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ISOLATION OF TOXIGENIC STRAINS OF *CLOSTRIDIUM NOVYI* FROM SOIL

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ABSTRACT

NISHIDA, S. (Kanazawa University, Kanazawa, Japan), AND G. NAKAGAWARA. Isolation of toxigenic strains of *Clostridium novyi* from soil. J. Bacteriol. **88**:1636-1640. 1964.—The cultural conditions for toxin production by *Clostridium novyi* were investigated, and the optimal constituents of a proper medium were determined. A comparison of the value of this medium with other media in regard to toxin production by wild strains of *C. novyi* type A revealed that in this medium the organism could produce more consistent yields of potent toxin than in the other media. Isolation of *C. novyi* was attempted from 62 soil samples, and all were found to contain this organism. Toxigenicities of strains isolated under various conditions were examined in the above-mentioned medium, with the following results. (i) The longer the duration of the sample incubation, the less toxigenic were the strains isolated. (ii) When higher temperatures were employed to preheat the sample, fewer toxigenic strains were obtained.

Preheating of specimens is one of the requisites for efficient isolation of clostridia. Recently, Yamagishi, Ishida, and Nishida (1964) and Nishida, Tamai, and Yamagishi (1964) demonstrated with species of clostridia other than *Clostridium novyi* that the toxigenicity of isolated strains is significantly influenced by the preheating conditions employed.

To date, researchers engaged in isolating strains of *C. novyi* have not reported a similar phenomenon for this organism. Zeissler and Rassfeld-Sternberg (1930) and Sasaki (1932) isolated a number of *C. novyi* strains from soil to an extent of more than 60%, but they gave no clue to the relationship between toxigenicity and sporulating ability. However, in the light of recent findings in our laboratory, we have attempted to reinvestigate this problem with strains of *C. novyi* isolated according to the method of Zeissler and Rassfeld-Sternberg (1930).

We found that the usual media described for toxin production by *C. novyi* were not applicable to strains isolated from soil. We were, therefore, faced with the problem of preparing a proper medium to attain our objective.

MATERIALS AND METHODS

Samples. Soil samples were collected from various places, such as vegetable gardens, ricefields, and the playgrounds near our Medical School.

Isolation procedure. Each soil specimen was divided into two pea-sized portions, and each portion was suspended in 30 ml of liver broth contained in a 50-ml screw-capped bottle. Immediately, one suspension was heated at 70 C for 10 min; the other was heated at 100 C for 10 min. The heated suspensions were then incubated at 37 C. Isolation of *C. novyi* from the two bottles was attempted on the 3rd, 7th, 14th, and 21st days of incubation. On the given day of isolation, 0.5 ml of each cultural suspension was transferred into fresh liver broth in a test tube (15 by 150 mm), and the suspensions in liver broth were immediately heated to 70 or 100 C for 10 min; a series of tube subcultures obtained from the cultures of specimens heated at 70 C were heated again at the same temperature for 10 min; another series of tube subcultures obtained from the cultures of specimens heated at 100 C were also heated again at that temperature for 10 min. These heated suspensions were incubated for 48 hr, and then a loopful of the culture was plated on Nagler's modified V.F. blood-agar plate (Nagler, 1944). Suspected colonies were fished from the agar plate and committed for further serial subcultures to avoid contamination, which occurs frequently during isolation of clostridia. The isolated strains were stored in liver broth.

Identification and type determination of C. novyi. The biological nature of the isolated strains was examined according to the description of Sterne and van Heyningen (1958). However, we did

not exclude the strains showing slight indole formation, because Sasaki (1932) stated that some of the strains of *C. novyi* he had isolated produced a small amount of indole. The final identification and typing of strains was based on the method of Oakley, Warrack, and Clarke (1947).

The medium for toxin production will be described in Results.

Estimation of toxicity. A 0.5-ml amount of the culture filtrate was intravenously injected into two mice, and the minimal lethal dose (MLD) was read after 3 days of observation. The tenfold dilution method was usually adopted for estimation of toxicity of a cultural filtrate. However, when a more exact value was required, the twofold dilutions within the adjacent tenfold dilutions were employed.

RESULTS

Reinvestigation of the medium for toxin production by C. novyi. Among the media tested, such as Walbum and Reyman's (1937) medium, Wildführ's (1950) medium, and liver broth, potent toxin production could not be obtained with *C. novyi* type A strain 140, a potent toxin producer. Walbum and Reyman (1937) recommended complete removal of fermentable substances from the medium and adjustment of the pH to 8.0. Proteose peptone medium containing 2, 4, 6, or 10% peptone, and the digest broth of Pope and Smith (1932), were fermented with *Escherichia coli* as described by Walbum and Reyman (1937). These media were inoculated with *C. novyi*, and the culture filtrates were examined for toxicity on the first, second, third, and fourth days of incubation. The results showed that only undiluted solutions of the culture filtrates could kill mice. Variation of incubation temperatures, such as 34 to 35 C, 31 to 32 C, and 25 to 26 C, could not improve the toxin yield of the organism in these media.

The digest broth of Pope and Smith (1932), containing 3% peptone (Poli peptone, Daigo Co., Osaka, Japan) and a piece of liver as described by Wildführ (1950), was also examined. The toxicity of the 1- to 5-day culture filtrates ranged between 0 and 10 MLD/ml.

During the course of our experiments, we noticed that the strain could produce toxin of 10^2 to 10^3 MLD/ml in 5 to 10% chopped meat broth, provided that glucose was added to the medium. Further experiments were undertaken to analyze the cultural conditions favorable for toxin production.

Effect of phosphate. Phosphate ($Na_2HPO_4 \cdot 12H_2O$) was added in concentrations of 0, 0.1, 0.2, 0.4, and 0.8% to a 1% peptone broth containing 1% glucose and 5% chopped meat particles. The toxicity obtained was 400, 800, 1,600, 1,400, and 2,000 MLD/ml in respect to the phosphate concentrations mentioned above. Since the findings revealed the effectiveness of phosphate in toxin production by *C. novyi*, a 0.5% concentration was used in all media for the following experiments.

Effect of meat particles. To a 1% Poli peptone broth containing 0.5% glucose and 0.5% phosphate, 5, 10, and 20% concentrations of meat particles were added. The result showed that higher toxin yields were always obtained in the presence of meat particles than in their absence. However, still higher toxin yields could not be obtained by increasing the concentration of meat particles to more than 5%. The presence of 5% meat particles in the medium seemed to be significant in securing the growth.

Effect of peptone concentration. Effect of peptone was examined at the concentrations of 1 to 5% in the 5% chopped meat broth containing 1% glucose and 0.5% phosphate. Toxin yields obtained were 1,600, 4,000, 20,000, 20,000, and 8,000 MLD/ml in the order of peptone concentrations mentioned above.

Effect of energy source. Effects of glucose, maltose, or fructose on toxin yields were examined. The sugars were added at a concentration of 0.5% to the 5% chopped meat broth containing 0.5% phosphate and 3% peptone. The toxin yields obtained were 8,000, 20,000, and 160 MLD/ml in the order of sugars mentioned above. If the sugar concentration was raised to 1%, the toxin yields were 16,000, 40,000, and 200 MLD/ml respectively.

Effect of incubation temperatures. The optimal medium was prepared according to the results obtained above. It contained 5% chopped meat particles, 3% peptone, 0.5% phosphate, and 1% maltose in meat infusion broth; the pH was adjusted to 8.0. The pH of the medium, when autoclaved, fell to 7.2 to 7.4. The toxigenicity of the organism was measured after the cultures had been incubated at 37 C for 24, 48, 72, and 96 hr. Results showed that, at this incubation

TABLE 1. *Ability of type A strains of Clostridium novyi to produce lethal toxin on various media*

Medium examined	Strains used			
	No. 140 (stock strain)	37101	37105	37110
Author's medium	10^{5}*	10^{2}	10^{2}	10^{0}
Liver broth	10^{1}	10^{1}	10^{1}	10^{1}
Walbum and Reyman's medium	10^{0}	10^{0}	10^{0}	10^{0}
Wildführ's medium	10^{1}	10^{1}	10^{0}	10^{0}

* Numbers represent the LD_{100} per milliliter for two mice.

TABLE 2. *Effect of length of incubation of specimen on toxigenicity of isolated strains**

Incubation time	Strains isolated†	Toxigenicity (MLD/ml)‡	Type determined‡
days			
3	11/42	10^{3} (11)	A (11)
7	11/31	10^{2} (11)	A (11)
14	17/20	10^{1} (13)	A (14)
		10^{0} (1)	
		0 (3)	C (3)
21	3/3	10^{0} (1)	A (1)
		0 (2)	C (2)

* Samples were heated at 70 C for 10 min before incubation.

† Number of strains isolated per number of soil specimens examined.

‡ Numbers in parentheses indicate the number of strains to which the toxigenicity level or type applies.

temperature, the toxicities of the filtrates were 10^{4}, 10^{5}, 10^{5}, and 10^{4} MLD/ml, respectively, after 24, 48, 72, and 96 hr of incubation. However, when the incubation temperature was 30 C, the toxicity was 10^{5} MLD/ml in all cultural filtrates harvested after the above periods of incubation.

Effect of lipid fraction. Nishida, Murakami, and Yamagishi (1962) stated that the lipid fraction released from meat particles autoclaved at alkaline pH inhibited toxin production of *C. perfringens*. It was, however, demonstrated in this study that the toxin production of *C. novyi* was not influenced by the lipid fraction. This suggests that consistent yields of potent toxin can be obtained in the medium we have described and that it is useful for routine toxin production by *C. novyi*.

Production of lethal toxin by wild-type A strain of C. novyi in the medium devised. Our medium appeared particularly suited to toxin production by *C. novyi* type A strain 140 (Table 1). However, we also found that the wild-type A strains of *C. novyi* can usually produce more potent lethal toxin in our medium than in the others.

Toxigenicity of strains isolated at different periods of sample cultivation. Isolation was carried out on 42 samples of soil. Each of the 42 samples was divided into two parts; one was heated at 70 C for 10 min and the other was heated at 100 C for 10 min, as described above.

Toxigenicity of strains isolated from soil specimens heated at 70 C for 10 min. Isolation was attempted from 42 samples of soil incubated for 3, 7, 14, and 21 days (Table 2). When the cultures were positive, subcultures were made on modified V. F. blood-agar. Colonies suspected to be *C. novyi* were picked and identified by the methods described. Among the strains isolated from a sample on each given period of incubation, only one strain was subjected to toxigenic analysis.

C. novyi was isolated from 11 of 42 samples of soil after 3 days of incubation. All 11 strains had toxigenicity of 10^{3} MLD/ml, and they all belonged to type A according to the method of classification of Oakley et al. (1947). Another 11 samples of the remaining 31 samples of soil yielded positive cultures of *C. novyi* after 7 days of incubation. The toxigenicity of these 11 strains was 10^{2} MLD/ml, and none of them possessed the toxigenicity of 10^{3} MLD/ml. They were also classified as type A. From the remaining 20 samples which had not been positive up to the seventh day, 17 strains of *C. novyi* were isolated when the samples were further incubated until the fourteenth day. Of these 17 strains, 14 were proven to be toxigenic and were classified as type A. Their toxigenicity ranged from 10^{0} to 10^{1} MLD/ml, whereas the remaining three strains were nontoxic and were consequently classified as type C. The last three samples which had not so far been positive were examined after the 21st day of incubation, and *C. novyi* was obtained from all of them. Of these three strains, two were nontoxic and were classified as type C;

the other possessed toxigenicity of 10^0 MLD/ml and was classified as type A.

Toxigenicity of strains isolated from soil specimens heated at 100 C for 10 min. Of 42 samples incubated for 3 days, only 5 were positive for *C. novyi* (Table 3). All the five strains obtained from these samples were toxic and were classified as type A. Their toxigenicities ranged from 10^1 to 10^2 MLD/ml. However, none of them exhibited toxigenicity of 10^3 MLD/ml. Further isolation after a 7-day incubation period revealed that, of the remaining 37 samples, 5 were positive for *C. novyi*. The five strains obtained were proven to possess toxigenicity ranging from 10^0 to 10^2 MLD/ml; one strain possessed a toxigenicity of 10^2 MLD/ml, three strains 10^1 MLD/ml, and one strain 10^0 MLD/ml. Further isolation was not carried out on cultures incubated for 14 and 21 days in this series. However, in another series, *C. novyi* was isolated on the 14th and 21st days of incubation when another 20 soil samples which had been heated to 100 C for 10 min were examined. All 20 samples were found to contain *C. novyi* whether they were examined on the 14th or 21st day of incubation. The toxigenicity of the 20 strains isolated on the 14th day varied from zero to 10^2 MLD/ml: 17 strains had a toxigenicity between 10^0 and 10^2 MLD/ml and were classified as type A, and three were found to be nontoxic and were thus classified as type C. In contrast, the toxigenicity of the 20 strains isolated on the 21st day of incubation was weak, varying from zero to 10^1 MLD/ml. Of these 20 strains, 11 were proven to be nontoxic, and the remaining 9 strains were slightly toxic (10^1 MLD/ml) and were classified as type A.

These findings not only confirm the above conclusion that the longer the incubation period, the less toxigenic were the strains isolated, but also suggest that when higher heating temperatures were applied to the soil samples fewer toxigenic strains of *C. novyi* could be isolated.

TABLE 3. *Effect of length of incubation of specimen on toxigenicity of isolated strains**

Expt no.	Incubation time	Strains isolated†	Toxigenicity (MLD/ml)‡	Type determined‡
	days			
I	3	5/42	10^2 (4)	A (5)
			10^1 (1)	
	7	5/37	10^2 (1)	A (5)
			10^1 (3)	
			10^0 (1)	
II	14	20/20	10^2 (6)	A (17)
			10^1 (7)	
			10^0 (4)	
			0 (3)	C (3)
	21	20/20	10^1 (9)	A (9)
			0 (11)	C (11)

* Samples were heated at 100 C for 10 min before incubation.

† Number of strains isolated per number of soil specimens examined.

‡ Numbers in parentheses indicate the number of strains to which the toxigencity level or type applies.

DISCUSSION

The *Escherichia coli* fermented medium of Walbum and Reyman (1937) is the only medium for toxin production of *C. novyi* cited in Smith's book. Oakley et al. (1947) stated that, before 1943, they also used prefermented infusion broth for toxin production by *C. novyi*. However, we found that the presence of sugar was indispensable for potent toxin production of our strains obtained from soil, most of which belonged to type A group. Although Walbum and Reyman (1937) did not refer to the type of the strain they used, the contradiction as mentioned above may be due to the difference in types of the strains employed.

In the light of the findings obtained in our laboratory concerning the relationship between toxigenicity and sporulating potency (Yamagishi et al., 1964; Nishida et al., 1964), we assume that, when longer incubation periods precede isolation, the sporulating potency of the isolated strains becomes stronger, thus influencing toxigenicity. It seems inexplicable, however, that the level of toxigenicity was always similar among strains obtained during each isolation and particularly that less toxigenic strains, possibly possessing a stronger sporulating ability, could not be found during the earlier periods of isolation. Future investigations will be required on this aspect.

It is generally accepted that a number of nontoxigenic as well as toxigenic strains of *C. novyi* exist in nature (Zeissler and Rassfeld-Sternberg, 1930; Sasaki, 1932). There is a possibility that the nontoxigenic strain, now classified as type C,

may be the type A strain which has a stronger sporulating potency. Investigation of this problem will be described in a forthcoming paper.

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LITERATURE CITED

NAGLER, F. P. O. 1944. Bacteriological diagnosis of gas gangrene due to *C. oedematiens*. Nature **153**:496.

NISHIDA, S., M. MURAKAMI, AND T. YAMAGISHI. 1962. Chopped meat broth as a medium for the preparation of toxins by clostridia. I. Production of toxins by *Cl. welchii*. Japan. J. Microbiol. **6**:33–40.

NISHIDA, S., K. TAMAI, AND T. YAMAGISHI. 1964. Taxonomic study of *Clostridium bifermentans* and *Clostridium sordellii*. I. Their toxigenicity, urease activity, and sporulating potency. J. Bacteriol. **88**:1641–1646.

OAKLEY, C. L., G. H. WARRACK, AND P. H. CLARKE. 1947. The toxins of *Cl. oedematiens* (*Cl. novyi*). J. Gen. Microbiol. **1**:91–107.

POPE, C. G., AND M. L. SMITH. 1932. The routine preparation of diphtheria toxin of high value. J. Pathol. Bacteriol. **35**:573–588.

SASAKI, S. 1932. Studies on clostridia. IV. Clostridia in soil. J. Juzenkai [in Japanese] **37**:3016–3218.

STERNE, M., AND W. E. VAN HEYNINGEN. 1958. The clostridia, p. 343–364. *In* R. Dubos [ed.], Bacterial and mycotic infections of man, 3rd ed. The J. B. Lippincott Co., Philadelphia.

WALBUM, L. E., AND G. C. REYMAN. 1937. The production of toxin by *Clostridium oedematiens* (*Cl. novyi*). J. Pathol. Bacteriol. **44**:379–387.

WILDFÜHR, G. 1950. Über die Bedeutung der Vorkulturen für die Toxinbildung der Gasoedemerreger in künstlichen Nahrsubstraten. Z. Hyg. Infektionskrankh. **130**:528–535.

YAMAGISHI, T., S. ISHIDA, AND S. NISHIDA. 1964. Isolation of toxigenic strains of *Clostridium perfringens* from soil. J. Bacteriol. **88**:646–652.

ZEISSLER, J., AND L. RASSFELD-STERNBERG. 1930. Anaerobenzüchtung, p. 35. *In* W. Kolle, R. Kraus, and P. Uhlenhuth [ed.], Handbuch der pathogenen Mikroorganismen, vol. 10, 3rd ed. Gustav Fisher, Jena.