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STUDIES ON CLOSTRIDIAL MICROORGANISMS IN RABBITS AND THE USE OF ELISA FOR DETECTION OF *CLOSTRIDIUM* PERFRINGENS TOXINS

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SUMMARY

A total of 300 samples were collected from the intestine, liver and feces of apparently healthy (150 samples) and dead rabbits were with intestinal pathological lesions (150 samples). 4-12 weeks old rabbits were obtained from private farms in Menoufiea governorate , and were bacteriologically examined at diagnostic laboratory of the Animal Health Research Institute, Shebin El-Kom-Menoufiea. *C. perfringens* was the most prevalent type recovered from apparently healthy rabbits with incidences of 30% from intestine , 18% from liver and 10% from feces samples. Followed by *C. difficile* 6% ,4% and 4% from intestine , liver and feces, respectively. Also *C. perfringens* was the most prevalent type from dead rabbits with incidences of 70% from intestine , 60% from liver and 36% from feces samples . Followed by *C. difficile* 10% ,10% and 8% from intestine, liver and feces, respectively. Typing of *C. perfringens* isolates revealed that type A was the most prevalent type followed by type D with incidences of 40% and 20% in intestine , 33.3% and 11.1% in liver and 40% and 20% in feces in apparently healthy rabbits, respectively. The incidences of type A and type D in dead rabbits were 42.9% and 25.7% in intestine ,36.6% and 23.3% in liver and 38.9% and 22.2% in feces, respectively.

Enzyme linked immunosorbent assay (ELISA) was compared with neutralization test in mice for typing of *C. perfringens* toxins. Results showed that ELISA can capture alpha and epsilon toxin from intestinal contents of rabbits . The ELISA gave excellent agreement with mouse protection test. Furthermore ELISA was sensitive and qualitative , it allowed the differential diagnosis of *C. perfringens* types A, B, C and D , enterotoxaemias from samples of intestinal contents .

The sensitivity test indicated that enrofloxacin , spectinomycin and flumequine were effective against the *Clostridium* isolates.

INTRODUCTION

Enteritis in rabbits mainly after weaning is the major cause of economic losses in the commerical rabbits . The role of anaerobic bacteria including clostridium microorganisms as etiological agents in necrotic enteritis in rabbits , many pathogenic anaerobic bacteria are normal inhabitants of the bacterial flora in animals Szemeredi et al. (1983).

All clostridia are potent producer of exotoxins which are the major virulence factors. C.perfringens is one of the most widely spread members in the genus *Clostridium* . The infection of this organism is considered as one of the most dangerous diseases which affect the animal industry including rabbits Timoney et al. (1988).

Clostridial involvement in rabbit diseases include *C. spiroforme* Carman and Boriello, (1984). *C. difficile* Fischetti et al. (1984). and *C. perfringens* types A and E Nagi et al. (1988) .

Percy and Barthold, (2001) reported that clostridial species, principally *C. difficile* and *C. spiroforme*, proliferate and produce toxins to induce necrotic enteritis in rabbits. Clostridial exotoxins induce secretory and vascular effects. A history of antibiotic therapy with broad spectrum antibiotics including oral ampicillin, clindamycin or lincomycin, may be associated with this disease. Clostridial enteritis may occur in rabbits that have not been treated with any antibiotics. Diets high in carbohydrates enhance the overgrowth of *Clostridium* species. Change in gut flora and other stress factors leading to anorexia may predispose to disease. It was recorded that the clinical signs were sudden death with no previous signs of illness or watery diarrhea 2 to 3 days prior to death . This disease affects all ages, but primarily targets recently weaned rabbits.

C. perfringens has been reported to cause necrotic enteritis Kunstyr et al. (1975). Matthes (1981) found outbreaks of severe diarrhea with purulent necrotic typhlitis and liver lesions in rabbits . Bacteriological examination of intestinal and fecal flora revealed sharp increases in spore- forming anaerobes particularly *C. perfringens*. The disease was reproduced in rabbits by giving them cecal contents containing *C. perfringens* from diseased rabbits.

Baskerville et al. (1980) recorded that rabbits of all ages from four weeks were affected by *C. perfringens* enterotoxamia. The disease which was characterised by a profuse watery diarrhea, was invariably fatal. Death occurred within a few hours of the onset. Post mortem examination was carried out in all rabbits which died or were

killed. Gross lesions were in all cases confined to the intestines , and consisted of a varying degree of inflammation of the caecum, which was distended with fluid contents.

The diagnosis of this disease in rabbits depends on the detection in the intestinal contents of the major lethal toxin α, β and /or epsilon. The most widely used method for detecting clostridial toxins is the neutralization test in mice Stern and Batty, (1975).

In recent years the enzyme linked immunosorbent assay (ELISA) has been developed as an alternative to neutralization test in mice to detect *C. perfringens* toxins in intestinal contents in animals. Naylor et al. (1987) reported that ELISA was sensitive enough to detect a level of 7.8 ng / ml of epsilon toxin and was specific and quick to perform. Also described a modified method of ELISA for the dection of B toxin. Martin et al. (1988) developed modification of ELISA for detection of α toxin. Nayler et al., (1997) it was found to be a simple and sensitive test for detecting *C. perfringens* α toxin.

Clostridium organisms cause severe losses in well nourished healthy rabbits, therefore it can hinder rabbits production . There is no doubt that clostridial diseases cause potential losses among rabbits in Egypt . So the present study was undertaken to throw light on the incidence and types of Clostridium species in the intestine , liver and fecal samples collected from apparently healthy and diseased rabbits. Besides, their susceptibility to chemotherapeutic agents as an aid to treat the disease and reduce losses. The use of ELISA to detect *C. perfringens* toxins in intestinal contents of rabbits was compared with mouse intravenous neutralization test (MINT).

MATERIALS AND METHODS

Samples:

The samples were collected from apparently healthy rabbits (50 intestine, 50 liver and 50 fecal samples), Similar number of samples was collected from rabbits with prominent gross lesions observed including fluid- filled odematous cecum with serosal congestion and hemorrhage and watery mucoid feces in the colon . Severe lesions include necrotic erosoins or ulcerative typhlitis with swelling . Large Gram-positive bacilli with spores were often observed on the mucosal surface.The livers were friable with yellowish brown colour and in some cases had demarcated necrotic focci.

The collected samples were obtained from private farms for rabbits and were bacteriologically examined at the diagnostic laboratory of the Animal Health Research Institute , Shebin El- Kom - Menoufiea.

Bacteriological examination :

All samples were subjected to bacteriological examination for anaerobes. Each sample was inoculated into two tubes of freshly prepared previously boiled and cooled modified Robertson's cooked meat medium .Both tubes were incubated anaerobically at 37°C for 24 hours, then a loopful from the first tube was streaked onto the surface of 10% sheep blood agar plate with neomycin sulphate (75ug/ml) .The plate was incubated anaerobically at 37°C for 24 hours. Suspected colonies of *C. perfringens* that were characterized by double zone of hemolysis , were reinoculated into cooked meat broth for further identification . The second tube was heated at 80°C for 10 minutes to eliminate the non spore forming anaerobes , then a loopfull was streaked onto sheep blood agar plate without neomycin and incubated anaerobically.

All isolates were identified morphologically and biochemically according to Koneman et al. (1992) and Collee et al. (1996). *C. perfringens* isolates were typed by mouse intravenous neutralization test Stern and Batty, (1975).

Sensitivity of isolates to chemotheraputic agents :

The disc diffusion method was used on a pure subcultures from the predominant isolates of clostridia from necrotic enteritis of rabbits as described by Koneman et al. (1992) and Quinn et al. (1994).

Enzyme linked immunosorbent assay (ELISA):

ELISA was performed according to Iacona et al. (1980). ELISA plates 96 flat bottom wells were coated each with 100µl (50µl of soluble intestinal content from diseased rabbit in 50µl carbonate buffer pH 9.6 separately). The soluble intestinal contents was clarified by centrifugation at 2000 rpm for 20 minutes. The plates were incubated overnight at room temperature. Following blocking with 0.1% bovine serum albumin (BSA) in coating plates, 100 ul of the antitoxin A and D diluted at 1: 100 in PBS were added and the plates were kept for 2 hours at 37°C in a shaking water bath . After washing the plates 5 times with PBS containing 0.05% tween 20 , 100 ul of alkaline phosphatase labeled anti- equine IgG antibodies diluted at 1:3000 in PBS were added and the plates were kept for 1 hour at 37°C in a shaking water bath . The chromogen paranitrophenyl phosphate, at 1mg per ml substrate buffer , pH 9.8 , was added and the absorbance of the colored reaction was read within 30 minutes at 405nm using a titertek multishan ELISA reader . The positively threshold value (cut off value) was determined as double fold of the mean value of negative sera .

RESULTS AND DISCUSSION

Results presented in (Table, 1) and (Fig.1) show that the rate of isolation of clostridial microorganisms from intestine , liver and feces of apparently healthy rabbits, the table demonstrates that *C.*

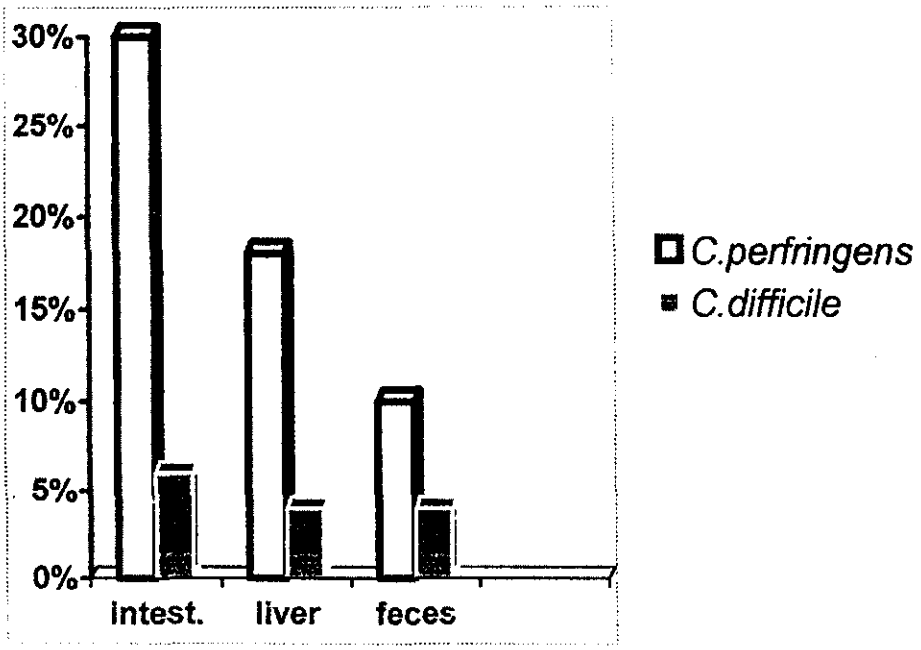

Fig. (1): Isolation rate of clostridia from intestine, liver and fecal samples of apparently healthy rabbits.

Table (1): Incidence of clostridia in the examined organs of apparently healthy rabbits.

The examined rabbit organs	No. of examined organs	*Clostridium* species isolates					
		C. perfringens		*C. difficile*		Total	
		No. of positive cases	%*	No. of positive cases	%*	No.	%*
Intestine	50	15	30	3	6	18	36
Liver	50	9	18	2	4	11	22
Feces	50	5	10	2	4	7	14
Total	150	29	19.3	7	4.7	36	24

%* was calculated according to the number of examined organs.

perfringens isolates were the most prevalent with incidences of 30% , 18% and 10%, respectively. Followed by *C. difficile* 6% , 4% and 4% from intestine , liver and feces, respectively .

These finding agreed with those recorded by Bernal et al. (1981) ; Hughes et al. (1983) and Lindsay and Dennison, (1986). *C. perfringens* has been found, in varying numbers in the intestinal contents of apparently normal rabbits Carman and Boriello, (1982).

The results in Table (2) and Fig. (2) show rate of isolation of clostridial microorganisms from the intestine, liver and feces of dead rabbit with enteritis. The Table demonstrates that *C. perfringens* isolates were the most prevalent with incidences of 70% , 60% and 36%, respectively. Followed by *C. difficile* 10% ,10% and 8% from intestine , liver and feces, respectively .

These results are in general agreement with that of McDonel and Duncan, (1975) who isolated *C. perfringens* in high incidence from diseased rabbits . Also Patton et al. (1981) recorded a result similar to but not identical with enteritis caused by *C. perfringens* type A in an incidence of 95% . Moreover, Szemeredi et al. (1983) examined the internal organs and intestinal contents of diseased rabbits and recorded that clostridia were present in high number in the gut of rabbits. All of organisms isolated were identified as *C. perfringens* . Furthermore, Peeters and Carlier, (1985) noticed digestive disorders and over 70% of losses of weaned rabbits due to *C. perfringens*. Also Nagi et al. (1988) compared between healthy and diarrheic rabbit cecal contents regarding the presence of *C. perfringens*. They found that diarrheic rabbits yielded obviously higher isolation rates of *C. perfringens* . It was found from the results of the present investigations that *C. difficile* was also isolated from diseased rabbits, but in lower incidence. Simlar findings were reported by Hughes et al. (1983) , Mitchell et al. (1986) and Carman and Borriello, (1983) who isolated *C. spiroforme* from feces of diseased rabbits.

Results given in Table (3) revaled that the isolated clostridia were mostly *C. perfringens*. Studying its toxigenicity, which is the important character , the highest rate were toxigenic in diseased rabbits showing 80% ,73.3% and 72.2% in intestine, liver and feces, respectively, while the toxigenic strains in apparently healthy animals were 66.6%, 55.6% and 60% in intestine , liver and feces, respectively.

Generally , toxigenic *C. perfringens* was probably more widely spread in the diseased rabbits than other toxigenic collected from apparentaly healthy rabbits . These results agree with the observations by Szemeredi et al. (1983) , Harris and Portas, (1985) and Wang (1985), who attempted to investigate the percentage of toxigenic *C. perfringens* in the fecal samples of diseased and apparently normal rabbits and found that toxigenic

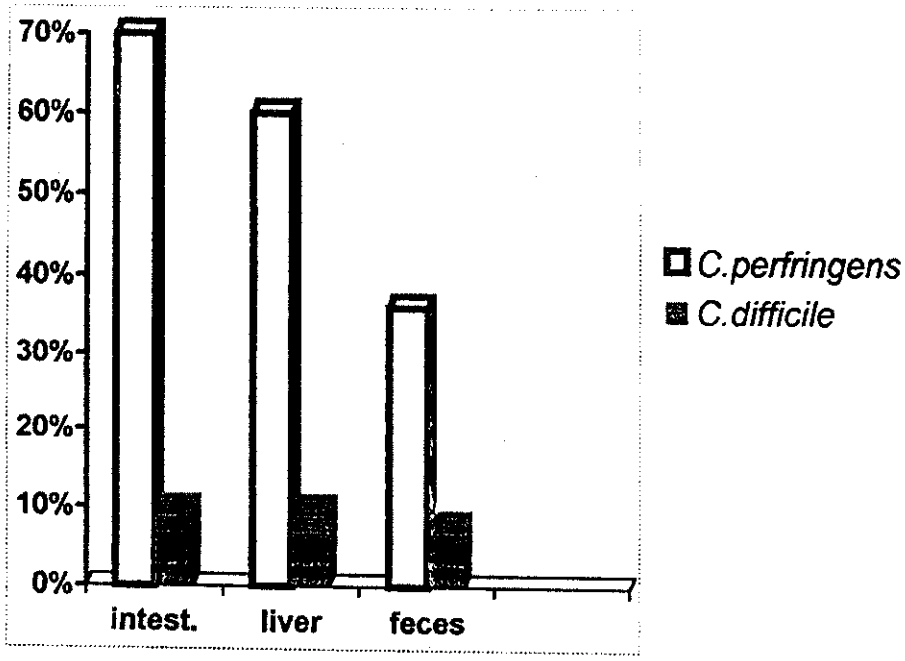

Fig. (2) : Isolation rate of clostridia from intestine, liver and fecal samples of diseased rabbits.

Table (2): Incidence of clostridia in the examined organs of diseased rabbits.

The examined rabbit organs	No. of examined organs	*Clostridium* species isolates					
		C. perfringens		*C. difficile*		Total	
		No. of positive cases	%*	No. of positive cases	%*	No.	%*
Intestine	50	35	70	5	10	40	80
Liver	50	30	60	5	10	35	70
Feces	50	18	36	4	8	22	44
Total	150	83	55.3	14	9.3	97	64.7

%* was calculated according to the number of examined organs.

Table (3): Typing of *C.perfringens* isolated from the examined intestine, liver and feces of apparently healthy and diseased rabbits.

General health status	Organ examined	No.of examined samples	No.of *C.perfringens isolates*		Types of *C. perfringens*									
					Toxigenic		Type (A)		Type (D)		Mixed (A&D)		Non-toxigenic	
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Apparentaly healthy rabbits	Intestine	50	15	30	10	66.7	6	40	3	20	1	6.7	5	33.3
	Liver	50	9	18	5	55.6	3	33.3	1	11.1	1	11.1	4	44.4
	Feces	50	5	10	3	60	2	40	1	20	0	0	2	40
Diseased rabbits	Intestine	50	35	70	28	80	15	42.9	9	25.7	4	11.4	7	20
	Liver	50	30	60	22	73.3	11	36.7	7	23.3	4	13.3	8	26.7
	Feces	50	18	36	13	72.2	7	38.9	4	22.2	2	11.1	5	27.8

No.: Positive number
%* was calculated according to no. of *C. perfringens* isolates

C. perfringens was higher among diarrheic than normal cases .

Typing of the different isolates of toxigenic *C. perfringens* toxins by the use of neutralization test in mice and dermonecrotic reaction of guinea pigs revealed that *C. perfringens* type A was the most prevalent isolate recovered from intestine , liver and feces of apparentaly healthy rabbits with incidences of 40% , 33.3% and 40%, respectively, followed by *C. perfringens* type D with incidences of 20%, 11.1% and 20% from intestine, liver and feces, respectively, then mixed types (Aand D) with incidences of 6.7% , 11.1% and 0% from intestine, liver and feces, respectively .This indicated that type A is widely distributed in apparently healthy rabbits . These findings agree with Kunstyr et al. (1975) who isolated *C. perfringens* type A from the liver of apparently healthy rabbits. In contrast, Nagi et al. (1988) compared between types of toxigenic *C . perfringens* isolated from both healthy and diarrheic rabbits and they found that toxigenic isolates were more prevalent , mostly of *C. perfringens* type E and a few were of type A.

Typing of different isolates of *C.perfringens* recovered from diseased rabbits revealed that *C. perfringens* type A was the most prevalent isolate recovered from intestine, liver and feces with incidences of 42.9% , 36.7% and 38.9% , respectively , followed by *C. perfringens* type D with incidences of 25.7% , 23.3% and 22.2% from intestine, liver and feces, respectively , then mixed types (Aand D) with incidences of 11.4% , 13.3% and 11.1% from intestine , liver and feces , respectively. These results agree with the observations done by Kunstyr et al. (1975) who isolated *C. perfringens* type A in pure culture from the liver of diseased rabbits . McDonel and Duncan, (1975) recovered *C. perfringens* type A in high incidence from necrotic liver of diarrheic rabbits . McDonel and Duncan, (1977) found that type A of *C. perfringens* constituted the main clostridial organism among rabbits. Also Szemeredi et al. (1983) recognized *C. perfringens* type A only from the internal organs and intestinal contents of diseased rabbits . Furthermore, Haffr et al. (1988) recorded 13% in 600 adult rabbits with fatal *C. perfringens* type A infection .

Fig. (3) illustrated the results of application of antitoxin to capture the α and epsilon toxins in intestinal contents. Enzyme Linked immunosorbent assay (ELSA) was used for this purpose. A total of 25 samples of diseased rabbits was examined. The ELISA was proved to be a reliable test with an excellent agreement with the mouse protection test . Twenty samples had a difference in optical density greater than 0.3. All of these samples were positive by the mouse protection test . Values below 0.3 were negative by mouse protection test. As a result, a value of 0.3 optical density units was regarded as the minimum for a positive test by the ELISA. Of the 20 positive samples, 13 were positive for α toxin alone, 7 were positive

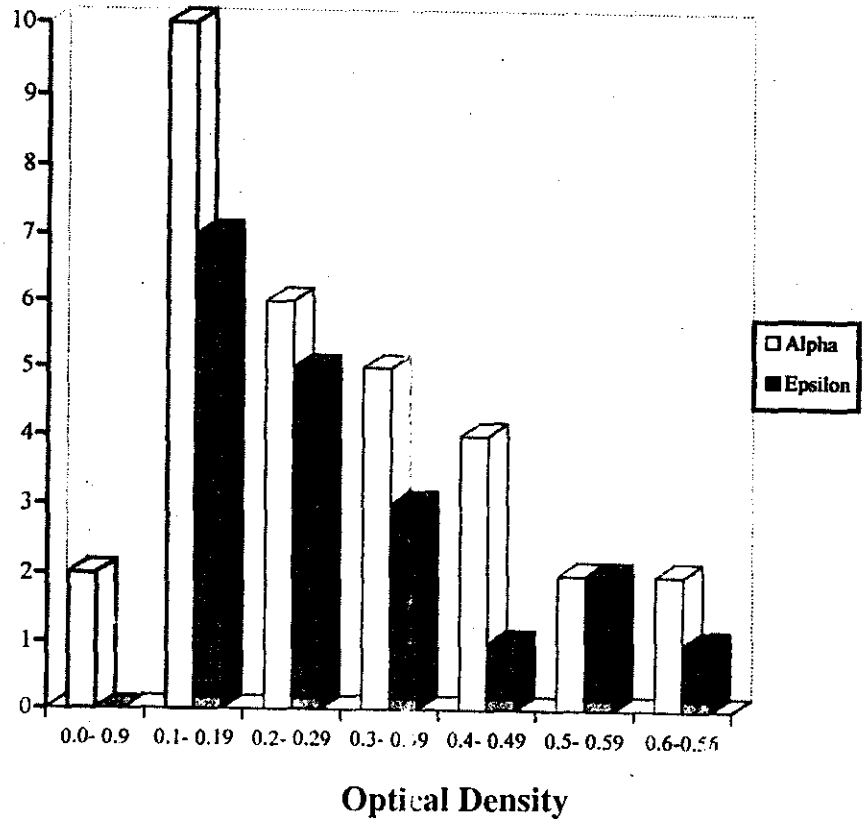

Fig (3): Optical density values of alpha and epsilon toxin as detected by ELISA in the intestinal contents of 25 samples .

for epsilon and 3 were positive for α and epsilon toxins. These results agreed with that of Naylor et al. (1987) as they detected eplison toxin in field smaples of intestinal contents from sheep and goats from enterotoxemia cases. Meanwhile, Naylor et al. (1997) developed an ELISA for the detection of *C. perfringens* α toxin in intestinal contents of animals which died of suspected *C. perfringens* type A enterotoxemia. They reported that α toxin was detectable down to a level of 25 ng/ml.

At present , the most commonly used test for the detection of *C. perfringens* alpha and epsilon toxin is the neutralization test in mice . The ELISA has proved to be a faster, a more sensitive test giving qualitative results within 4 hours in contrast to the 48 hours required for the mouse test. So, ELISA is considered as a reliable alternative to neutralization tests in mice for the detection of *C. perfringens* toxins and allows the differential diagnosis of *C. perfringens* type A , B , C and D enterotoxemias as well as the typing of *C. perfringens*.

In Table (4) results of vitro antibiotics sensitivity testing of recovered (8) *C. perfringens* and (6) *C. difficile* isolated from rabbits . Tested clostridial isolates were highly resistant to kanamycin , lincomycin and tetracycline. On the other hand,

Table (4): Sensitivity of clostridial isolates to different chemotherapeutic agents.

Antimicrobial discs	Potency	*C. perfringens*				*C. difficile*			
		No. (8)	%	IZ.(mm)	AA.	No. (8)	%	IZ.(mm)	AA.
Ampicillin	10ug	5/8	62.5	13	IS	4/8	50	12	IS
Chloramphenicol	30ug	5/8	62.5	15	IS	6/8	75	20	IS
Enrofloxacin	5ug	8/8	100	22	S	7/8	87.5	20	S
Erythromycin	15ug	6/8	75	16	IS	7/8	87.5	18	S
Flumequine	30ug	7 /8	87.5	17	S	6/8	75	13	IS
Kanamycin	30ug	0/8	0	--	R	1/8	12.5	--	R
Lincomycin	10ug	1/8	12.5	--	R	1/8	12.5	--	R
Penicillin-G	10U	5/8	62.5	18	IS	6/8	75	20	IS
Spectinomycin	100mg	8/8	100	20	S	6/8	75	14	IS
Tetracycline	30ug	2/8	25	--	R	0/8	0	--	R

IZ.: Inhibitory zone (mm).
AA.:Antibiogram activity.
No. Number of isolates.
% :Percentage of sensitive isolates.
S : sensitive.
IS: intermediate sensitive.
R: resistant.

the *C. perfringens* was sensitive to enrofloxacin , flumequine , and spectinomycin. *C. difficile* was sensitive to chloramphenicol, enrofloxacin and erythromycin . Other tested antimicrobial agents as ampicillin, and penicillin-G showed variable intermediate degree of sensitivity . These results agreed with the findings of Rood et al. (1978) who observed the resistant of *C. perfringens* to lincomycin, tetracycline and lindamycin, Sycasiu (1983), which showed that chloramphenicol and erythromycin were active against *C. perfringens* types A ,C, and E , and El.Bardisy (1999), who recorded that *Clostridium* species were sensitive to enrofloxacin and ampicillin.

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دراسات على ميكروبات الكلوستريديم في الأرانب مع إستخدام إختبار الآليزا لتشخيص سموم الكلوستريديم بيرفرنجينز

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** معهد بحوث صحة الحيوان - شبين الكوم - المنوفية

تم الفحص البكتريولوچى الاهوائى لعدد ٣٠٠ عينة من الأمعاء والكبد والبراز لأرانب سليمة ظاهرياً (١٥٠) عينة وأرانب مصابة ونافقة بإضطرابات معوية (١٥٠) عينة من مزارع خاصة بشبين الكوم - محافظة المنوفية.

تم عزل الكلوستريديم بيرفرينجينز من الأرانب السليمة بنسبة (٣٠٪) من الأمعاء ومن الكبد بنسبة (١٨٪) ومن البراز بنسبة (١٠٪) والكلوستريديم نيقسيل بنسبة (٦٪) من الأمعاء ومن الكبد بنسبة (٤٪) ومن البراز بنسبة (٤٪).

ومن الأرانب المصابة تم عزل الكلوستريديم بيرفرنجينز بنسبة (٧٠٪) من الأمعاء ومن الكبد بنسبة (٦٠٪) ومن البراز بنسبة (٣٦٪). والكلوستريديم ديفسيل بنسبة (١٠٪) من الأمعاء ومن الكبد بنسبة (١٠٪) ومن البراز بنسبة (٨٪).

وبتصنيف العترات المعزولة ثبت أن ميكروب الكلوستريديم بيرفرينجينز نوع (أ) هو أكثر العترات تصنيفاً يليه نوع (د) فى الأرانب السليمة والمصابة فكانت النسب فى الأرانب السليمة على النحو التالى من الأمعاء (٤٠٪ و ٢٠٪) من الكبد بنسبة (٣٣.٣٪ و ١١.١٪) ومن البراز بنسبة (٤٠٪ و ٢٠٪) على الترتيب. ولكن فى الأرانب المصابة كانت النسب من الأمعاء (٤٢.٩٪ و ٢٥.٧٪) ومن الكبد بنسبة (٣٦.٧٪ و ٢٣.٣٪) ومن البراز بنسبة (٣٨.٩٪ و ٢٢.٢٪) على الترتيب.

وقد تم إستخدام إختبار الأليزا وإختبار الحقن التعادلى فى الفئران لتعيين سموم الكلوستريديم بيرفرنجينز الموجودة فى محتويات أمعاء الأرانب حيث أثبتت النتائج أن إختبار الأليزا ذو كفاءة وحساسية عالية فى تعيين سموم الألفا والأبسيليون من محتويات الأمعاء.

وبإجراء إختبار الحساسية للعترات المعزولة أظهرت ميكروبات الكلوستريديم حساسية عالية إلى الأنروفلوكساسين والسبكتينومايسين والفلوميكين.