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EFFECT OF COLLOCALIA MUCOID MEDIA ON TOXICITY AND NEURAMINIDASE ACTIVITY OF VIBRIO CHOLERAE*

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ABSTRACT

A study on the effect of **Collocalia** mucoid mucin media on **Vibrio cholerae** culture was carried out. The result showed that the organism grew well in this plain mucin media and mucin seemed to induce the enzyme neuraminidase production and increase the culture filtrate toxicity observed in rabbit skin test. It was suggested to use **Collocalia** mucoid or other mucins as **Vibrio cholerae** culture media for studying its virulence and pathogenicity.

INTRODUCTION

It has been reported that cholera diarrhea is caused by an exotoxin elaborated by cholera vibrios in the small intestine (5, 7, 9, 23). Cholera toxin has been isolated and characterized (8, 10). It is mixture of protein and enzymes (2, 6, 10, 15). An important enzyme, which is abundantly present in the **V. cholerae** enterotoxin is neuraminidase or recepter destroying enzyme (1, 11). Neuraminidase catalytically cleaves sialic acid or N-acetylneuraminic acid from various glycoproteins (12), neuraminyl lactose (13) and cell membrances (14, 27, 28). The surface of the intestinal mucosa is composed mainly of sialic acid-containing glycoproteins (18).

Vibrio cholerae can differently grow in various media such as alkali peptone water (22), desoxy cholate citrate medium (26), trypticase tellurite taurocholate peptone (20). Several media has been employed

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for the purpose to get a higher percentage of positive culture results (21). In this study, the suitable media for enhancement of the pathogenicity was tested, according to the fact that subcultures of this pathogenic organism **in vitro** causes a decrease or loss of their virulense and toxicity. Virulence may be restored by, **in vivo**, passage through mice. Using **in vitro** subcultures to retain the virulence of **V. cholerae** has not been reported. Mucin has been used to increase virulence of some organisms in animal passage by mixed with the organisms before injection (24). This similar effect should be studied in cholera.

One of the common mucin in Thailand is **Collocalia** mucoid. **Collocalia** mucoid is a mucin secreted from salivary glands of swiflet, living along the Pacific coast of Indian Ocean. It acts as a cementing substance in the bird's nest. It is commercially available, since Chinese and Asian people have been eating this bird's nest as a delicacy for their good health. Chemically, **Collocalia** mucoid from the edible bird nest is composed mainly of glycoprotein (16).

In this report, we have investigated the effect of using **Collocalia** mucoid as a culture media of **V. cholerae** on the toxicity and the neuraminidase activity of the organism.

MATERIALS & METHODS

Collocalia mucoid : This was prepared from edible bird nest bought from a local market. The mucin was extraced from the powdered bird nest with warm water as described by Howe et al. (17).

Vibrio cholerae : The strain El Tor Inaba Chachoengsao which gives the lowest neuraminidase activity in all six stains (kindly recieved from SEATO Laboratories, Bangkok) was used for this study. The freeze-dried organism was suspended in 1% alkali peptone broth pH 8.4, incubated at 37° C for six hours, then streaked on blood agar plate to obtained isolated colony of **V. cholerae**.

Tryptone broth : Bacto tryptone was purchased from Difco Laboratories, Detroit 1, Michigan, U.SA.

Three kinds of Media were prepared :

1. **Tryptone Broth**. 2% Bacto tryptone in saline (0.5% NaCl) was adjusted to pH 7.5 with 4 M NaOH. The organism from isolated colony on blood agar plate was suspended in distilled water to reach Mac Farland No. 2.0 turbidity. The 0.05 ml. of cell suspension was inoculated into 20 ml. tryptone broth, incubated at 30° C for 18 hours. The culture was centrifuged to remove the cells, and the supernatant fluid was filtered through the millipore filter membrane by using filtering centrifuge tube, This filtrate was streaked on

blood agar plate in order to test the sterility. The culture filtrate, as control in this study, was stored at 4° C for determining the neuraminidase and toxicity.

.2. **Mucin in Tryptone** broth. This media was made by adding 0.2 gm. of **Collocalia** mucoid in 100 ml. tryptone broth prepared as above. The same strain of organism was subcultured into 5 ml. of this media for 10 times, at 24-hour intervals. The treated organism was streaked on blood agar plate to get pure isolated colony. The same turbidity and volume of cell suspension was used for culturing in tryptone broth to obtain culture filtrate as above. The culture was brought for determination of neuraminidase activity.

3. **Mucin Media** No tryptone was used in this media. The media contained 2.7 % **Collocalia** mucoid in 0.5 % NaCl. The pH of 7.5 was also adjusted. The same strain of **V. cholerae** was treated in 5 ml. of this media with seven subcultures at 24-hour intervals. Finally, the treated organism was cultured in tryptone broth after isolating on blood agar plate, to obtain a culture filtrate as described before. The culture filtrate was taken for determination of neuraminidase activity and toxicity.

Detection of Toxicity :

By using skin test of rabbit, the toxicity of the culture filtrate was indicated by the edema or swelling of the animal skin. An aliquot of 0.2 ml. of the culture filtrate was injected intradermally into rabbit back skin after hair removal. A 5-minute heated culture filtrate was also run as a control. Any visual change of the injected skin was recorded.

Determination of Neuraminidase Activity :

Disappearance of bound sialic acid in substrate was used as the enzymatic activity of neuraminidase. The action of neuraminidase is to hydrolyse bound sialic acid into free molecules. Orosomucoid or serum L-acid glycoprotein was used as the substrate. The substrate was dissolved in acetate buffer (1.25 gm. orosomucoid in 100 ml., 0.075 M acetate buffer containing 0.3% $CaCl_2$), pre-incubated at 37°C, then the substrate mixture and the cholera culture filtrate were mixed together with a volume ratio of 4 to 1 respectively.

As soon as the cholera culture filtrate was well mixed with the substrate mixture, 0.2 ml. aliquots of the incubation mixture were withdrawn at 0, 5, 10, 20, 30, 60 and 90 minutes. The aliquots were heated at 100°C in boiling water for 3 minutes to stop enzymatic action. Bound sialic acid content in the incubation mixture was analysed by periodate - resorcinol method (19). A decrease of bound sailic acid within 20 minutes of incubation time indicated the initial activity of the enzyme.

RESULTS

Rabbit Skin Test for Toxicity. The result is summarized in **Table 1.** The culture filtrate obtained from the original organism gave no sign of swelling on the rabbit skin within 24 hours. It showed a little swelling in 48 hours, and on the third day the local lesion was observed.

In the case of mucin media presubcultures, the organism produced the culture filtrate which showed higher toxicity than that from the original untrated one. For it gave the observation of skin swelling within 24 hours and the local lesion occured at 48 hours after injection.

TABLE 1

Toxicity of Vibrio cholerae culture filtrate from different culture media by rabbit skin test.

Culture media	Swelling observation in	
	24 hrs.	48 hrs.
1. Tryptone broth (as control)	−	+
2. Mucin presubcultures	+	++

Neuraminidase Activity Table 2 shows the activity of neuraminidase in the culture filtrates using orosomucoid as a substrate. The activity of enzymes is expressed as Units per ml. culture filtrate. One Unit of enzyme is expressed as the enzyme that can liberate one ugm. of sialic acid from orosomucoid at pH 5.5 in 15 minutes at 37°C (25). Each initial velocity from the plot shown in Figure 1. is used in calculation.

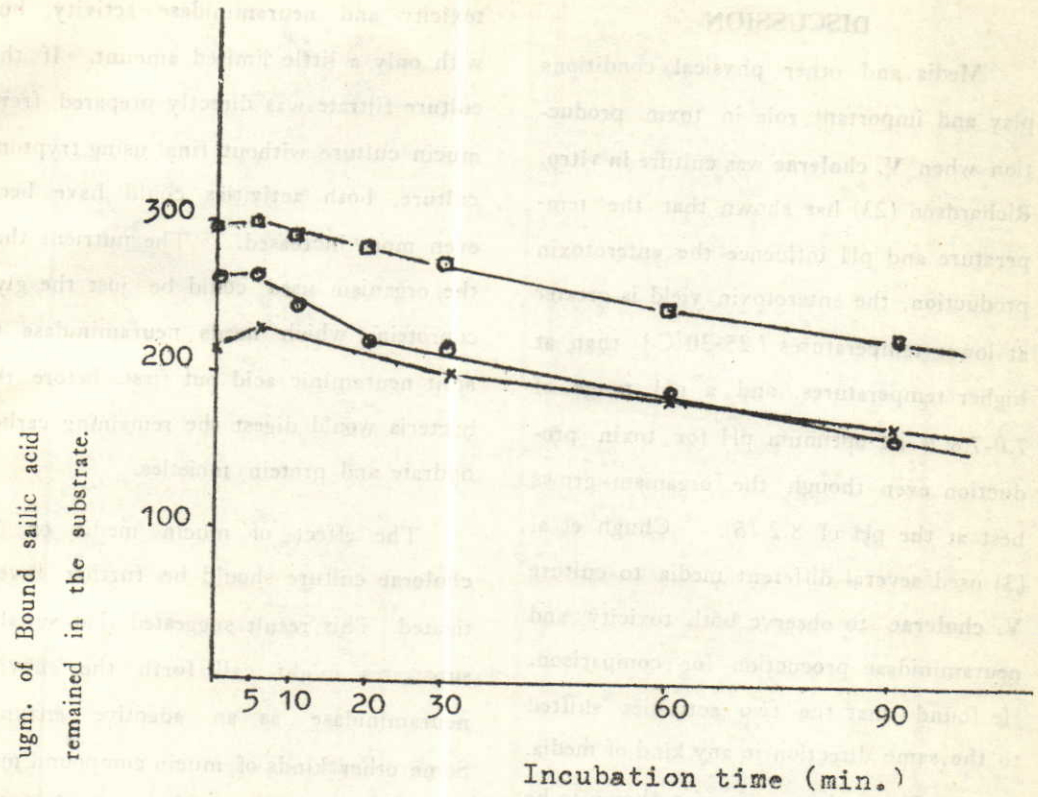

Fig. 1. Enzymatic activity of neuraminidase in **V. cholerae** culture filtrates obtained from different types of subculture media; ×—×, control, □—□, mucin tryptone subculture media, 0—0, mucin subculture media.

TABLE 2

Neuraminidase activity of **V. cholerae** culture filtrates obtained from different culture media. Numbers in parentheses are numbers of subcultures.

Culture media in subculture	Enzymatic activity (Units/ml,)
Tryptone broth (0)	75
2% Mucin in tryptone broth (10)	75
Mucin only (7)	200

DISCUSSION

Media and other physical conditions play and important role in toxin production when **V. cholerae** was culture **in vitro.** Richardson (23) has shown that the temperature and pH influence the enterotoxin production, the enterotoxin yield is greater at lower temperatures (25-30°C) than at higher temperatures, and a pH range of 7.0-7.8 is an optimum pH for toxin production even though the organism grows best at the pH of 8.2 (5). Chugh et al (3) used several different media to culture **V. cholerae** to observe both toxicity and neuraminidase procuction for comparison. He found that the two activities shifted to the same direction in any kind of media.

In this study, mucin was shown to be a very simple media for **Vibrio cholerae.** No other integradient was needed to make it enriched. This media was made to imitate the nature of intestinal epithelium which contains mucins or glycoproteins (18). It was observed that even the organism was cultured in plain mucin solution, it was able to grow quite well. The result showed that after the organism was incubated several times in mucin media it tended to give a higher degree of both toxicity and neuraminidase activity, but with only a little limited amount. If the culture filtrate was directly prepared from mucin culture without final using tryptone culture, both activities could have been even more increased. The nutrient that the organism used could be just the glycoprotein, which needs neuraminidase to split neuraminic acid out first, before the bacteria would digest the remaining carbohydrate and protein moieties.

The effect of mucin media on **V. cholerae** culture should be further investigated. This result suggested that suitable substrates might call forth the enzyme neuraminidase as an adaptive enzyme. Some other kinds of mucin compound may be better or perhaps some other suitable substance and some physical conditions are needed for more production of neuraminidase and toxin. These conditions should be similar to that in the intestine of the cholerae patient. Finally this might be an economical means to enhance the vilurence or induce the enzyme production of **Vibrio cholerae** by **in vitro** subcultures, instead of using **in vivo** orderliness as generally employed by microbiologist.

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