MOLECULAR AND IMMUNOLOGICAL STUDIES ON SOME C. PERFRINGENS TYPE D STRAINS

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ABSTRACT

Six polyvalent clostridial vaccines of sheep were prepared. Each contain toxoid of C. perfringens type D strains from different origins. These vaccines were injected in sheep. The epsilon antitoxin titers were not correlated with the toxicity of the strains. SDS-PAGE test and western blotting were applied for both cells and toxins. Common protein bands with molecular weights between 50 - 10 KDa were appeared in all strains. It was found that there was no a marked relation between toxicity, immunogenicity and the current molecular studies.

INTRODUCTION

C. perfringens type D produces two major lethal toxins and a number of minor lethal or non lethal toxins (**Worthington et al., 1973**). The principal toxin is epsilon toxin, which causes enterotoxaemia, a disease of great economic importance in all sheep breeding countries.

Production of high yield of bacterial toxin is of practical importance in the preparation of effective toxoids for purpose of immunization (**Gilroy, 1967**). The antibody titres in sera of sheep immunized with toxoids prepared from highly toxigenic cultures were higher than those prepared from low toxigenic cultures (**Diab et al., 2000**).

In recent years, rapid advances in the genetics of C. perfringens virulence factors have allowed performance of extensive surveys of the strains isolated in various diseases (**Daube et al., 1996**).

It is evident that more information is needed about effectiveness of vaccination in sheep by polyvalent or bivalent vaccines containing type D-toxoid using strains of different origins. In some prepared routine batches of polyvalent and bivalent vaccines, epsilon (ε) antitoxin titres was not related to toxins produced, so that the aim of this study is to find a relation between

molecular structure of C. perfringens type D strains, toxogenicity and its effect on immune response.

MATERIAL AND METHODS

*** Strains :**

1. Six lyophilized strains of C. perfringens type D, were obtained from different origins. 8346 j31 (UKI), 8504 141 (UKIII) strains were obtained from National Collection of Type Culture (NCTC), London; Syrian strain; Neuzeland strain; Turkey strain and England strain.
2. Lyophilized strains of C. perfringens type B, C. septicum, C. chauvoei., C. oedematiens type B and C. tetani were obtained from Anaerobic Departement, Veterinary Serum and Vaccine Research Institute (VSVRI), Egypt. Used for preparation of polyvalent clostridial vaccine in which C. perfringens type D is one of its component.

Standard toxins and antisera :

Standard toxins and antisera against C. perfringens type B and D. C. oedematiens type B and C. septicum were obtained from Wellcome Research Laboratories, England. C. tetani toxin and antitoxin was obtained from WHO.

Hyperimmune serum :

Sheep hyperimmune serum against epsilon toxin was obtained from Anaerobic Research Department (VSVRI), Abbassia, Cairo; and used for western blotting.

Vaccines preparation :

Six polyvalent vaccines containing antigens of C. perfringens type D (from each strain), C. perfringens type B, C. chauvoei, C. septicum, C. oedematiens type B in equal amounts and C. tetani (25 Lf/dose/sheep) were prepared according to **Gadalla et al. (1974)** except C. tetani toxoid was prepared according to **Rijks Instructions (1980)**.

Sterility, safety and potency tests (in rabbits) were carried out in accordance with the regulation of **British Veterinary Codex (1970)**.

Animals :

Thirty sheep, 9-12 months of age, were divided into six groups and used for evaluation of C.

perfringens type D as a component of the polyvalent vaccines prepared.

Vaccination schedules :

Each type of polyvalent vaccine was injected s/c in a group of 5 sheep in two doses (5 ml and 3 ml) 4 weeks intervals.

Serum sampling and antitoxin assays:

All sheep were bled before vaccination and 2 weeks after the second dose. Sera were separated, pooled for each group and stored at -20°C until used. The antitoxic values for C. perfringens type D, expressed in (IU/ml) of all pooled serum samples were determined in Swiss white mice by method described by **British Veterinary Pharmacoepia (1985).**

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for C. perfringens type D :

1. Cultures of the six strains of C. perfringens type D which obtained from different origins were centrifuged then the supernatant toxins were collected as well as the cell precipitate. The supernatant toxins were concentrated by glycerin to 1/3 of the original volume for each toxin.
2. Each of the cells and toxins of C.perfringens type D strains were subjected to Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Jin et al. (1996) on a minislab gel electrophoresis containing 5% stacking polyacrylamide gel and 12% separating gel. The samples were heated in sample buffer consisting of 62.5 mM Tris-HCL (pH 6.8), 1% 2-mercaptoethanol, 1% SDS, 10% glycerol, and 0.001% bromophenol blue at 100°C for 3 minutes. Electrophoresis was performed at 100 V until the dye front reached the bottom of the gel. The gel was stained with 0.5% Coomassie brilliant blue R by the conventional method. Prestained protein molecules weigh standard high range (GIBCO-BRL) from 14.3 to 200 KDa was used as standard marker.

Western blotting (immunoblotting) :

This was done according to the method deeribed by Hunter et al. (1992). The proteins of the toxin separated by SDS-PAGE were transfered onto a polyvinylidine difluoride membrane (Immobilon P, Millipore Corp.) and the membrane was blocked with a 5% (wt/vol) solution of dried

skim milk in Tris-buffered saline. The membrane was probed with a 1:1000 dilution of sheep anti-epsilon-toxin, as a first antibody and the antigen antibody complexes bound to alkaline phosphatase conjugated antisheep IgG (Sigma USA) as a secondary antibody, then the reaction was detected by using BCIP/NBT substrate.

RESULTS AND DISCUSSION

C. perfringens is the causative agent of a wide variety of diseases. It has been associated with enterotoxemic diseases in many species of domestic animals (**Hunter et al., 1992**). To prevent these diseases, production of immune response against toxoids of the different toxins play an important role in protection of animals. Results of neutralizing antibody level against epsilon toxoid of different strains of C. perfringens type D in sheep are presented in Table (1). The antibody titres were obtained in sera of vaccinated animals so that different toxicity of the strains was not correlated with antibody titre, as in the Neuzeland strain which gave a toxicity of 4000 MLD/ml, produced antibody titre of 12 IU/ml which was almost resemble that of UKIII strain (12 IU/ml and 6000 MLD/ml), also UKI strain (15 IU/ml and 10.000 MLD/ml). While low antibody titre was obtained in the other vaccines in which England strain gave 5 IU/ml titre and 2000 MLD/ml toxicity. Syrian strain gave 3 IU/ml and 3000 MLD/ml and Turkey strain gave 3 IU/ml and 2000 MLD/ml. . Table (1). These results were disagree with that of (**Sato et al., 1972**) who found that immunogenicity of highly purified alpha toxoid of C.perfringens was higher than crude one when injected in guinea pigs and (Diab et al., 2000), who said that the production of epsilon toxoid in fermentor produced epsilon toxin of high MLD and consequently afforded good protection in vaccinated animals. This disagreement may attributed to the use of different strains from different origins while the previous authors used only one strain.

C. perfringens type D synthesized different forms of epsilon prototoxin which showed electrophoretic heterogenicity on disc electrophoresis. Activation of epsilon prototoxin by trypsin, resulted in the formation of epsilon toxin which was electrophoretically heterogenous. Moreover, epsilon prototoxin (mol. wt. 23.200 - 25.000) and epsilon toxin showed antigenic identity (Habeeb, 1969). So protein profiles for toxins and cells of C. perfringens were studied. So electrophoretic analysis of protein profiles of cells of different C.perfringens type D strains as shown in Table (2) and Fig. (1) revealed about 9-18 protein fractions with molecular weight ranged from 12 to 200 KDa. The major protein bands of molecular weight 37, 24, 22, 19 KDa were found in all strains. In the England strain there are extra bands of molecular weight 200 and 163.77 KDa, while there are another bands between 100 and 50 KDa in the Neuzeland strain. The majority of the protein bands located between 12 - 47 KDa.

The protein profile analysis for the toxin was revealed 8 - 12 bands in the different strains with molecular weight ranged from 13 - 101 KDa as shown in Table (3) and Fig. (2). The protein fractions with molecular weight 77.3, 55.8, 42.7, 39.7, 32.9, 27.6, 25.2, 21.6 were prominant in all strains, while there are two extra bands at 101.3 and 13.177 in the England, UKI, and UKIII and one extra band at 70 KDa in Neuzeland.

For the western blot the hyperimmune serum against epsilon toxin was detected proteins at molecular weight from 11.9 to 281.7 for the toxin which cannot be detected by the Coomassie Blue stain, but can be appeared by the western blotting. These findings agree with Sambrook et al. (1989) who said that Coomassie Blue stain can detect protein up to 0.1 (g (100 ng), but the lowest amount of proteins that can be detected by western blotting is approximately 1 - 5 ng.

Since type D strains can produce both the alpha and epsilon toxins in addition to several other extracellular antigens, this may explain the results obtained by electrophoresis of the used crude filtrates, which contains these toxins in trace amounts.

From the present study, it could be concluded that there is no a marked correlation between toxicity, immunogenicity and the current molecular studies of C. perfringens type D epsilon toxin of different strains. So a further advanced studies should be done on the pure epsilon toxin to estimate the specific relation between its toxicity and immunogenicity to the molecular structure.

Table 1 : Toxicity and immunogenicity of different strains of Clostridium perfringens type D.

Strain	MLD/ml	Epsilon antitoxin titre (IU/ml)	Molecular weight summation	
			Cells	Toxin
England	2.000	5	66.8	72.9
Neuzeland	4.000	12	53.9	54.3
Syrian	3.000	3	47.4	56.5
Turkey	2.000	3	54.3	51.7
UK I	10 000	15	57.6	53.9
UK III	6.000	12	58 4	51.7

MLD/ml = Minimum Lethal Dose / ml.
IU/ml. = International Unit / ml.
* Sera tested before vaccination were free from epsilon antibodies.

Table 2 : Protein profile analysis of cells of different strains of C.perfringens type D separated by SDS-PAGE.

M.W. (KDa)	C. Perfringens type D strains					
	England	Neuzeland	Syrian	Turkey	UK I	UK III
200-150	200 163.77				151.19	
150-100	109.81 73.386	145.26 114.29	145.26	145.26	123.8	
100-50	66.379	86.406 81.827 72.591 65.584 50.91	50.299	54.074		
50-10	47.365 37.371 29 24.345 22.436 19.392 15.705 14.393 12.937	47.93 38.81 37.371 31.786 29.157 24.345 22.436 19.392 14.77 13.712 12.895	47.93 39.443 37.371 24.487 22.436 19.392 15.554 14.722 13.756	47.93 39.231 37.371 31.495 24.345 22.436 19.392 15.756 14.818 13.801	47.356 41.183 37.371 33.01 29 24.354 22.436 19.392 15.691 13.712	47.356 37.371 29 24.354 22.436 19.392 15.705 13.98 12.895
Sum.	66.8	53.9	47.4	54.3	57.6	58.4

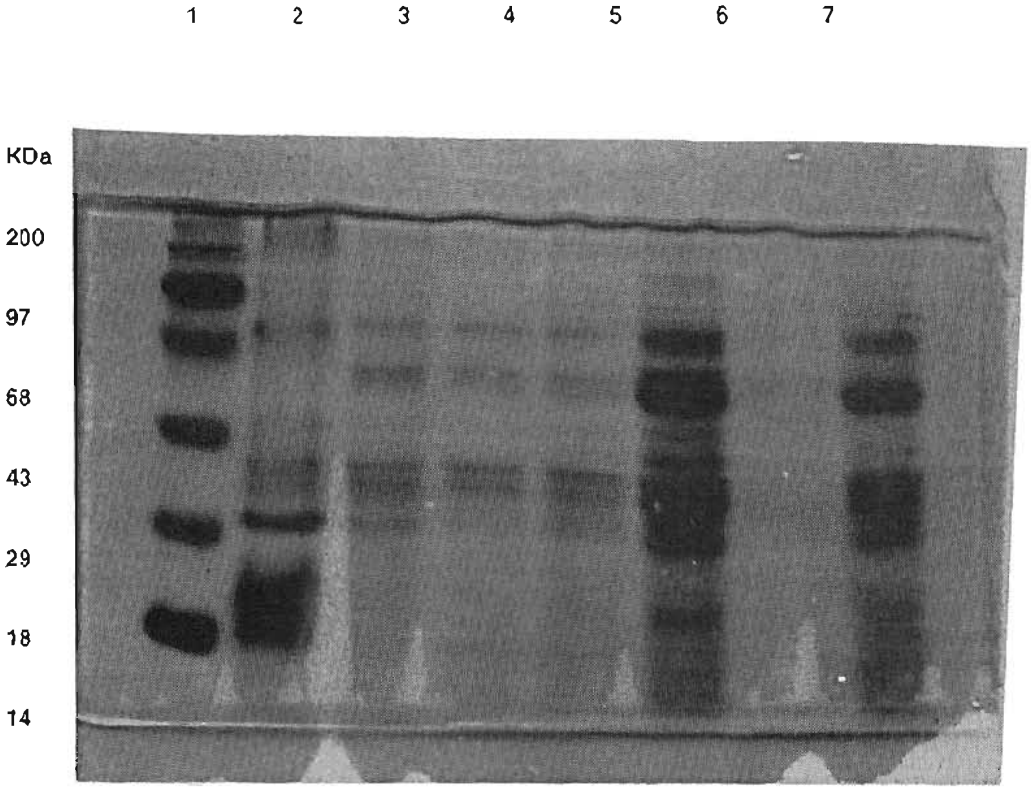
Table 3 : Protein profile analysis of toxins of different strains of C. perfringens type D separated by SDS-PAGE

M.W. (KDa)	C. Perfringens type D strains					
	England	Neuzeland	Syrian	Turkey	UK I	UK III
200-150	-	-	-	-	-	-
150-100	101.37	-	-	-	101.37	101.37
100-50	77.311	77.313	77.311	77.311	77.311	77.311
	68.878	70.669	55.873	55.873	55.873	55.873
	55.873	55.873	-	-	-	-
50-10	42.723	42.723	42.723	42.723	42.423	42.723
	39.794	39.794	39.794	39.794	39.794	39.794
	32.998	32.998	32.998	32.687	32.687	32.786
	27.692	27.692	27.692	27.692	27.692	27.692
	25.25	25.250	25.250	25.250	25.250	25.250
	21.697	21.697	21.697	21.297	21.697	21.697
	13.449	13.449	13.634	-	13.495	13.449
	13.177	-	-	-	13.177	13.177
Sum.	72.9	54.3	56.5	51.7	53.9	51.7

Table 4 : Western blot analysis of toxin filtrates of different strains of C.perfringens type D

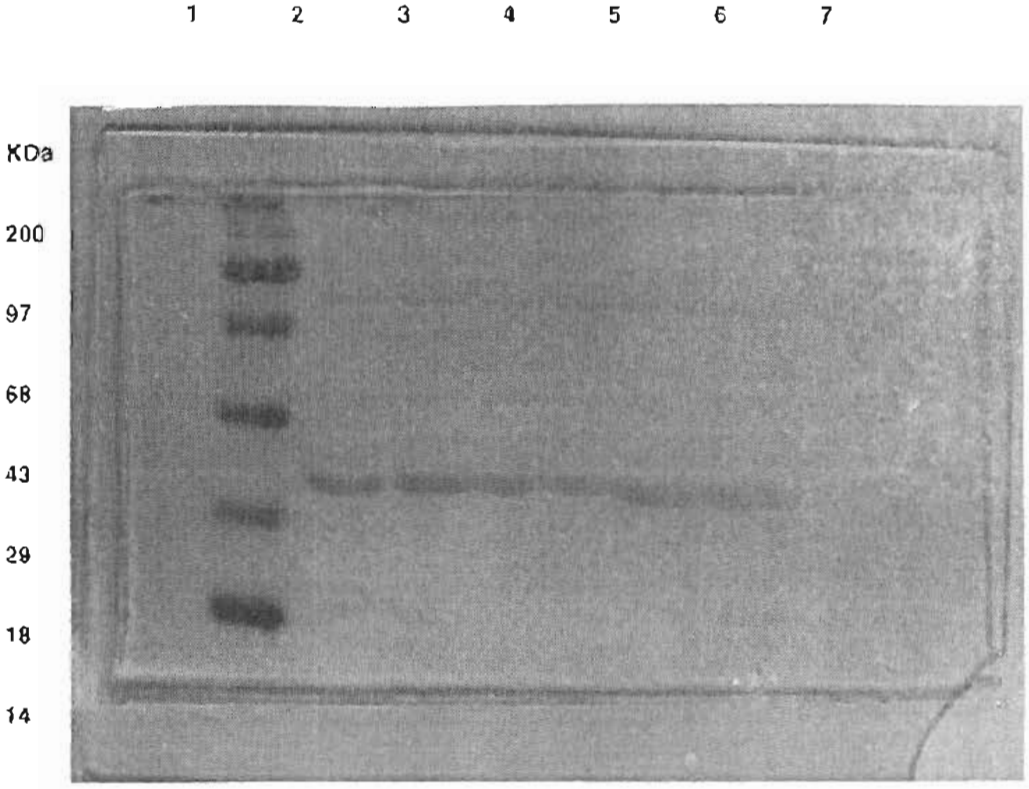
M.W. (KDa)	C. Perfringens type D strains					
	England	Neuzeland	Syrian	Turkey	UK I	UK III
300-250	-	272.24	-	263.07	263.07	281.73
250-200	221.65	214.19	245.64	214.9	-	-
	-	-	206.97	-	-	-
200-150	174.039	-	162.84	162.84	186.75	186.75
	-	-	-	-	-	162.84
100-50	95.095	90.646	89.567	96.24	84.36	96.24
	88.5	83.356	85.377	84.36	77.576	90.646
	77.576	68	77.576	77.576	68	77.576
	64.878	55.036	68	68	55.036	68
	-	-	61.175	64.878	-	55.036
	-	-	55.036	61.175	-	-
	-	-	-	55.036	-	-
50-10	49.512	46.141	46.141	41.441	41.441	41.441
	46.141	38.254	41.441	38.254	41.187	41.187
	38.254	32.598	41.187	35.75	38.254	38.254
	35.75	27.224	38.254	32.598	32.598	32.598
	32.598	15.515	32.598	27.397	27.224	29.906
	29.906	11.969	29.906	25.396	25.396	27.224
	27.224	-	25.396	18.752	21.278	25.396
	23.691	-	21.278	13.678	18.634	18.634
	21.287	-	18.752	11.969	16.771	16.771
	18.4	-	16.771	-	15.515	15.515
	16.771	-	15.515	-	13.678	13.678
	15.515	-	13.678	-	11.969	11.969
	13.678	-	11.969	-	-	-
	11.969	-	-	-	-	-

Fig. 1 : Protein profile analysis of cellular antigens of different strains of C. perfringens type D separated by SDS-PAGE.



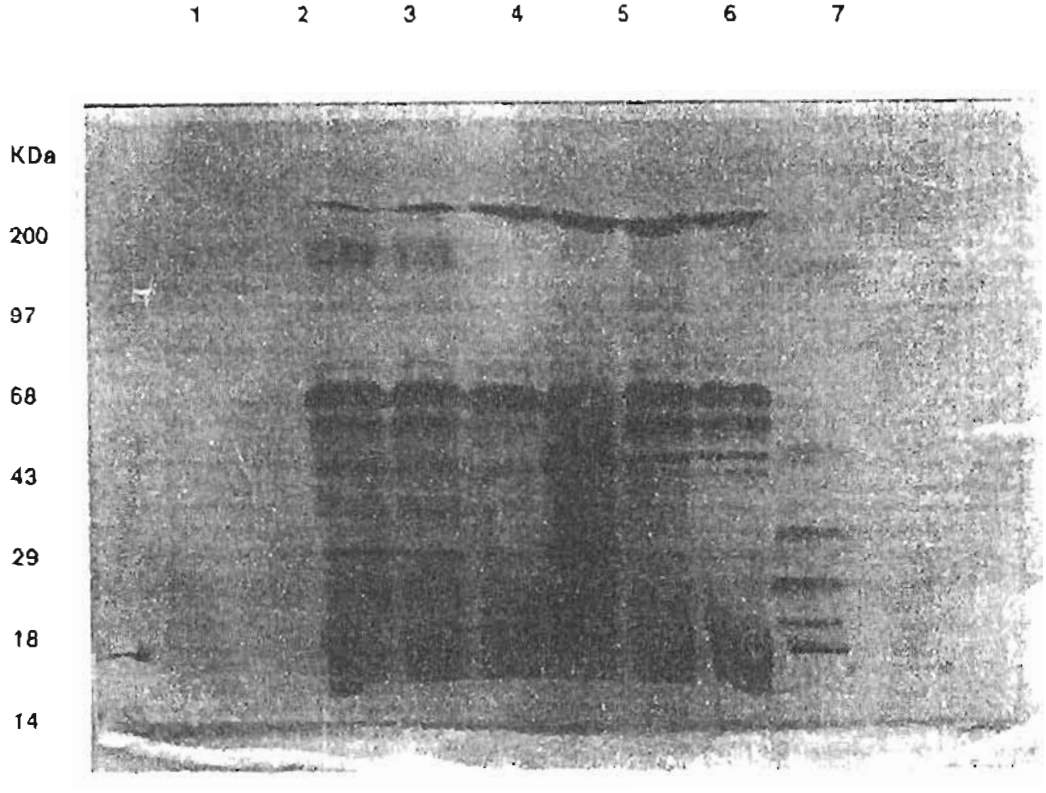
Lane (1) Molecular weight marker.
Lane (2) England strain.
Lane (3) Neuzeland strain.
Lane (4) Syrian strain.
Lane (5) Turkey stain
Lane (6) UKI
Lane (7) UK III.

Fig. 2 : Protein profile analysis of toxin filtrates of different strains of C. perfringens type D separated by SDS-PAGE.



Lane (1) Molecular weight marker
Lane (2) England strain.
Lane (3) Neuzeland strain.
Lane (4) Syrian strain.
Lane (5) Turkey stain
Lane (6) UK I
Lane (7) UK III.

Fig. 3 : Western blot analysis of toxin filtrates of different strains of C. perfringens type D



Lane (1) Molecular weight marker.
Lane (2) England strain.
Lane (3) Neuzeland strain.
Lane (4) Syrian strain.
Lane (5) Turkey stain.
Lane (6) UKI
Lane (7) UK III.

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الملخص العربى

دراسات جزيئية ومناعية لبعض عترات الكلوستريديم برفرنجنز نوع (د)

المشتركون فى البحث

فتحيه شافعى رسمى عبدالغفار دياب علاء عبدالفتاح المنيسى

ناديه مصطفى عماره سهام عبدالرشيد الزيدى

تم تحضير وحقن ستة لقاحات من اللقاح الجامع للأغنام كل منها يحتوى على توكسيد عترة من عترات ميكروب الكلوستريديم برفرنجنز نوع "د" المختلفة المصدر وتم حقن هذه اللقاحات فى الأغنام، وكانت النتائج تشير إلى عدم وجود علاقة طردية قاطعة بين قوة السمية والاستجابة المناعية، وباستخدام إختيار التحليل الكهربائى الرأسى (SDS-PAGE) والتحليل الكهربائى اللطعى (Western blott) لكل من الخلايا والسموم للعترات المختلفة تبين وجود بروتينات مشتركة يتراوح الوزن الجزيئى لها من ٥٠ إلى ١٠٠ كيلو دالتون فى كل العترات ونتيجة لتلك الدراسة وجد أنه لاتوجد علاقة محددة بين قوة السمية والاستجابة المناعية للاختيارات البيوجزيئية المستخدمة فى تلك الدراسة.