Journal of Applied Veterinary Sciences, 7 (2): 53 – 57 (April, 2022).
ISSN: Online: 2090-3308, Print: 1687-4072
Journal homepage : https://javs.journals.ekb.eg

Factors Affecting on Production of Clostridium novyi type (B) Alpha Toxin

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ABSTRACT

Clostridium novyi type B is an anaerobic bacterium that causes black disease (infectious necrotic hepatitis). The alpha toxin is considered as a major virulence factor. The disease has been controlled using toxoid vaccines. Production of a high yield of bacterial toxin is very important in the preparation of effective toxoids. The present study aimed to follow the most satisfactory growth requirements to produce a high yield of alpha toxin. This study tested the effect of different concentrations of meat pieces (0. 0.5, 1.5, 2 and 2.5%), adding yeast extract in concentration of (0.5%), variable incubation times (24, 48, 72, 96 and 120 hours) and different pH values (7, 7.5 and 8). The results showed that the best media used for producing alpha toxin of *Clostridium novyi* type B was the one that contains yeast extract in the concentration of 0.5% with absence of meat pieces and adjusting pH between 7.5- 8 and incubation for 24- 48 hrs. that leads to high bacterial growth and toxin yield production optimum physical condition as incubated period 24- 48 hrs. and favorable pH for growth production and toxin yield of *Clostridium novyi* type B which lead consequently to produce a high potent *Clostridium novyi* vaccine.

Keywords: Alpha toxin, *Clostridium novyi type B*, incubation time, meat pieces, pH, yeast extract.

Original Article:
DOI:https://dx.doi.org/10.21608/javs.2022.123292.1129
Received :22 February, 2022.
Accepted :02 April, 2022.
Published in April, 2022.

J. Appl. Vet. Sci., 7(2): 53-57.

INTRODUCTION

Clostridium novyi (C. novyi) is a strict anaerobic, endospore-forming, gram-positive bacterium. Previously known as *Clostridium oedematiens*, causes disease in humans and animals and can be classified into three different types, namely A, B, and C, based on toxin production. Major virulence factor of *C. novyi* type B is an alpha toxin principally causing infectious necrotic hepatitis (black disease) in ruminants, pigs, and horses (**Whitfield *et al.*, 2015 ; Navarro and Uzal, 2016**). Alpha toxin has lethal, oedematizing and cytotoxic activities (**Oksche *et al.*, 1992 ; Ball *et al.*, 1993**).

The disease associated with *C. novyi* is peracute which clinical signs are rarely seen, it is fatal disease and is controlled with toxoid vaccines (**Underwood *et al.*, 2015**). In vitro toxin production evaluation is an important tool not only for diagnostic purposes but also for the study of pathogenesis of *C. novyi* and vaccine production. Inactivated alpha toxin in these vaccines is regarded as the major protective antigen of *C. novyi*. The estimation of toxicity of *C. novyi* supernatant indicates that the culture medium is an important factor in the determination of the toxigienicity of the microorganism (**Miyakawa *et al.*, 2007**). Another factor related to the strain itself when grown under favorable, controlled conditions, it may yield crude toxic filtrates with several hundred MLD/ml (**Ispolatovskaya, 1971**).

In the current study a media with different ratio of meat particles or without meat particles that subjected to different factors as pH, incubation time and addition of yeast extract was used in comparison with the current used media for production of C. novyi type B toxin.

MATERIALS AND METHODS

1. Bacterial strain:

C. novyi type B standard strain was obtained from the Medical School, University of Edinburgh. United Kingdom.

2. Media:

2.1. Primary culture media:

Cooked Meat Medium (Oxoid, United Kingdom) was used for the propagation of *C. novyi type B* strain as a primary culture medium.

2.2. Basal Production media:

Basal production media used for toxin production described before by (**Gadalla *et al.*, 1971)** which consisted of 2.5% meat pieces; 3% peptone; 0.5% Lab Lemco; 0.5% sodium chloride; 0.2% disodium hydrogen phosphate;2% glucose and 0.05% Thioglycollate. This media was adjusted at pH 7.5. The culture is incubated at 37°C for 5 days. This basal medium was used as a control medium.

3. Effect of some factors on production of *C. novyi* type B alpha toxin as shown in table (1):

Table 1: Ingredients of different media used for production of C. novyi type B alpha toxin:

Item	The currently used media as described by (Gadalla *et al.*, 1971)	Modified media under study
Meat pieces	2.5%	0, 0.5, 1.5 and 2%
Pepton	3%	3%
Lab lemco	0.5%	0.5%
Sodium chloride	0.5%	0.5%
Di sodium hydrogen phosphate	0.2%	0.2%
Glucose	2%	2%
Thioglycolate	0.05%	0.05%
pH	7.5	7, 7.5 and 8
Incubation time	120 hrs (5days)	24, 48, 72, 96 and 120hrs
Incubation temperature	37°C	37°C

3.1. Effect of different meat pieces concentrations:

Meat pieces were used in concentrations of (0, 0.5; 1.5; 2, and 2.5%) to the basal production media on production of alpha toxin of *C. novyi* type B.

3.2. Effect of adding yeast extract:

Adding yeast extract (Oxoid, United Kingdom) in a concentration of 0.5% to the basal production media on production of alpha toxin of *C. novyi* type B.

3.3. Effect of different pH values:

Through adjusting the pH at 7, 7.5 and 8 of the basal production media on production of alpha toxin of *C. novyi* type B.

3.4. Tracing the effect of incubation time:

at 37°C for 24, 48, 72, 96 and 120 hrs.

4. Toxin assay:

For the determination of lethal values, white Swiss mice, weighing about 20 g, were used. Different dilutions of C. novyi type B alpha toxins were inoculated 0.1 ml intravenous. Two mice for each dilution were inoculated and the highest dilution of sample causing death of mice within 24 hours was designated as the minimum lethal dose (MLD).

5. Statistical analysis:

All parameters are presented were analyzed for the statistical difference by analysis of variance (ANOVA). Differences were considered significant at $p<0.05$.

RESULTS

According to Table (2) there was a significant difference ($p<0.05$) between different concentration of meat pieces, the maximum yield of alpha toxin was achieved at 0% meat pieces (900 MLD (Minimum Lethal Dose)) after one day incubation, at 0 and 0.5% (800 MLD) after second day incubation, at 0% (800 MLD) after third day incubation, at 0% (800 MLD) at fourth day incubation and at 0 and 0.5 % (600 MLD) after fifth day incubation. The highest MLD (900) was achieved at 0% meat concentration after one day incubation time while the lowest MLD (500) was at 2.5% meat concentration after 5 days incubation at pH 7.5 and 37°C.

Table 2: Effect of different concentrations of meat pieces in basal toxin production medium on Alpha toxin production from *C. novyi* type B expressed as Minimum Lethal Dose (MLD) .

Meat concentration	Alpha toxin production from *C. novyi* type B expressed as Minimum Lethal Dose (MLD)				
	1st day	2nd day	3rd day	4th day	5th day
2.5%	600	600	500	500	500
2%	700	600	600	500	500
1.5%	700	700	600	600	500
0.5%	800	800	700	700	600
0%	900	800	800	700	600

Table (3) illustrated that when adding 0.5% yeast extract on basal production media, the maximum yield of alpha toxin was achieved after 1st and 2nd day of incubation than that prepared without yeast extract. There is an inversely proportional relationship between incubation time and alpha toxin production as upon the addition of yeast extract in which the MLD was 700 after one day incubation and reached to 600 after 5day incubation. Toxin production yield from media without yeast extract was 600 MLD after 1 day incubation and reached to 400 after 5 days incubation. So, the addition of 0.5% yeast extract with short incubation time has a great effect on alpha toxin production.

Table 3: Effect of adding yeast extract in basal toxin production medium on Alpha toxin production from *C. novyi* type B expressed as Minimum Lethal Dose (MLD).

Effect of yeast extract	Incubation time at 37°C				
	1st day	2nd day	3rd day	4th day	5th day
Basal production media without yeast extract	600	600	500	500	400
Basal production media with yeast extract	700	700	600	600	600

When using 0.5% yeast extract with different concentrations of meat pieces at pH 7.5 and 37°C we found that the highest yield of alpha toxin (1000MLD) was achieved after one day incubation by using 0.5% yeast extract with 0 or 0.5% of meat pieces. Highest MLD was achieved at 0% meat concentration with 0.5% yeast extract along 5 days incubation. The lowest toxin production (500MLD) was achieved after 5 days incubation by using 2.5% meat pieces with 0.5% yeast extract as showed in T able (4).

Table 4: Effect of interaction of different concentration of meat particles with 0.5% yeast extract on alpha toxin production expressed as MLD (Minimum Lethal Dose).

Meat concentration	Yeast extract	Incubation time at 37°C				
		1st day	2nd day	3rd day	4th day	5th day
2.5%	0.5% yeast extract	700	700	600	600	500
2%		700	700	600	600	600
1.5%		800	800	700	700	600
0.5%		1000	900	800	700	700
0%		1000	1000	900	900	900

Table (5) showed that final basal modified media (by using 0% meat concentration with 0.5% yeast extract) achieved the highest alpha toxin production when pH adjusted between 7.5 and 8 after 1 and 2 days incubation time while at pH 7 yield of toxin production was decreased.

Table 5: Effect of different pH value on production of alpha toxin expressed as MLD (Minimum Lethal Dose):

pH values	Incubation time at 37°C				
	1st day	2nd day	3rd day	4th day	5th day
7	800	700	700	600	600
7.5	1000	1000	900	900	900
8	1000	1000	900	800	800

Effect of incubation time:

The toxigenicity of the organism was measured after the modified cultures had been incubated at 37° C for 24, 48, 72, 96 and 120 hr. The results showed that the MLD of alpha toxin was at its highest level after 24- 48 hrs. Incubations as showed in tables no. (2 3, 4 and 5).

After studying the effect of the previous factors, the results showed that removing of meat pieces from the basal media with the addition of yeast extract in concentration of 0.5%, incubating them at a temperature of 37° C, and adjusting the pH between 7.5 and 8 led to the production of the highest MLD of alpha toxin after 24- 48 hours of incubation as showed in tables no. (2 3, 4 and 5).

DISCUSSION

Clostridium novyi type B alpha toxin is a major virulence factor that causes necrotic hepatitis, known as black disease. Black disease is an acute toxemic disease especially in sheep and cattle (**Navarro and Uzal 2016**). Black disease symptoms include hemoglobinuria, reduced appetite, fever, lethargy, decreased milk production and blood-stained feces, all of which reduce productivity in sheep, horses, pigs and cattle. The main method for combating diseases caused by *C. novyi* is vaccination. These vaccines normally contain toxoids (or toxoids and bacterins) in their composition. The Production of high yield of bacterial toxin is very important in the preparation of effective toxoid.

The present study targeted to follow the most satisfactory growth requirements to produce high yield of alpha toxin. This includes effect of different concentration of meat pieces, adding yeast extract, different pH value and different incubation time. In the light of the findings obtained in our study we assume

that, the fewer pieces of meat in the media, the higher the production of toxin. There is an inversely proportional relationship between meat concentration, growth rate and toxin production. It was also noted that when the pieces of meat were completely removed, the production of toxin increased. These results may be due to the release of lipid fractions from meat pieces autoclaved at alkaline pH which leads to inhibition of Clostridial toxin production as explained by (**Nishida *et al.*, 1962**).

Also, (**Aquino *et al.*, 2016**) discovered that in media containing cooked meat, growth of *C. novyi* is slow, so, delayed production of alpha toxins. Also, results of (**Gadalla *et al.*, 1974**) come in agreement with our findings as he noticed that the alpha toxin of *C. novyi* type B became more toxic when using toxin production media without meat pieces compared to the media prepared previously by (**Gadalla *et al.*, 1971**), which contains pieces of meat. Also agrees with (**Ardehali *et al.*, 1992**) who found that meat pieces cause some difficulties in mass production so he tested several media with different ingredients for production of *C. oedematiens* vaccine. The medium without meat pieces was selected and used for a large-scale production.

Bacterial growth with direct proportional to the presence of yeast extract in the media. The results showed that adding yeast extract at a ratio of 0.5% in toxin production media has an influence on both growth and toxin production. The increase in the yield of alpha toxin (up to 1000 MLD) with yeast extract indicated that was not only due to an increase in growth but also, due to the direct effect on toxin production as a result of the presence of fermentable organic compounds in the yeast extract such as glucose and- or amino acids. Also yeast extract provides nitrogenous compounds, carbon, sulfur, trace nutrients, vitamin B complex and other essential growth factors, which are essential for bacterial growth (**Marion Leclerc *et al.*, 1998; Tofalo and Suzzi 2016**). Yeast extract is usually used as growth stimulants or growth factors for bacteria. (**Bard and McClung, 1948**) used a media containing yeast extract to study the biochemical properties of the toxin of *C. novyi*.

The effect of varying pH values on the growth rate and toxin yield was studied. The result revealed that the favorable pH for bacterial growth and alpha toxin production was 7.5- 8. This result was agreed with (**Ardehali *et al.*, 1992**) who obtained high yield of alpha toxin production when kept pH of medium at 8 and (**Gadalla *et al.*, 1974**) who found that the optimum pH for toxin production of *C. novyi* type B was 7.5. Also (**Nishida and Nakagawara, 1964**) stated that the pH of media should be adjusted to 8.

The incubation period of microbe is one of the most essential factors for bacterial growth as well as toxin production, so the effect of the incubation period on *C. novyi* and toxin production was studied, and the results showed that the favorable incubation time was 24- 48 hours. This result is agreed with (**Mohsen Fathi Najafi *et al.*, 2019**) who found that decreasing the bacterial growth conditions to 24 hrs. has a great effect on alpha toxin production of cultivation of *C. novyi* for vaccine production. Also, when (**Nishida and Nakagawara 1964**) found that the longer the duration of the sample incubation, the less toxigenic for the *C. novyi* isolation from soil.

CONCLUSION

We concluded that removal of meat pieces with the addition of 0.5% yeast extract, adjusting the pH 7.5- 8 with short incubation time (24- 48hrs.) has a great effect on the alpha toxin production yield of *C. novyi* type B which consequently lead to production of highly potent *C. novyi* vaccine.

Authors' contribution

All authors designed the overall study, performed the experiment, determined of lethal values of *C. novyi* type B alpha toxins, performed statistical analysis and wrote the paper.

Declaration of Conflicting Interests:

The authors revealed that there is no potential conflicts of interest.

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How to cite this article:
Marwa M Ahmed; Marwa Yehia and Hala ElSawy Ahmed, 2022. Factors Affecting on Production of Clostridium novyi type (B) Alpha Toxin. Journal of Applied Veterinary Sciences, 7 (2): 53– 57.
DOI:https://dx.doi.org/10.21608/javs.2022.123292.1129