension	72	1 min.	
Lin(B) and *Tet(*K)	Initial denaturation	94	5 min	1
	Denaturation	94	45 sec	35
	Annealing	54	45 sec	
	extension	72	1 min	
	Final extension	72	5 min	1

In vitro Antibiotic Susceptibility Test:
Fourty (40) *C.perfringens* isolates (field isolates) were subjected to antibiotic sensitivity test against 10 commonly used antibiotics in poultry farms. The antimicrobial susceptibility profile Ampicillin, Amoxicillin, Penicillin, Florfenicol, Enrofloxacin, Vancomycin, Bacitracin, Oxytetracyclin, Lincomycin and Clindamycin was tested by disk diffusion methods according to Clinical and Laboratory Standards Institute **(**CLSI, 2012).

RESULTS

Table 3: Prevalence of *C. perfringens* in examined samples collected from turkeys.

Samples	No. of examined samples		No. of positive samples	%
Apparently healthy birds (20)	Intestine	20	3	15%
	Liver	20	0	0%
Clinically diseased birds (40)	Intestine	40	24	60%
	Liver	40	14	35%
Freshly dead birds (40)	Intestine	40	26	65%
	Liver	40	15	37.5%
Total	200		82	41%

Table 4: Results of multiplex PCR and PCR assay for detection of some *C. perfringens* toxin and antimicrobial resistance genes.

Isolate	Results					
	Toxin genes				Antimicrobial resistance genes	
	Cpa	*Cpb*	*Etx*	*NetB*	*Tet*(K)	*Lin*(B)
	Alpha toxin gene	Beta toxin gene	Epsilon toxin gene	NE toxin B gene	Tetracycline resistance gene K	Lincomycin resistance gene B
1	+	+	-	-	+	-
2	+	-	-	+	+	+
3	+	+	-	+	-	+
4	+	-	-	-	-	+
5	+	-	-	-	+	-
Total	100%	40%	0%	40%	60%	60%

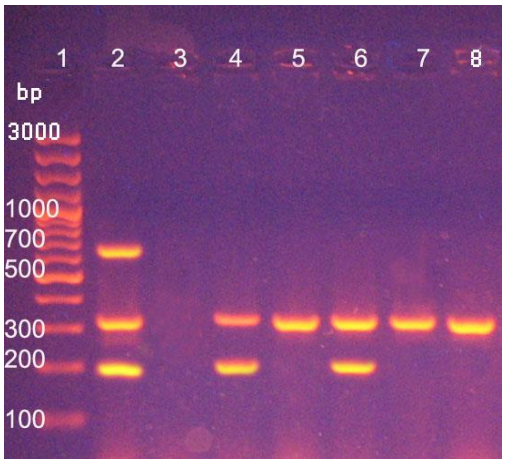

Fig. 1: Agarose gel (1.5%) electrophoresis of multiplex PCR products obtained with various *C. perfringens* toxin genes (*Cpa* gene (324bp), *Cpb* gene (196bp) and *Etx* gene (655bp).
Lane 1: DNA marker (GeneRuler 100 bp DNA Ladder)
Lane 2: Control Positive (mix of various toxin types).
Lane 3: Control Negative
Lane 4-8: PCR products of toxin genes from *C. perfringens* field isolates.

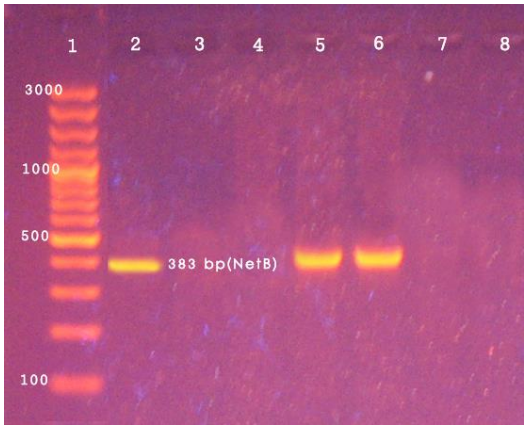

Fig. 2: Agarose gel (1.5%) electrophoresis of PCR products showing amplification of 383 bp fragment using *NetB* gene primer.
Lane 1: DNA marker (Gene Ruler 100 bp DNA Ladder)
Lane 2: Control Positive.
Lane 3: Control Negative
Lane 4-8: PCR products of *NetB* gene from *C. perfringens* field isolates.

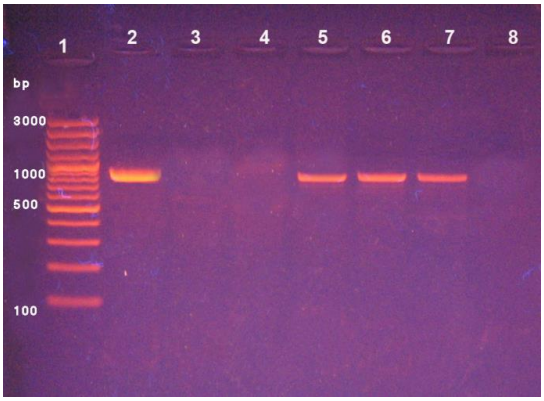

Fig. 3: Agarose gel (1.5%) electrophoresis of PCR products showing amplification of 906 bp fragment using *LinB* gene primer.
Lane 1: DNA marker (Gene Ruler 100 bp DNA Ladder)
Lane 2: Control Positive.
Lane 3: Control Negative
Lane 4-8: PCR products of *Lin*(B) gene from *C. perfringens* field isolates.

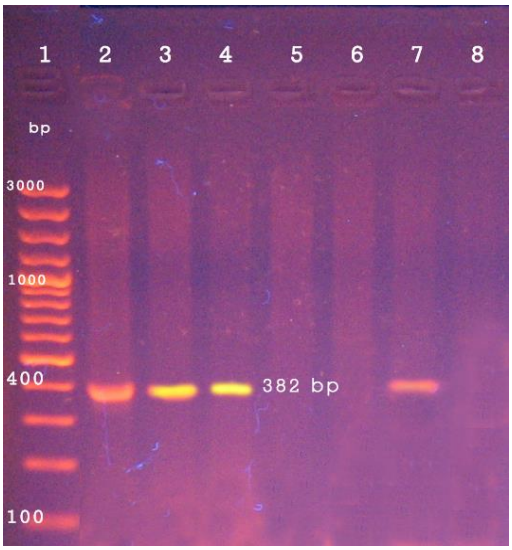

Fig. 4: Agarose gel (1.5%) electrophoresis of PCR products showing amplification of 382 bp fragment using *Tet(*K) gene primer.
Lane 1: DNA marker (Gene Ruler 100 bp DNA Ladder)
Lane 2: Control Positive.
Lane 8: Control Negative
Lane 3-7: PCR products of *Tet*(K) gene from *C. perfringens* field isolates.

Table 5: Antibiotic sensitivity and resistance pattern for (40) *C. perfringens field isolates.*

Antibiotic	No. of tested isolates	Sensitive		Resistant	
		No.	%	No.	%
Ampicillin	40	34	85	6	15
Amoxicillin	40	34	85	6	15
Penicillin	40	33	82.5	7	17.5
Florfenicol	40	29	72.5	11	27.5
Enrofloxacin	40	27	67.5	13	32.5
Vancomycin	40	26	65	14	35
Bacitracin	40	24	60	16	40
Oxytetracyclin	40	13	32.5	27	67.5
Lincomycin	40	9	22.5	31	77.5
Clindamycin	40	6	15	34	85

DISCUSSION

Necrotic enteritis in turkeys has emerged in Egypt in the last years as an economic problem causing great losses and great concern for the breeders. The present study was undertaken to study the incidence of the disease, antimicrobial susceptibility and detection of some toxin and antimicrobial resistance genes of *C. perfringens* field isolates involved in apparently healthy and diseased turkeys.

In the present investigation, turkeys from affected flocks with *C.perfringens* showed clinical signs including a sudden increase in mortality observed in the flock, depression, ruffled feathers, diarrhea, dehydration and emaciation with decrease in feed consumption and sometimes an increase in water consumption. These findings agreed with that observed by Saif *et al. (*2003). While, postmortem findings were consistent with necrotic enteritis (NE) with the small intestines which most frequently and severely affected. Small and demarcated lesions were found in the duodenal loop, hepatitis and choleocystitis were also observed. Unopened intestines were gas-filled and enlarged and their wall appeared thin. The intestinal contents were dark due to necrotic material. The opened intestines showed necrotic lesions of varying severity of the mucosa. In severe cases, the mucosa was covered with a typically thick greenish or yellowish diphtheric pseudomembrane ("Turkish towel"). These findings agreed with that observed by Lyhs *et al.* (2013).

In general, the investigation of 200 samples collected from apparently healthy, clinically diseased and freshly dead turkeys revealed that, only 82 samples were positive to *C. perfringens* the prevalence rate was 41% (Table 3). Nearly similar results were recorded by Heidy *et al.* (2015) who recorded that the prevalence rate of *C. perfringens* in turkeys was 45.9%. On the other hand, Gad *et al.* (2011) and Parvaiz *et al.* (2017) recorded that the prevalence

rates were 29.1% and 31.01%, respectively. These differences may be due to age, immune status of birds, nutrition and management.

PCR technology is considered to be a convenient and highly reliable tool for molecular detection of the major toxin genes such as (alpha, beta, epsilon, and iota toxin genes) (Yoo *et al.,* 1997).

In the present study, five *C. Perfringens* isolates (field isolates) were analyzed by multiplex PCR in order to detect the presence of some toxin genes. A mixture of primers of *Cpa* (alpha toxin) gene (324bp), *Cpb* (beta toxin) gene (196bp) and *etx* (epsilon toxin) gene (655bp) were used. Also, these Five *C. Perfringens* isolates were analyzed by PCR in order to detect the *NetB* (necrotic enteritis toxin B) gene (383 bp).

All toxigenic types of *C.perfringens* are able to produce alpha toxin which has lecithinase activity and causes tissue necrosis especially in small intestine so alpha toxin is mainly responsible for necrotic enteritis in birds Keyburn *et al.* (2010).

Our results revealed that the *Cpa* gene (alpha toxin) was detected in all tested isolates (100%) confirming the isolates as *C. perfringens* (Table 4 and Figure 1). This was in the same direction with the result of Engstrom *et al.* (2003) and Lyhs *et al.* (2013) who detected *Cpa* gene (alpha toxin) in all tested isolates of *C. perfringens*.

Enterotoxins are frequently cytotoxic and kill cells by altering the apical membrane permeability of the mucosal cell of the intestinal wall. They are mostly pore-forming toxins, secreted by bacteria that led to form pores in cell membrane causing cell necrosis (Cooper and Songer, 2009).

With respect to *Cpb* gene (beta toxin), it was detected in two of the tested isolates (40%) (Table 4 and Figure 1) indicating that *Cpb* has a role in development of necrotic enteritis. This was agreed with that recorded by Ahsani *et al.* (2010).

The *Etx* gene (epsilon toxin) was not detected in any of the tested isolates (0%) (Table 4 and Figure 1). These results agreed with that reported by Baums *et al.* 2004.

NetB gene (necrotic enteritis toxin B) is located on a plasmid and encodes a pore-forming toxin, which perforates the plasma membrane and thereby damages host cells (Keyburn *et al.,* 2010).

In the present investigation, *NetB* gene was detected in two of the isolates (40%) that were recovered from turkeys with necrotic enteritis (Table 4 and Figure 2). These results were nearly similar with that recorded by Abildgaard *et al.* (2010) who reported that 52% of the *C. perfringens* strains isolated from NE-affected birds were *netB*-positive. On the other hand, Keyburn *et al.* (2010*)* reported that 70% of the *C. perfringens* strains isolated from NE-affected birds were *netB*-positive and Lyhs *et al.* (2013) reported that only 8% of *C. perfringens* isolates recovered from turkeys with NE were *netB*-positive.

Antimicrobial susceptibility of 40 *C.perfringens* isolates recovered from turkeys showed high sensitivity to penicillin group (Ampicillin, Amoxicillin and Penicillin, 85%, 85% and 82.5%, respectively). This was in the same direction with the findings of Mona and Abdelhafez, (2017). While, *C. perfringens* isolates showed high resistance to Clindamycin, Lincomycin and Oxytetracyclin (85%, 77.5% and 67.5%, respectively). These results run parallel with that recorded by Martel *et al.* (2004).

C. perfringens isolates were also screened by PCR assay for the detection of some antibiotic resistance genes, Tetracycline resistance gene, *tet*(K) (382bp) and Lincomycin resistance gene, *lin*(B) (906bp). In three of the tested isolates (60%), the *tet*(K) and *lin*(B) genes were detected (Table 4 and Figures 3 and 4). These findings support the antimicrobial susceptibility test results. These results were in agreement with Ahmadreza *et al.* (2009) who detected *tet*(K) and *lin*(B) genes in *C.perfringens* isolates.

CONCLUSION

The presence of some toxin genes confirmed the pathogenic potential of the isolated *C. perfringens* strains and their association with clinical manifestations and postmortem findings. Antibiotic sensitivity and resistance pattern showed high antibiotic resistance of *C. perfringens* isolates which require strict regulations on the use of antibiotics in veterinary therapy to minimize the emergence of resistant bacteria in turkeys which may increase the public health problem.

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التوصيف الجزيئي للكلستريديم برفرنجنز المعزوله من دجاج الرومي

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أجريت هذه الدراسه علي عدد ٢٠٠ عينه من الأعضاء الداخليه (١٠٠ من الكبد و ١٠٠ من الأمعاء) لعدد ١٠٠ دجاجه رومي عمر من ٤ الي ٦ أسابيع (٢٠ سليم ظاهريا ، ٤٠ مصاب ، ٤٠ نافق حديثا) تم تجميعها من مزارع دجاج رومي مختلفه بمحافظه الدقهليه. وباجراء الفحص الظاهري للطيور المصابه ظاهريا تبين وجود خمول ، ضعف عام ، اسهال مع علامات جفاف ، انخفاض معدلات استهلاك العلف مع زيادة استهلاك مياه الشرب وحدوث نفوق مفاجئ وزيادة معدلات النفوق بين القطيع. وباجراء الصفه التشريحيه تبين وجود علامات التهاب الأمعاء التنكرزي خاصة في الأمعاء الدقيقه مع ظهور انتفاخ في الأمعاء مع صغر في سمك جدارها وعند فتحها ظهر محتوي الأمعاء داكن اللون نتيجة وجود الأغشيه المتنكرزه والأنزفه مع الغازات والتهاب الكبد والحوصله المراريه. أظهر الفحص البكتريولوجي أن إجمالى عدد العينات الأيجابيه للكلستريديم برفرنجنز ٨٢ عينه بنسبه عامه ٤١%. تم اجراء اختبار البلمره المتسلسل المتعدد لعدد ٥ معزولات حقلية من الكلستريديم برفرنجنز لتحديد والكشف عن بعض الجينات المسئوله عن افراز السموم. وقد تبين وجود الجين المسئول عن افراز سم الألفا (*Cpa* gene) في جميع العينات المفحوصه (١٠٠%) وهذا يؤكد أن المعزولات للكلستريديم برفرنجنز. كما تبين وجود الجين المسئول عن إفراز سم البيتا (*Cpb* gene) بنسبة ٤٠% ولم يتبين وجود جين سم الابسيلون (*Etx* gene) في أي من المعزولات بينما وجد الجين المسئول عن افراز NE toxin B (*NetB* gene) بنسبة ٤٠%. وباجراء اختبار الحساسيه لعدد ٤٠ معزوله من للكلستريديم برفرنجنز لقياس نسبة مقاومتها لعدد ١٠ مضادات حيويه شائع استخدامها في مزارع دجاج الرومي تبين أن نسبة حساسية المعزولات كانت ٨٥% لكلا من الأمبسيلين والاموكسيسلين، ٨٢,٥% للبنسيلين، ٧٢,٥% للفلورفينيكول ، ٦٧,٥% للانروفلوكساسين ، ٦٥% للفانكومايسين ، ٦٠% للباستراسين ، ٣٢,٥% للأوكسي تتراسيكلين ، ٢٢,٥% للينكومايسين و ١٥% للكليناميسين. ودعم اجراء اختبار انزيم البلمره المتسلسل لعدد ٥ معزولات هذه النتائج بالكشف عن وجود جينات مقاومه للتتراسيكين *Tet*(K) gene بنسبة ٦٠% واللينكومايسين *Lin*(B) gene بنسبة ٦٠%. وقد خلصت هذه الدراسة لوجود علاقة بين وجود الجينات المفرزة للسموم وشدة وضراوة الكلستريديم برفرنجنز ونظرا لارتفاع نسب مقاومة ميكروب الكلستريديم برفرنجنز لأغلب المضادات الحيويه توصي هذه الدراسه بالحد من استخدام المضادات الحيويه بطرق غير علميه في مزارع دجاج الرومى واجراء الإختبارات المعمليه المناسبه لاختيارالمضادات الحيويه الفعاله لتفادي حدوث مقاومه من الميكروبات لها .