Phenotypic Identification of *Clostridium Perfringens* Associated With Necrotic Enteritis of Broiler Chickens in Sharkia Governorate

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Abstract

A total of 314 samples of liver and intestinal loop were collected from broiler chickens suffering from diarrhea during 2013-2014 .The tissue samples were examined for the prevelance of *C.perfringens* revealed 91 (66.93%) of them were toxinogenic and 44(31.06%) non toxinogenic isolates .One hundered and thirty two samples were positive for *Clostridium perfringens* typing by intradermal inoculation in guinea pig. The higher incidence of *C.perfringens* was recorded in winter (53.12%-57.5%) followed by autumn (50%) then summer (32.60%-37.93%) and the lower incidence was recorded in spring (30%-31.25%).The incidence of *C.perfringens* from intestinal samples was(65.85%-58.66%)and liver samples was(21.95%-21.33%). The most effective antibiotic was recommended for treatment of necrotic enteritis were amoxicillin, Ampicillin, Piperacillin, ceftraxione, cefruxime, Cefotaxime which was applied by sensitivity test to 91 isolates of toxinogenic *Clostridium perfringens.* Our study recorded that there is the isolation rate of *Clostridium perfringens* from intestine was exteremly higher than liver samples and ranged from(58.66%-65.85%) and(21.95 %-21.33) ,respectively that subtyped into type A 76 (57.57%) and typeD 15 (11.36%).

Introduction

Necrotic enteritis(NE),is one of the most important infectious disease affecting poultry,where the birds lose the ability to digest nutrients from feed ***(Cooper et al, 2009).*** According to the production of four major toxins alpha,beta, epsilon and iota**.** *C. perfringens* is classified into five types A,B,C,D and E ***(Cato et al, 1986).*** All types of *C.perfringens* produced a multifunctional phospholipase leathal toxin called alpha toxin ***(Songer, 1996)***. The understanding of disease progression of necrotic

enteritis has been very difficult and usually challening due to its complexity, many clostridial species can be normal inhabitants of the gut ,making it difficult to determine these role in virulenc ***(Cooper et al, 2013).*** As a normal inhabitantin the intestinal tract of healthy birds activated *C.perfringens* when several predisposing factors as coccidiosis ,high protein in ration,food stuff rich in Zinc ***(Baba et al, 1992a)***, poor hygenic condition ***Fram and Bickfordn (1986)*** and ***Jakson et al (2003)*** were included.They are required to elicit the clinical signs and lesions of NE ***(Kaldhusdal et al, 1999).*** The most widely used method for detection clostridial toxins is the mice neutralization test **(*Stern and Batty, 1975).*** So,the aim of this study was isolation,identification, typing of *C. perfringens* and Serological identification of *C. perfringens* strains by toxin- antitoxin neutralization test.This work was studying the *in vitro* antibiotic sensitivity pattern of clostridial species to different chemotherapeutic agents as amoxicillin, Ampicillin, Piperacillin, ceftraxione, cefruxime, Cefotaxime, chloramphenicol, erythromycin trimethoprim+ sulphamethazole, tetracycline, Deoxycycline, lincomycin, enrofloxacin, and Streptomycin in order to check the highly potent ones recommended to eliminate such conditions

Material and methods

samples

A total number of 157 intestinal and 157 liver samples were collected from 75 flocks have broiler chickens suffering from diarrhea,ruffled feathers and stunted growth respectively during 2013-2014.The recent dead broiler were affecting part of intestine showed ballowing ,thining ,velvety appearance and liver showed necrosis some of diseased chicken were suffering from coccidiosis the samples were collected from private flocks of different ages(20-50 days) in sharkia governorat . The collected birds represented (75)flocks.

Bacteriological examination

Each sample was inoculated onto tubes of freshly prepared cooked meat medium (CMM) then incubated anaerobically at 37c for 48h. A loopful from each one was streaked onto the surface of 10% sheep blood agar with neomycin sulphate (200mg/ml). The plates were incubated anaerobically at 37c for 48hr the suspected colonies of *C. perfringens* were picked up and examined for morphological, Microscopical appearance ***Wilson and Miles (1975).*** and biochemical characters according to ***Koneman et al (1992).*** Activity of lecithinase of *C.perfringens* alpha toxin agler's test by half antitoxin plate as described by ***Smith and Holdeman (1968).*** The toxins of *C. perfringens* were typed by dermonecrotic test in

albino guinea pigs ***(Bullen, 1952)***. The results were interpreted by the degree of the dermonecrotic reaction ***(Stern and Batty, 1975)***.The neutralization tests were performed by using toxin antitoxin of different types of *C.perfrenges* in albino guinea pig ***(Smith and Holdeman, 1968)*** by using diagnostic *C.perfringens* antitoxin typeA,B, C,D and E (Burroguns, Welcome, Beckenham, London, England)

Sensitivity of *C.perfringens* isolates to chemotheraputic agents

the disc diffusion method was used on a pure sub cultures from 91 isolates of clostridia causing necrotic enteritis of broilers as described by ***Koneman et al (1992).***The antibiotic discs were purchased from oxoid LTD, london , england. Briefly , one milliliter of 24 hrs broth culure was spread on the surface of muller hinton agar (oxoid No337). Antibiotic disc were placed on the surface of seeded agar plates and were incubated anaerobically at 37c for 24 hrs the sensitivity was judged according to the diameter of inhibition zone arround each disc and compared with standered figures.

Results

Incidence of C. perfringensin

The Incidence of C. perfringensin among different flocks during (2013-2014)in Sharkia Governorate **was** ulseratted in **tables (1, 2**) and figure (1,2) respectively.

Identification of C. perfringens isolates:

Regarding to traditional methods for identification of *C.perfringens* recovered from diseased broilers, the obtained results revealed that *C.perfringens* is Gram positive short plumb rarely sporulated and non motile bacilli (Photo 2) . The *C.perfringens* revealed double zone of haemolysis on sheep blood agar with neomycin sulphate (200 µg/ml) (Photo 1).

All the isolates were fermentative to different sugars as glucose, sucrose ,lactose,mannose and maltose with production of acid and gases, catalase, oxidase, gelatin liquefiers, litmus milk positive, and indole tests negative.

Nagler's test (lecithinase activity) represented the action of *C. perfringens* alpha toxin on lecithin of egg yolk onto enriched egg yolk agar medium which appeared as pearly opalescence zone surround the colonies while this reaction was inhibited by *C. perfringens alpha toxin antiserum (Photo 3*)

C.perfringens isolates recovered from diseased broilers were identified by dermonecrotic reactions in albino guinea pigs into 91 strains were toxigenic (68.93%) (76 type A, 15 type D) and 41 strains were non toxigenic with an incidence of 31.06% respectively (Table 5) photo(4).

Sensitivity of *C. perfringens* isolates derived from diseased broilers to different antimicrobial agents

C.perfringens was highly sensitive to amoxicillin, Ampicillin, Piperacillin, ceftraxione, cefruxime, Cefotaxime, Fuscidic acid and Bacitracin. while chloramphenicol and erythromycin were of moderate effect . On the other hand *C.perfringens* isolates were resistance to trimethoprim+ sulphamethazole , tetracycline, Deoxycycline, lincomycin, clindamycin, enrofloxacin, and Streptomycin (table 6)

Table (1): *Incidence of C. perfringens from broiler chickens in 2013*

Flocks	Age in days	No. of samples			No. of positive sample			No. of+ve samples in different seasons	%
		Int.	L.	Total	Int.	L.	Total		
1	25	2	2	4	1	1	2	Spring (10)	31.25%
2	20	3	3	6	2	-	2		
3	26	4	4	8	2	1	3		
4	30	2	2	4	1	-	1		
5	32	1	1	2	-	-			
6	19	4	4	8	1	1	2		
7	22	3	3	6	3	-	3	Summer (22)	37.93%
8	45	2	2	4	-	-			
9	33	4	4	8	3	1	4		
10	31	1	1	2	1	-	1		
11	25	2	2	4	2	-	2		
12	22	4	4	8	4	1	5		
13	37	2	2	4	-	-	-		
14	43	3	3	6	2	1	3		
15	36	5	5	10	2	1	3		
16	34	3	3	6	-	1	1		
17	29	1	1	2	-	-	-	Autumn (17)	50%
18	27	2	2	4	1	1	2		
19	21	3	3	6	3	1	4		
20	24	1	1	2	1	1	2		
21	22	1	1	2	2	-	2		
22	33	2	2	4	-	1	1		
23	49	5	5	10	3	1	4		
24	37	2	2	4	1	1	2		
25	27	3	3	6	2	1	3	Winter (23)	57.5%
26	24	2	2	4	2	-	2		
27	35	1	1	2	-	-			
28	40	4	4	8	3	1	4		
29	23	2	2	4	3	1	4		
30	36	2	2	4	2	1	3		
31	27	3	3	6	3	-	3		
32	20	2	2	4	3	-	3		
33	22	1	1	2	1	-	1		
Total		82	82	164	54 (65.85%	18 (21.95%	72	72	43.90%

No.: Number *Int.: Intestine* *L.: Liver*

Table (2): *Incidence of C. perfringens from broiler chickens in 2014*

Flocks	Age in days	No. of samples			No. of positive sample			No. of+ve samples in different seasons	%
		Int.	L.	Total	Int.	L.	Total		
1	25	1	1	2	1	-	1	**Spring (12)**	30%
2	22	3	3	6	1	1	2		
3	36	2	2	4	1	-	1		
4	33	1	1	2	-	-	-		
5	20	3	3	6	1	1	2		
6	25	2	2	4	1	-	1		
7	40	1	1	2	-	-	-		
8	37	3	3	6	1	1	2		
9	51	2	2	4	1	1	2		
10	29	1	1	2	1	-	1		
11	32	1	1	2	-	-	-		
12	27	4	4	8	2	1	3	**Summer (15)**	32.60%
13	40	1	1	2	1	-	1		
14	42	2	2	4	2	-	2		
15	21	1	1	2	-	1	1		
16	41	2	2	4	2	-	2		
17	35	1	1	2	1	-	1		
18	36	1	1	2	1	-	1		
19	50	2	2	4	1	1	2		
20	42	2	2	4	1	1	2		
21	46	3	3	6	2	1	3	**Autumn (16)**	50%
22	28	1	1	2	-	-	-		
23	27	2	2	4	1	-	1		
24	33	1	1	2	-	-	-		
25	28	1	1	2	1	-	1		
26	20	3	3	6	2	-	2		
27	34	1	1	2	-	-	-		
28	32	2	2	4	1	-	1		
29	30	3	3	6	2	1	3		
30	23	1	1	2	-	-	-		
31	25	1	1	2	1	-	1		
32	31	1	1	2	-	-	-		
33	23	3	3	6	3	1	4		
34	27	1	1	2	1	-	1	Winter (17)	53.12%
35	20	1	1	2	2	-	2		
36	33	3	3	6	3	1	4		
37	44	1	1	2	-	-	-		
38	22	1	1	2	1	-	1		
39	23	4	4	8	3	2	5		
40	43	2	2	4	1	-	1		
41	22	1	1	2	1	-	1		
42	29	2	2	4	1	1	2		
Total		75	75	150	44 (58.66%)	16 (21.33%)	60	60	40%

No.: Number *Int.: Intestine* *L.: Liver*

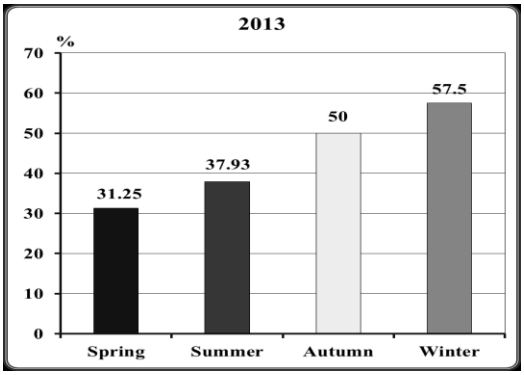


Fig. (1): *Occurence of C. perfringens from broiler farms in 2013*

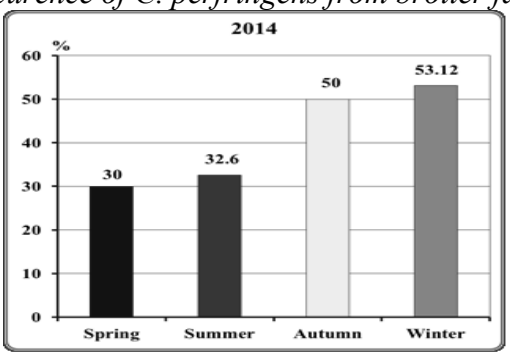


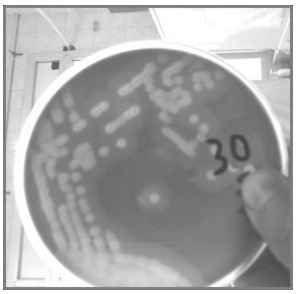
Fig. (2): *Occurence of C. perfringens from broiler farms in 2014*

Table (3) *Recovery rate of C.perfringens isolates from broilers in relation to different ages 2013*

Age	Number of examined samples	Number of +ve samples	%
20-30 days	90	46	51.11
30-40 days	52	18	34.6
40-50 days	22	8	36.3
Total	164	72	43.90

Table (4): *Recovery rate of C.perfringens isolates from broilers in relation to different ages 2014*

Age	Number of examined samples	Number of +ve samples	%
20-30 days	80	35	43.75
30-40 days	34	10	29.41
40-50 days	36	15	41.66
Total	150	60	40



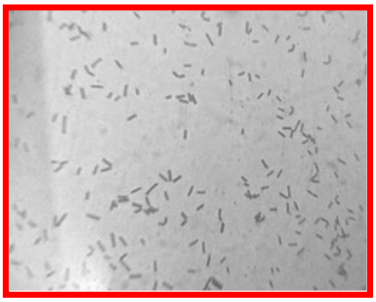


Photo (1): *C. perfringens* induced double zone of haemolysis onto neomycin sulphate sheep blood agar.
Photo (2): *C.perfringens* show Gram positive short bacilli stained with Gram's stain .

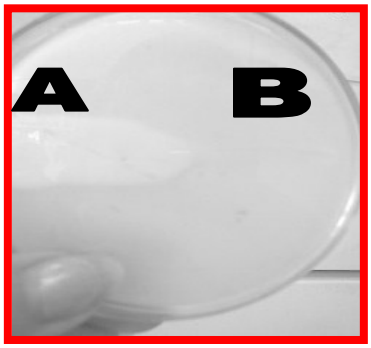


Photo (3): Nagler's test

A)*C. perfringens* gave opalescence appearance due to licithinase effect of alpha toxin On egg yolk agar medium

B) Neutriliation of C.perfringens α-toxin by its specific antitoxin

Table (5): *Typing of toxigenic C.perfringens isolates recovered from diseased broilers*

Number of examined]samples	**Number of positive samples**	**Types of *C. Perfringens***					
		Toxigenic isolates 91(68.93%)				**Non–toxigenic isolates**	
		A		**D**		**Number**	**%**
314	132	76	57.57%	15	11.36%	41	31.06

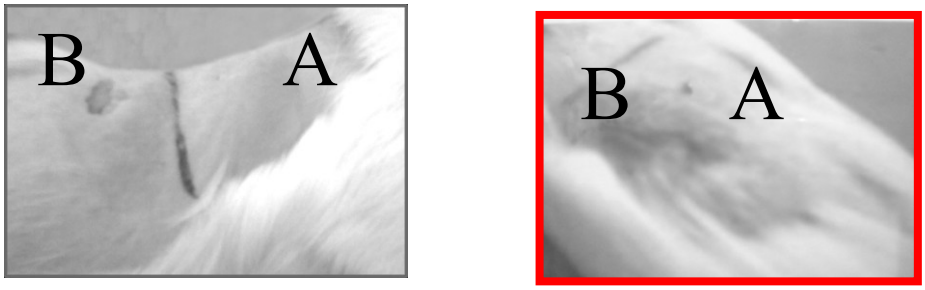


Photo (4): *Dermonecrotic reaction used for typing of C.perfringens A and D.*
(A) Alpha toxin shows an irregular area of yellowish green on the skin of albino guinea pig.
(B) Epsilon toxin produces a circular whitish green necrosis A few small of purplish haemorrhagic areas.

Photo (5): *Toxin antitoxin neutralization test on the skin of albino guina pig*
(A) Action of C.perfringens alpha toxin on necrosis and the lesion tends to spread downward.
(B) Neutralization of C.perfringens alpha toxin with its specific antiserum.

Table (6): *Sensitivity of C. perfringens isolates from diseased broilers to different antimicrobial agents*

Antimicrobial agents	Code	R	I	S	Average of Iz (mm)	No. of S .isolates / total (91)	%	A.A
Amoxicillin	Ax 25	≤15	16-22	≥23	24	82	90.10	S
Ampicillin	AM10	≤13	14-16	≥17	23	73	80.21	S
Piperacillin	PRL100	≤17	18-20	≥21	25	79	86.81	S
Ceftraxione	CRO30	≤14	15-22	≥23	28	79	86.81	S
Cefruxime	CXM 30	≤14	15-22	≥23	27	82	90.10	S
Cefotaxime	CTX 30	≤14	15-22	≥23	30	88	96.70	S
Doxycycline	DO 30	12	13-15	16≥	5	12	13.18	R
Tetracycline	TE 30	≤ 14	15-18	≥19	3	16	17.58	R
Bacitracin	B 10	≤8	9-12	≥13	27	77	84.61	S
Erythromycin	E 15	≤13	14-17	≥18	16	33	36.26	S
Lincomycin	L 2	≤13	14-21	≥22	4	10	10.98	R
Clindamycin	DA 2	≤11	12-20	≥21	8	23	25.27	R
Trimethoprim-sulphamethoxazol	SXT30	≤10	11-15	≥16	3	10	10.98	R
Chloramphenicol	C 30	≤12	13-17	≥18	13	31	34.06	S
Streptomycin	S 10	≤11	12-14	≥15	5	22	24,17	R
Enrofloxacin	ENR 5	_	_	_	6	15	16.48	R
Fuscidic acid	FA 10	≤12	13-20	≥21	24	69	75.82	S

Iz:Inhibitory zone
No.:Number S :Sensitive I : Intermediate sensitive R: Resistant
%:Percentage of sensitive isolates A.A.:Antibiogram activity

Disscusion

Clostridium perfringens plays an important role in the development of NE disease in broiler chickens ***(Broussard et al, 1986).*** Signs of necrotic enteritis involved reluctant to move, diarrhea and decrease in appetite ***(Ficken and Wages, 1997).***

Atotal of 314 intestinal and liver samples were collected from 75 broiler flocks in different seasons and ages from Sharkia Governorate.Table(1,2).The incidence of *C. perfringens* in this study was 43.90% in 2013 and 40% in 2014.Comparable percentage of *C.perfringens* isolates were reported in Egypt *by* ***Abd El-Salam (2000), Abd El-Gwad and Abd El-Kader (2001) and Ahmed (2010)*** who reported incidence of 50%, 51.4%, 48.5%, 40%, 44.4% and 45% ,respectively, from different localities in Egypt.

However higher occurance was recorded by ***Dosoky (1990)*** *and* ***Afify and Nasr (2009)*** who succeded in detect *C.perfringens* in chicken with 79% and 75.55% respectively.In the present study the isolation rate of *C.perfringens* from diseased chickens in 2013 and 2014 was 65.85% and 58.66%,respectively in intestine and 21.95% and 21.33%, respectively in liver .There are rise in incidence of *C.perfringens* from intestinal samples compared with liver samples and this could be indicated that *C.perfringens* is predisposing factor to necrotic enteritis and normal inhabitant in intestine ***(Silva et al, 2009)***.These results were nearly similar to the results obtained by ***El-Refay (1999), Ahmed (2010) and Ali (2010)*** who reported that the incidence of *C. perfringens* isolation from intestine was 33.3%, 53.08% and 41.7% respectively. Also, the results coinside with ***Awad (2012)*** who stated that the incidence of *C.perfringens* isolated from intestine and liver was 47.4% and 12.3%, respectively. Concerning the incidence of *C. perfringens* in different seasons, the results in the present study revealed that the higher incidence of *C.perfringens* was noted in winter (57.5% in 2013 and 53.12% in 2014) autumn (50% in 2013 and 50% in 2014) then summer (37.93%) in 2013 and 32.60% in 2014) and the lower incidence was recorded in spring (31.25% in 20013 and 30% in 2014). These results due to adverse environmental condition in cold seasons . These results agree with that reported by ***Kaldhusdal and Skjerve (1996) and Ahmed (2010)*** who reported high incidence in cold seasons (October to March) and low incidence in worm season (April to September), while disagree with that reported by**,** ***Berinier et al. (1974a) and Cygan and Nawak (1974)*** who reported high incidence in July, August, September and October (summer and autumn).

Johansson (2006) who stated that, in 2-4 weeks old chickens necrotic enteritis occurs as an acute clinical disease and causing high mortality.

The relationship between the incidence of *C.perfringens* dietary factors ,coccidiosis, and bad hygienic condition shown in the incidence is little pit lower at 30-40 days and increased in 40-50 days and this may due to increase the protein content in the diet. These results agree with that reported by **Knarreborg *et al.* (2002)**.

C.perfringens recovered from diseased broiler identified by microscopical examination, culture characteristics, biochemical tests and Nagler`s test ***(Smith and Holdeman, 1968); Peter et al, 1986;Han et al, 1997; Vaikosen and Muller, 2001 and Assis et al, 2002)***. isolates were identified by dermonecrotic reactions of *C. perfringens* isolates in albino guinea pigs classified into toxigenic (68.93%) [type A 57.57% , type D was 11.36%] and non toxigenic (31.06%) .These results were nearly in agreement with the result obtained by ***Songer and Dale (2005)*** who typed *C.perfringens* as toxigenic and non toxigenic strains in an incidence of 36.1 % and 15.3% respectively.

In this work ,toxin antitoxin neutralization test on the skin of albino guinea pigs was done by using specific antitoxin to identify the toxigenic strains of *C.perfringens* recovered from diseased broilers . These results are in agreement with ***Songer and Dale (2005)***.

In the present work, sensitivity of *C.perfringens* isolates to antimicrobial agents in vitro was studied, as shown in table (6) it was noted that *C. perfringens* isolates were highly sensitive(80-96%) to amoxicillin, ampicillin, pipracillin, ceftraxione, cefruxime and Bacitracin followed by fuscidic acid similar results were reported by Jansen and ***(Jansen and Bermmelgaard, 1988; Traub, 1990; Tansuphasiri et al, 2005 and Silva et al, 2009)*** who reported highly susceptible of *C.perfringens* to amoxicillin, ,ceftraxione,cefruxime and Bacitracin. while chloramphenicol and erythromycin were of moderate effect (40%) , On the other hand *C.perfringens* isolates were resistance to trimethoprim+sulphamethazole tetracycline, Deoxycycline, lincomycin, clindamycin, streptomycin, enrofloxacin ,tobramycin, trimethoprime, streptomycin and kanamycin(9.09-13.2%).These findings are in general agreement with those of ***(Abdel-Rhman et al, 2006; Ali, 2010; Mohammed, 2013 and Farag et al, 2013)***.These results are in accordance with ***Johansson et al (2004)*** and ***Silva et al (2009)*** they reported that *C.perfringens* isolates were resistant to tetracycline. Also, disagree with ***Afify and Nasr (2004)*** Who indicated that enroflxacin, and erythromycin were highly effective against colestridum isolates.This study was cocluded that *C. perfringens* plays a serious role in necrotic enteritis through

proliferation in the intestine and production of several exotoxins . Higher incidence of *C. perfringens* was recorded in winter in age of 20:30day that attributed to environmental stress and unhygienic conditions then 40:50day and the intestine appeared to be the most common site for isolation of *C. perfringens* followed by liver . Type A was the most predominant one. *C.perfringens* strains recovered from chicken suffering from necrotic enteritis in broilers indicated that *C.perfringens* was highly sensitive to amoxicillin, Ampicillin, Piperacillin, ceftraxione, cefruxime, Cefotaxime, Fuscidic acid and Bacitracin. while chloramphenicol and erythromycin were of moderate effect . On the other hand *C.perfringens* isolates were resistance to trimethoprim+sulphamethazole , tetracycline, Deoxycycline, lincomycin, clindamycin, enrofloxacin, and Streptomycin.

Reerence

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التوصيف الظاهرى للكولستريديم بيرفيرنجينز المصاحبة للنزلات المعوية التنكرزية فى بدارى التسمين فى محافظه الشرقية

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****وحدة اللاهوائيات - قسم بحوث البكتريولوجيا معهد بحوث صحة الحيوان-الدقى-الجيزه*

تم اجراء هذة الدراسة على ٣١٤ عينة من الامعاء والكبد جمعت من بدارى تسمين تعانى من اسهال خلال عام ٢٠١٣-٢٠١٤.وبعد فحص انسجه العينات التى تم عزلها تم فحصهم و وجد ٩١عتره ممرضه (٦٦,٩٣%)وعترات غير ممرضه ٤٤(٣١,٠٦%). وجدت ١٣٢ عينه ايجابيه لعزل الكوليستريديم بيرفرينجينز ونوعت عن طريق الحقن داخل الجلد فى خنازير غينيا الألبينو.
وسجلت أعلى نسبه عزل للكوليستريديم بيرفيرينجينز في فصل الشتاء (٥٧,٥٪ في عام ٢٠١٣ و ٥٣,١٢٪ في عام ٢٠١٤) ثم في الخريف (٥٠ ٪ في عام٢٠١٣ و ٢٠١٤) ثم في فصل الصيف (٣٩,٦٥٪ في ٢٠١٣ و ٣٤,٧٨٪ في عام٢٠١٤) وسجلت معدلات أقل في فصل الربيع (٣١,٢٥ ٪ في عام ٢٠١٣و ٣٠٪ في ٢٠١٤).
كانت نسبة الكوليستريديم بيرفرينجينز المعزوله من عينات الامعاء هى(٥٨,٦٦%-٦٥,٨٥)) ومن الكبد هى و(٢١,٩٥%-٢١,٣٣٪).
لتحديد المضادات الحيويه الاكثر تاثيرا فى علاج التنكرز المعوى تم عمل اختبار الحساسية على ٩١ معزول من الكولسيتريديم بيرفرنجينز الممرضه والمضادات الموصي بها هي اموكسي سيللينوببراسيكلين وامبسلين و سيفاتراكزيون سيفاروكسيم و سيفوتاكسيم .
وقد سجلت دراستنا ان نسبه عزل الكوليستريديم بيرفرينجينزمن عينات الامعاء اكثر منها من عينات الكبد وتراوحت من٦٥,٨٥:٨٥,٦٦) و(٢١,٣٣ %-٢١,٩٥%) بالتتابع وصنفت الي نوع A ٧٦ (٥٧,٥٧%) ونوع D١٥(١١,٣٧%)