A STUDY ON CLOSTRIDIUM SPECIES IN CHICKENS AND TURKEYS WITH SPECIAL REFERENCE TO THE EFFICACY OF ELISA FOR DETECTION OF CLOSTRIDIUM PERFRINGENS TOXINS

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ABSTRACT

110 intestinal and liver samples from dead broiler chickens (4- 8) weeks and dead adult turkeys were obtained from the diagnostic laboratory of poultry at the Animal Health Research Institute, Dokki, Giza and from private farms. C. perfringens, the most prevalent clostridium species, was isolated from 34 samples (75.55%) of chicken and 7 samples (70%) of turkeys. Typing of C. perfringens isolates revealed that type A was the most prevalent type followed by type D with incidences of (44.11 and 29.41) respectively in chicken and (71.4 and 28.5) respectively in turkeys.

The in vitro sensitivity test indicated that enrofloxacin, penicillin G, chloramphenicol and erythromycin were highly effective against these clostridium isolates. Enzyme linked immunosorbent assay (ELISA) was used in typing of C. perfringens toxins with neutralization test in mice. Results showed that ELISA can capture alpha and epsilon toxin from intestinal contents of chicken and turkeys. 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.

INTRODUCTION

Clostridia are common in the environment and can be isolated from the intestinal contents of birds and animals **(Timoney et al., 1988)**.

Certain members of genus Clostridium can contribute in significant diseases in poultry under certain circumstances. There are many reports concerning the importance of clostridial organisms in poultry which have been known to be the causes of many diseases **(Onderka et al., 1990)**.

Reports of clostridial involvement in poultry diseases include C. novyi **(Peterson, 1964)**, C. sporogenes **(Peterson, 1967)**, C. fallax **(Ellwood and Halliwell, 1973)**; C. septicum **(Fowler and Hussaini, 1975)** and C. perfringens type A **(Nillo, 1976)**.

C. perfringens has been reported to cause necrotic enteritis **(Parish, 1961)**. Fukata et al. (1986) found that when the number of C. perfringens increased due to reduction of major intestinal microflora, alpha-toxin by C. perfringens increased and outbreak of necrotic enteritis might be induced. The disease is accompanied with severe fatal diarrhoea due to production of toxins which destruct the epithelial lining of the intestinal mucosa followed by the invasion of clostridia especially the exotoxins into the blood stream **(Cowen et al., 1987; Baba et al., 1992)** because C. perfringens is usually found in healthy chicks.

Laboratory diagnosis of these diseases depends on the demonstration in the intestinal contents of the major 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)**.

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 **(Olsvik et al., 1982)**. It was found it to be a simple and sensitive test for detecting C. perfringens type A toxin **Weddell and Worthinghton (1984)** 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 and **Naylor et al. (1987)** described a modified method of ELISA for the deteetion of B toxin.

Clostridium organisms causes severe losses for well nourished healthy birds, therefore it can hinder poultry production **(El-Seedy, 1990)**. There is no doubt that clostridial diseases cause potential losses among poultry in Egypt. So the present study was undertaken to throw the light on the incidenee and types of clostridium species in the intestine and liver samples collected from diseased broiler chickens and adult turkeys, their susceptibility to chemotherapeutic agent as an aid to avoid their pathogenicity, the use of ELISA to detect C. perfringens toxins in intestinal contents of chickens and turkeys and to compare this technique with mouse intravenous neutralization test (MINT).

MATERIAL AND METHODS

Samples:

A total number of 110 liver and intestinal samples were collected from chickens and turkeys with gross lesions showing distension of the intestine with gases, focal necrotic lesions in the small intestinal mucosa, marked catarrhal enteritis and formation of a thick pseudomembrane.

The livers were friable with yellowish brown colour and in some cases had demarcated necrotic foci.

These samples were obtained from the diagnostic laboratory of poultry at the Animal Health Research Institute, Dokki, Giza and from private farms from adult birds (4- 8 weeks).

Bacteriological examination:

All samples were subjected to bacteriological examination for anaerobes. Each sample was inoculated into two tubes of freshly prepared modified, Robertson's cooked meat medium. One tube was heated at 80°C for 10 minutes to eliminate the non spore forming acrobes while the other was left without heating. Both tubes were incubated anaeroieally at 37°C for 48 hours then, a loopful from the heated and unheated inoculated tubes were streaked onto the surface of 10% sheep blood agar and neomycin sulphate sheep blood agar plates (75µg/ml) respectively. The plates were incubated anaerobically at 37°C for 48 hours. The suspected colonies were reinoculated into cooked meat broth for further identification. 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 test:-

The antibiogram of the recovered pathogens was done using the disc diffusion method described by **Koneman et al. (1992) and Quinn et al. (1994)**.

Enzyme linked immunosrobent assay (ELISA):

ELISA was performed according to Iacona et al. (1980). ELISA plates, 96 flat bottom wells (Linbro, ICN Inc, USA) were coated each with 100 µl of 50 µl of intestinal content (intestinal contents, clarified by centrifugation at 2000 g for 20 minutes) in 50 µl carbonate buffer, pH 9.6, separately. The plates were incubated overnight at room temperature. Following blocking with 0.1% bovine serum albumin (BSA) in coating plates, 100 µl of the antitoxin A and D diluted at 1:50 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 µl of alkaline phosphatase labeled anti-horse IgG antibodies diluted at 1:2000 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 1 mg per ml substrate buffer, pH 9.8, was added and the absorbance of the coloured reaction was read within 30 minutes at 405 nm using a titertek multishan ELISA reader. The positively threshold value was determined as double fold the mean of negative sera cut off value.

RESULTS AND DISCUSSION

Fig (1) shows the rate of isolation of clostridial microorganisms from the intestine of ill birds with necrotic enteritis in chicken and turkeys. The figure demonstrates that C. perfringens isolates were the most prevalent for both chickens and turkeys with an incidence of 75.55% and 70% respectively, followed by C. sordelli (13.33%), C. bifermentans (11.1%), C. sporogenes (8.8%) and C. tertium (6.66%) for chicken. Meanwhile, the incidence of C. sporogenes and C. tertium was 20% for both in turkeys.

These findings are in general agreement with that of **Ficken and Wages (1997)** and Azzam and **El- Bardisy (1998)**, who reported that clostridia spp. were the main cause of necrotic and ulcerative enteritis in chickens.

On the other hand, **Gazdzinski and Julian (1992) and Droual et al. (1994)** isolated C. perfringens by anaerobic culture from the intestinal contents of turkey hens between 5-12 weeks of age with experienced outbreaks of necrotic enteritis.

The results in table (1 and 2) proved that the frequency of C. perfringens isolation was higher in the intestinal samples (91.11% and 70%) than the liver specimens (73.33% and 60%) from diseased chicken and turkeys respectively. These results agreed with the finding of **Cowen et al. (1982)**, who isolated C. perfringens more frequently from intestine of diseased chickens by necrotic enteritis. The present results are also in harmony with those reported by **El- Bardisy (1999)**.

The data illustrated in tables (1 and 2) showed that C. perfringens was isolated from the examined intestinal samples either in pure form (66.65%, 30%) or mixed with other Clostridium species (24.42%, 40%) for chickens and turkeys respectively. These findings agree with data published by **Abd El- Gabber et al. (1994)** who concluded that two types of clostridial infection in ill chickens with necrotic enteritis were recorded. The first type was single infection that represented by the highest rate (70.97%), the second type was mixed infection that represented by the lowest rate (29.03%)

A study on the typing of C. perfringens toxins by the use of neutralization test in mice and dermonecrotic reaction of guinea pigs was investigated. Table (3) revealed the isolated biovars were mostly C. perfringens (75.55%, 70%) for chicken and turkey respectively, upon its toxigenicity which is the important character, the highest rate were toxigenic (88.22%, 99.99%) for chicken and turkey respectively. Typing of toxigenic isolates revealed that C. perfringens type A as the most prevalent isolate recovered from diseased chicken and turkey with an incidence of (44.11%, 71.42%) respectively, followed by C. perfringens type D with an incidence of (29.41%, 28.57%) for chickens and turkeys respectively, then mixed types (A and D) with an incidence of

(14.70%) for chicken only.

In this concern, **El-Seedy (1990) and Abd El-Gaber et al. (1994)** concluded that type A was the most prevalent type followed by D. On the other hand, **Hofshagen and Stenwing (1992)** found that all isolates were C. perfringens type A. Alpha toxin was produced in significantly larger amounts by isolates from birds with necrotic enteritis.

The sensitivity of the application of antitoxin to capture the α and epsilon toxins in intestinal contents was studied by ELISA. A total of 29 samples was examined, the mean optical density values obtained by ELISA was illustrated in Fig. 2). The ELISA gave reliable test reproducibility and excellent agreement with the mouse protection test. Twenty five samples had a difference in optical density greater than 0.4 and all these samples were positive by the mouse protection test. Values below 0.4 were negative by mouse protection test. As a result, a value of 0.4 optical density units was regarded as the minimum for a positive test by the ELISA. Of the 25 positive samples, 15 were positive for α toxin alone, 10 were positive for epsilon and 5 were positive for α and apsilon These results agreed with that of **Naylor et al. (1987)** as they detected epsilon toxin in field samples of intestinal contents from sheep and goats from enterotoxaemia 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 enterotoxaemia. 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 and more sensitive test giving qualitative results within 4 hours in contrast to the 48 hrs 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 enterotoxaemias as well as the typing of culture of C. perfringens.

In vitro sensitivity of the recovered clostridial species to different antimicrobial agents was done on isolates obtained from necrotic intestinal samples from chickens and turkeys. Table (4) shows that, tested clostridial isolates were highly resistant to flumequine, nalidixic acid, oxytetracycline, streptomycine and sulpha methoxazole + trimethoprime. On the contrary the same strains were sensitive to ampicillin, chloramphenicol, enrofloxacin and penicillin G. On the other hand, other tested anti microbial agents showed variable degrees of sensitivity. These results agreed with the findings of **El- Bardisy (1999)**, who stated that clostridium species are sensitive to ampicillin and enrofloxacin but disagree with the same author in the fact that C. perfringens is sensitive to streptomycin. At the same time, **Abd El- Gaber et al. (1994)** stated that the use of chloromphenicol as food additive or in drinking water appears to be essential to avoid or decrease the infection with necrotic enteritis.

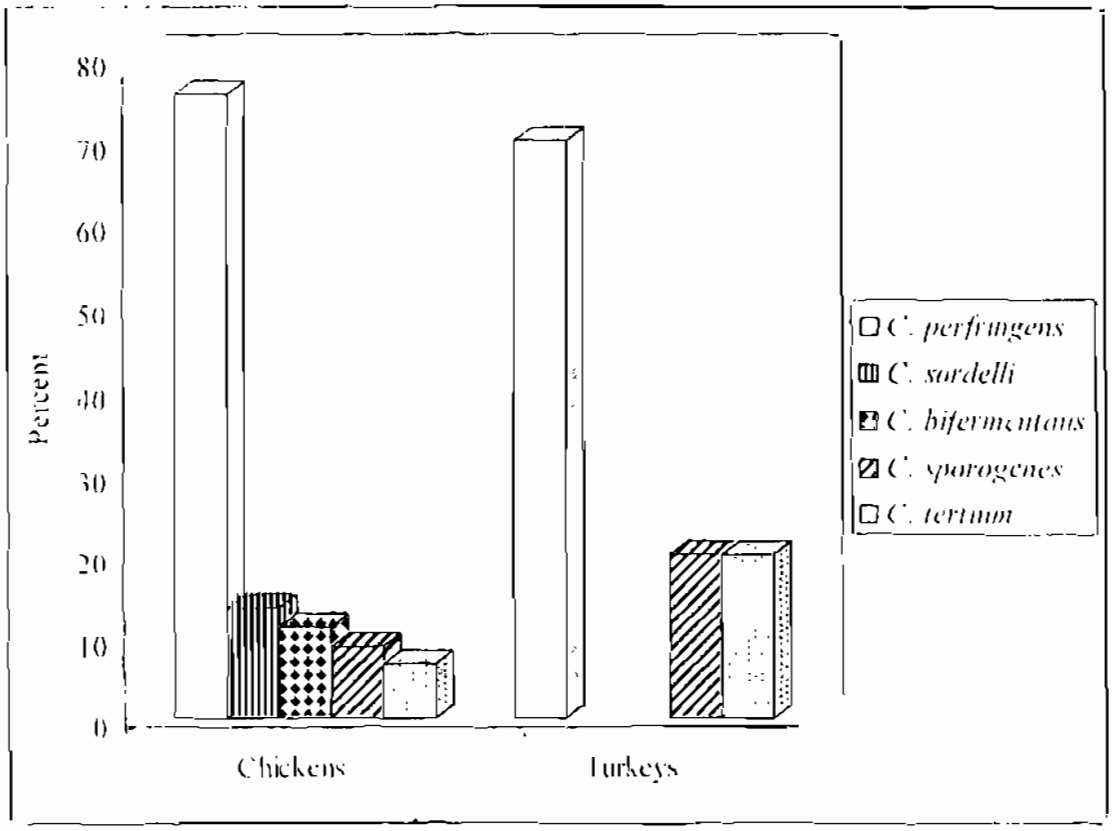

Fig. (1): Isolation rate of clostridial microorganisms from necrotic enteritis of chickens and turkeys.

Table (1): Distribution of the recovered clostridia among liver and intestine of chickens suffered from necrotic enteritis.

Organ examined	No. of examined samples	No. of samples positive for clostridia		Clostridia species single isolates	No.	%*	Clostridia species mixed isolates	No	%*
		No.	%*						
Intestine	45	41	90.11	*C. perfringens*	23	51.11	*C. perfringens + C. sordelli*	2	4.44
				C. sordelli	4	8.88	*C. perfringens + C. sporogenes*	3	6.66
				C. sporogenes	1	2.22	*C. perfringens + C. tertium*	3	6.66
				C. bifermentans	2	4.44	*C. perfringens + C. bifermentans*	3	6.66
Liver	45	33	73.33	*C. perfringens*	21	46.66	*C. perfringens + C. sordelli*	2	4.44
				C. sordelli	4	8.88	*C. perfringens + C. sporogenes*	3	6.66
				C. sporogenes	1	2.22	*C. perfringens + C. tertium*	2	4.44

* On the basis of number of organs examined

Table (2): Distribution of the recovered clostridia among liver and intestine of turkeys suffered from necrotic enteritis.

Organ examined	No. of examined samples	No. of samples positive for clostridia		Clostridia species single isolates	No.	%*	Clostridia species mixed isolates	No.	%*
		No.	%*						
Intestine	10	7	70.00	*C. perfringens*	3	30.00	*C. perfringens* + *C. sporogenes*	2	20.00
							C. perfringens + *C. tertium*	2	20.00
Liver	10	6	60.00	*C. perfringens*	2	20.00	*C. perfringens* + *C. sporogenes*	2	20.00

* On the basis of number of organ examined.

Table (3): Typing of *C. perfringens* isolates recovered from the examined intestines of chickens and turkeys.

Species	No. of examined samples	No. of *C. perfringens* isolates		Types of *C. perfringens*							
				Type A		Type D		Mixed (A, D)		Non-toxigenic	
		No.	%*	No.	%**	No.	%**	No.	%**	No.	%**
Chickens	45	34	75.55	15	44.11	10	29.41	5	14.70	4	11.76
Turkeys	10	7	70	5	71.42	2	28.57	-	-	-	-

* On the basis of number of examined samples

** On the basis of number of *C. perfringens* isolates

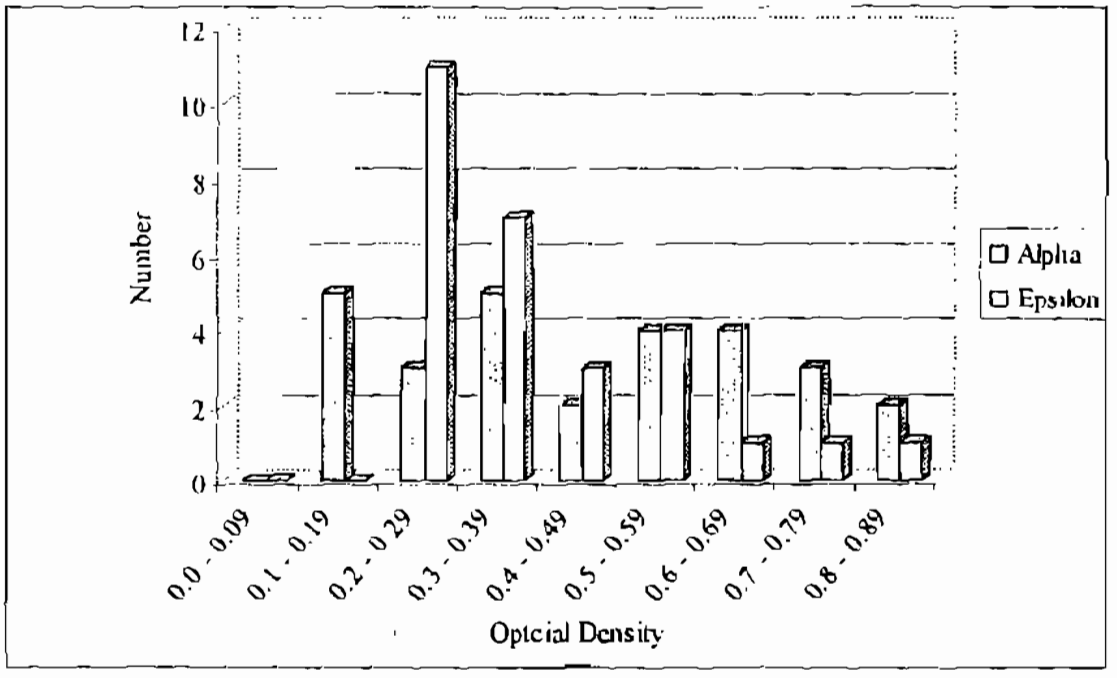

Fig. (2): Optical density values of α and epsilon toxin as detected by ELISA in the intestinal contents of 29 samples.

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

Antimicrobial agent	Potency	*C. perfringens*		*C. tertium*		*C. sporogenes*	
		Inhibitory zone (mm)	AA	Inhibitory zone (mm)	AA	Inhibitory zone (mm)	AA
Ampicillin	10 µg	20	S	20	S	10	IS
chloramphenicol	30 µg	24	S	15	S	8	IS
Enrofloxacin	5 µg	10	S	10	S	10	S
Erythromycin	15 µg	22	S	12	S	6	R
Flumequine	30 µg	-	R	-	R	-	R
Nalidixic acid	30 µg	-	R	-	R	-	R
xytetracycline	30 µg	9	R	6	R	7	R
Penicillin G	10 U	15	S	12	S	9	S
Streptomycin	10 µg	-	R	-	R	-	R
Sulphamethoxazole 25 + trimethoprim	25 µg	-	R	-	R	-	R

S: sensitive

IS: intermediate sensitive

R: resistant

AA: Antibiogram aetivity

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الملخص العربى

دراسة على ميكروبات الكلوستريديم فى الدجاج والرومى مع إشارة خاصة إلى كفاءة إختبار الاليزا لتعيين سموم ميكروبات الكلوستريديم بيرفرنجينز

المشتركون فى البحث

د/ منى مغـــاورى د/ إيمـــان مصطفى نصـــر

قسم البكتريولوجى - معهد بحوث صحة الحيوان

تم فحص عدد ١١٠ عينة أمعاء وكبد من دجاج تسمين نافق ودجاج رومى وكانت العينات تعانى من أعراض التنكرز المعوى.

تم عزل ميكروب بيرفرنجينز من ٣٤ عينة أمعاء بنسبة ٧٥ر٥٪ لدجاج التسمين ومن ٧ عينات أمعاء بنسبة ٧٠٪ لدجاج الرومى، بتصنيف هذه المعزولات ثبت أن ميكروب الكلوستريديم بيرفرنجينز نوع (أ) هو أكثر العترات تصنيفاً ويليه نوع (د) بنسبة ١١ر٤٤٪، ٤١ر٢٩٪ على التوالى فى دجاج التسمين وكانت النسبة ٤ر٧١٪ ، ٥ر٢٨٪ على التوالى فى الرومى. أظهرت ميكروبات الكلوستريديم حساسية عالية إلى أينروفلوكساسين، بنسبللين ج، كلورامفينيكول وايربشرومايسين، تم إستخدام إختبار الإليزا واختبار الحقن التعادلى فى الفئران لتعيين سمو الكلوستريديم بيرفرنجينز الموجودة فى محتويات أمعاء دجاج التسمين، حيث أثبتت النتائج أن إختبار الإليزا ذو كفاءة وحساسية عالية فى تعيين سموم الألفا والإبسيلون من محتويات الأمعاء.