Influence of Some Various Factors for Enhancement of Epsilon Toxin Yield from *C. perfringens* Type D

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Abstract
Enterotoxaemia (pulpy kidney) is a severe condition that has a very brief clinical course in small ruminants (young sheep and goats) and results in unexpected deaths. Therefore, therapeutic interventions often do not work. It is brought on by the absorption of the *Clostridium perfringens (C. perfringens)* type D toxin from the intestinal tract. This bacterium has five toxinotypes, and type D, which produces epsilon toxin, is primarily linked to enterotoxaemia in caprine and ovine species. This study aimed to improve the efficacy of the immunization by investigating the toxigenicity of *C. perfringens* type D and the impact of each element of its production media on the potency of the epsilon toxin, three types of sugars (glucose, sucrose and Dextrin) were tested with four types of proteins (peptone, tryptone, protease peptone and N-Z casein) with different incubation periods (18, 24 and 48 hours) to improve the media to reach to maximum toxigenicity. Among all these trials, sucrose with N-Z casein with 24 hours' incubation has been ascended the throne.

Key words: *C. perfringens* type D, enterotoxaemia, MLD (Minimal Lethal Dose

Introduction

C. perfringens is a rod-shaped, spore-forming, gram-positive bacteria. Based on the generation of main toxins, these anaerobic bacteria can be categorized into five toxinotypes (A, B, C, D, and E) ***(Petit et al., 1999).*** Numerous animals, including sheep, goats, and cattle, have normal gut flora that contains *C. perfringens* type D isolates. ***(Uzal et al., 2004).*** The main predisposing factor of proliferating large amount of epsilon protoxins (which is rapidly activated by digestive enzymes such as trypsin which converts the protoxin into active toxin that cause the disease) is sudden change of diet ***(Uzal and Kelly, 1996).***

For sheep and goat the Enterotoxaemia, overeating or

pulby kidney disease induced by *C. perfringens* type D is very important economically. and it may cause sudden death at various ages ***(Miyashiro et al., 2007).***

Epsilon toxin hinders the function of venial micro-vessel endothelium; irreversibly that increases the wall permeability ***(Adamson et al., 2005).***

The toxin accumulates in brain and renal distal collecting tubes and causes bulby kidney and deaths ***(Chassin et al., 2007).***

The same lesions of sheep type D enterotoxaemia have been observed in cattle ***(Fairley, 2005).***

To optimize *C. perfringens* growth in vitro, cultivate the bacteria in a medium supplemented with 0.2% sucrose and 0.2% vitamin combination. After 8 hours of incubation, adjust the medium's pH to 7.5-8.0. This will assist in lowering the cost of production ***(Rai et al., 2013b).***

In vitro evaluation of toxin produced is an important tool for studying diagnosis, pathogenesis and vaccine production of *C. perfringens*. By analyzing the toxigenicity of *C. perfringens*; the culture media is found a very effective factor in determination of this toxigenicity ***(Miyakawa et al., 2007).***

This work has been done to study the effect of some factors as (source of proteins, sugars and different incubation periods) and interaction of each other for maximal growth and stability of epsilon toxin produced from *C. perfringens* type D which is essential in vaccine potency.

Material and Methods

Ethical approval

This study was approved by the Agricultural Research Center-Institutional Animal Care and Use Committee (ARC- IACUC). It was examined and overseen by the Ethical Committee of Veterinary Serum and Vaccine Research Institute (VSVRI) (protocol number ARC- VSVRI- 51- 24).

1. Experimental animal

Swiss mice, 18-22 g weight, were obtained from the Mice Farm, VSVRI, Abbasia, Cairo and used for in- vivo testing of Minimum Lethal Dose (MLD) of the toxin.

2. Strain

C. perfringens type D strain (8346) was supplied from National Collection of Type Culture (NCTC), London, UK.

3. Toxin production media

3.1. Primary culture media

The principal culture media utilized for the propagation of the *C. perfringens* type D strain was Cooked Meat media (Oxoid, United Kingdom).

3.2. Basal Production media

According to ***(Gadalla et al., 1971)*** this medium consists of Peptone 3 %, Sodium chloride 0.5 %, Yeast 0.5%, Beef extract 0.5%, Di-sodium hydrogen phosphate anhydrous 0.2% and Dextrin 2%. They were dissolved in distilled

water and the pH was adjusted to 7.5.

- Trypsin TPCK treated from bovine pancreas.
- pH was monitoring during growth and regulated at 7.5 which used as previously described ***(Philippe et al., 2006).*** All cultures were grown under anaerobic conditions in anaerobic jars containing Gas Packs (BD Biosciences, USA) at 37 ◦C ***(Mendez et al., 2008).***

4. Impact of some factors on production of *C. perfringens* type D Epsilon toxin

4.1. Source of proteins

Peptone 3% was replaced by Tryptone, Proteose peptone and N-Z casein with the same percentage.

4.2. Source of Carbohydrates

Dextrin 2% was replaced by Glucose and sucrose with the same percentage.

4.2. Tracing the effect of incubation time

Incubation time of 18, 24 and 48 hrs. at 37◦C had been done.

5. Toxin assays

After an hour from putting trypsin, 1 ml of filtered supernatants from several *C. perfringens* type D cultures were sampled and assayed for Minimum Lethal Dose (MLD) according to **(Fu *et al.*, 2004).**

6. Statistical analysis:

Data obtained in this study were analyzed using statistical difference by analysis of variance (ANOVA) test using Statistix software (version 9). Differences were considered significant at $P<0.05$.

Result

As illustrated in Table (1) Glucose with Proteose peptone in 24 and 48 hrs. gave the highest MLD (21000.67 and 21000, respectively) compared with those with other proteins and there was a significant difference ($p<0.05$) when using glucose with different proteins, the high yield of epsilon toxin was achieved when using glucose with proteose peptone (21000.67± 1.53MLD) after 24 hrs. incubation at 37◦C.

As illustrated in Table (2) Sucrose with N-Z casein in 24 and 48 hrs. gave the highest MLD (29000 and 26000, respectively) compared with those with other proteins and casein gave highest yield of epsilon toxin (29000 MLD) with sucrose when incubated 24 hrs. at 37◦C.

As illustrated in Table (3) Dextrin with Proteose peptone in 24 and 48 hrs. gave the highest MLD compared with those with other proteins. Also, there was a significant difference when using dextrin with different proteins, as it was found that using dextrin with proteose peptone and incubating it for 24 hrs. gave high epsilon toxin production (26000.67±1.53MLD).

The organism's toxigenicity was assessed following the cultures' 18–24, and 48-hour incubation periods at 37°C. The findings indicated a significant difference ($P< 0.05$) between results of 18 and 24 hrs. incubation times and there is no difference between results of 24 and 48 hrs. incubation times. From

these results it was found that the best incubation time of epsilon toxin production is 24 hrs. Also, through the previous results, it was found that there is significant difference ($P < 0.05$) when using different types of sugars (glucose, sucrose and dextrin) with different types of proteins (peptone, tryptone, proteose peptone and N-Z casein) interchangeably. It was found that using sucrose with N-Z casein gave the highest epsilon toxin yield production.

Table 1: *Mean value of Minimum Lethal Dose (MLD) of modified media using Tryptone, Proteose peptone and N-Z casein compared with Peptone using Glucose as source of carbohydrates in different incubation periods.*

Source of proteins	Glucose		
	Incubation period		
	18 hours	**24 hours**	**48 hours**
Peptone	19 ± 1^{Aa}	20 ± 1^{Aba}	20 ± 1^{ABa}
Tryptone	20 ± 1^{Aa}	20.33 ± 1.53^{Aba}	20.33 ± 1.53^{Aa}
Proteose peptone	20 ± 1^{Aa}	21.67 ± 1.53^{Aa}	21 ± 1^{Aa}
N-Z casein	15 ± 1^{Bb}	18 ± 1^{Ba}	18 ± 1^{Ba}

Means $\pm$ SD in the same row and carrying different superscript are significantly different at ($P < 0.05$)
(Means= x 1000)

Table 2: *Mean value of MLD of modified media using Tryptone, Proteose peptone and N-Z casein compared with Peptone using Sucrose as source of carbohydrate in different incubation periods.*

Source of proteins	Sucrose		
	Incubation period		
	18 hours	**24 hours**	**48 hours**
Peptone	20 ± 1^{Ca}	20 ± 1^{Ca}	18 ± 1^{Bb}
Tryptone	20 ± 1^{Ca}	20 ± 1^{Ca}	18 ± 1^{Bb}
Proteose peptone	26 ± 2^{Ba}	26 ± 2^{Ba}	24.33 ± 1.53^{Aa}
N-Z casein	29 ± 1^{Aa}	29 ± 1^{Aa}	26 ± 1^{Ab}

Means $\pm$ SD in the same row and carrying different superscript are significant different at ($P < 0.05$) (Means= x 1000)

Table 3: *Mean value of MLD of modified media using Tryptone, Proteose peptone and N-Z casein compared with Peptone using Dextrin as source of carbohydrate in different incubation periods.*

Source of proteins	Dextrin		
	Incubation period		
	18 hours	24 hours	48 hours
Peptone	19.67 ± 0.58^{Ba}	18.33 ± 1.53^{Ca}	17.33 ± 1.53^{Ca}
Tryptone	21 ± 1^{Ba}	22 ± 2^{Ba}	18 ± 1^{Cb}
Proteose peptone	26.6 ± 1.53^{Aa}	26.67 ± 1.53^{Aa}	25 ± 1^{Aa}
N-Z casein	25 ± 1^{Aa}	25 ± 1^{Aa}	22.33 ± 1.53^{Bb}

Means $\pm$ SD in the same row and carrying different superscript are significant different at ($P < 0.05$)

(Means= x 1000)

Discussion

One of the bacterial infections that affect small ruminants that is economically significant is enterotoxaemia, which is caused by *C. perfringens* type D. Enterotoxin (ETX), a powerful and deadly neurotoxin that damages endothelium cells and induces edema, is generated by *C. perfringens* type D ***(Adamson et al., 2005).*** The main cause of type D enterotoxaemia is this toxin. Large volumes of ETX are produced by the overgrowth of *C. perfringens* D in the intestines of susceptible animals. ETX is absorbed via the intestinal mucosa, resulting in edema and neurological dysfunction ***(Songer, 1996).***

Optimization of the in vitro growth of *C. perfringens* type D under various physical and chemical conditions was attempted in an effort to lower the cost of large-scale enterotoxaemia vaccine production and increase the antigenic mass in bacterial culture ***(Rai et al., 2013b).*** In this work, the effect of some factors as (source of proteins, sugars and different incubation periods) and interaction of each other for maximal growth and stability of epsilon toxin produced from *C. perfringens* type D was studied to improve the vaccine potency

Through the obtained results it was found that Proteose peptone and N-Z casein gave much better Minimal Lethal Dose (MLD) than peptone and tryptone and these results agreed with ***(El-Helw et al., 2017)*** who showed that using of proteose peptone as a source of amino acids in production medium of *C. perfringens* type A alpha toxin gave high yield of toxin. While the obtained results disagreed with ***(Demain et al., 2007)*** that reported that using tryptone not only

supported growth but also provided higher toxin titer.

Additionally, this data demonstrated that sucrose outperformed the other sugars evaluated and this agreed with ***(Rai et al., 2013b)*** who mentioned that Growing *C. perfringens* in a production medium enriched with sucrose will maximize its in vitro growth. the results shown in previous tables also come in agreement with the results of ***(Pivnick et al., 1964)*** who conducted three trials using sucrose at pH 7.5 and obtained milliliter-level yields of epsilon toxin ranging from 40,000 to 120,000 MLD which means that Sucrose is better than other sugars in *C. perfringens* type D toxin production, but disagreed with ***(Maaroufi et al., 2000)*** who had suggested that Dextrin improved the rate of biosynthesis of the epsilon toxin by 10 times.

The yield of toxin was less with glucose than dextrin ***(Nishida et al., 1962).***

By using different incubation periods for toxin production (18, 24 and 48hrs) it was found in this work that 24hr incubation time was the best time for yield of epsilon toxin which was better than 18 hr. and similar to 48hr. So, it is better to keep culture only 24 hr.; as leaving it for 48 hrs with the same MLD will be time consuming and this come parallel to ***(Rai et al., 2013a)*** who had reported that yield of epsilon toxin of *C. perfringens* type D was achieved after 24hrs incubation.

Conclusion

According to the obtained results, it could be concluded that the ideal production medium to be used to improve the yield of epsilon toxin of *C. perfringens* type D for vaccine manufacturing was one that had sucrose as a source of carbohydrates and N-Z casein as a source of protein. After a 24-hour incubation period, this combination reached maximal toxigenicity and increased the yield of C. perfringens type D epsilon toxin.

Authors' contribution

The study was conceived by all authors, who also carried out the experiment, calculated the lethal concentrations of *C. perfringens* type A alpha toxins, assessed the vaccinations, carried out statistical analysis, and authored the publication.

Declaration of Conflicting Interests

There are no potential conflicts of interest, according to the authors

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تاثير بعض العوامل المختلفة لتحسين انتاج سم الابسيلون من الكلوستريديم بيرفرينجيز نوع د

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الملخص

كان هدف دراستنا هو دراسة السمية الميكروبية لنوع د من بكتيريا الكلوستريديوم بيرفرنجنز (C. perfringens)وتأثير كل مكون في وسائط إنتاجها على فعالية سم الإبسيلون، وذلك لتحسين فعالية لقاح التسمم المعوي في المجترات الصغيرة الناتج عن بكتيريا C. perfringens ، وهو حالة قاتلة تتميز بمدة سريرية قصيرة و نتيجة لذلك، غالبًا ما تفشل الأساليب العلاجية. من بين خمسة أنواع سامة موجودة في هذه البكتيريا، يرتبط التسمم المعوي في الأنواع الماعز والأغنام بشكل أساسي بالنوعD ، الذي ينتج السم الإبسيلون. تم اختبار ثلاثة أنواع من السكريات (الجلوكوز، السكروز والدكستين) مع أربعة أنواع من البروتينات (الببتون، التربتون، ببتون البروتياز و N-Zكازين) مع فترات حضانة مختلفة (18، 24 و48 ساعة) لتحسين الوسط للوصول إلى أقصى درجة من السمية. وقد تبين من نتائج هذه التجارب؛ إن استخدام السكروز مع كازين N-Z بعد 24 ساعة كان له أفضل النتائج.

الكلمات الدالة: الكلوستريديوم بيرفرنجنز النوعD ، التسمم المعوي، الحد الأدنى للجرعة القاتلة.