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CHARACTERISTICS OF BORIC ACID TOLERANT *VIBRIO CHOLERAE*

M.O. OJO

Department of Veterinary Microbiology and Parasitology, University of Ibadan, Ibadan, Nigeria

Summary

Two strains of boric acid tolerant *Vibrio cholerae* have been studied in order to identify the characteristics which distinguish them from the 'wild-type' virulent strains. Strain Ib5 requires either amino acid cysteine or methionine while strain Ib5S requires deoxyribonucleic acid (DNA) for growth. Although the strains did not grow on desoxycholate citrate agar (DCA) they grew well on chocolate agar. Morphologically filamentous, bacillary and comma-shaped forms were seen, depending on the media and incubation period. The strains failed to cause accumulation of fluid in gut ligation and permeability factor tests. With the single radial immunodiffusion test, strain Ib5S gave positive reaction in 83% of the tests when DNA was added to the growth medium. On the other hand only 25% of the tests were positive when no DNA was added to the growth medium, even though the antigen was concentrated ten-fold. Strain Ib5 only gave 50% positive tests when the antigen was concentrated ten-fold. It is therefore suggested that Ib5S strain is a choleragenoid producer in the presence of DNA, and may be useful as an effective vaccine against cholera.

Résumé

Deux souches de *Vibrio cholerae* tolerantes à l'acid borigue ont été étudiées pour quon puisse identifier les caracteres qui les distingue de souches virulentes. La sourche Ib5 a besoin d'ou cysteine d'ou methionine tandis que la souche Ib5S n'a que besoin l'acid deoxyribonucleic (DNA) pour la croissance poussent bien sur l'agar à chocolat mais pas sur l'agar à désoxycholate citrate (DCA). Au point de vae de la morphologie, on a identifier, selon le milieu de croissance et la periode d'incubation, des formes filamenteuses, bacillaires et en virgules. A les epreuves de la ligature de l'anse intestinale grêle et de la facteur de permeabilité, les souches n'ont pas causé l'accumulation de l'eau.

A l'epreuve d'immunodiffusion, unique et radiale, la souche Ib5S a donné des resultats positifs dans 83% des cas quand on a ajouté le DNA au milieu de la croissance. Au contraire, il n'y avait que 25% des cas positifs quand on à pas ajouté le DNA au milieu de la croissance meme si l'antigen a été concentré dix fois. Ib5 n'a que donne 50% des resultats positifs quand l'antigen a été concentre dix fois. Il est donc suggeré que la souche, Ib5S produce le choleragenoid en presence du DNA, et pêut être utile comme une vaccine contre le choléra.

Correspondence: Dr M.O. Ojo, Department of Veterinary Microbiology and Parasitology, University of Ibadan, Ibadan, Nigeria

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Introduction

The biological and physical properties, pathogenicity and immunogenicity of boric acid tolerant *Vibrio cholerae* strain have been reported (Ojo, 1975; Ojo & Smith, 1979). Choleragenoid (natural toxoid) consists of L-subunits of choleragen (Holmgren *et al.*, 1977). These workers pointed out that the excessive diarrhoea in cholera is due to the action of exotoxin produced by *V. cholerae* on the small intestine epithelium. On this basis, the possible use of toxoid for improved immunoprophylaxis is considered a better alternative to

the vaccine in use (Holmgren *et al.*, 1977).

Formalin-derived toxoid, although reasonably antigenic, proves unsatisfactory because of reversion to toxicity. On the other hand, gluteraldehyde-derived toxoid, though stable, is poorly immunogenic. The L-subunits of the enterotoxin do not revert to toxicity and possess high protective immunity in experimental animals (Holmgren *et al.*, 1977).

The W.H.O. Scientific Group (1972) on oral bacterial vaccines considered effective immunization of the population at risk as the alternative to sanitation, public health and personal hygiene in order to limit intestinal infections, particularly in the developing countries where the problems of environmental hygiene may not be solved within the next four or five decades. The vaccines must however be stable when given orally and possess characteristics that readily distinguish them from the 'wild-type' virulent strains in the natural state.

The present work was therefore carried out to determine the morphology of the strains under test when cultured in different media, their biochemical reactions, growth requirements, sensitivity to antibacterial agents, shelf life of gelatine discs of the organisms and their ability to produce choleragen or choleragenoid. These were undertaken in order to identify a choleragenoid producer because it is believed this will be a cheaper and easier method of producing toxoid and that the other characteristics will aid the identification of the strains which produce choleragenoid and hence distinguish them from 'wild-type' virulent strains.

Materials and methods

Morphology

Strains Ib5 and Ib5S were cultured on blood agar, nutrient agar, MacConkey agar, desoxycholate citrate (DCA) agar (Oxoid) and chocolate agar incubated at 37°C and room temperature (22–28°C) for 20-120 h. The growth rates were noted and their colonial morphology examined macroscopically and recorded. Gram stain of the cultures on different media was carried out at different stages of growth. The Gram stain was slightly modified by counterstaining with dilute carbol fuchsin because, from personal experience, it is a better differential stain than safranin.

Biochemical reactions

Indole test after 24 and 48 h, oxidase reaction and ability to grow in Koser's citrate were carried out. Fermentation tests for glucose, maltose, sucrose, galactose, mannose, arabinose, lactose, mannitol, fructose, xylose, dulcitol, inositol and glycerol were carried out by conventional methods. Other tests included urease production, methyl red (MR), Voges-Proskauer (V-P) and litmus milk reactions. Two sets of reaction were set up for each strain; a set was incubated at room temperature and the other set at 37°C for up to 21 days in the case of fermentation tests, litmus milk reaction, urease production and ability to grow in Koser's citrate.

Sensitivity test

The sensitivity pattern was determined by the disc sensitivity test. A loopful of each strain, including the 'wild' strain and microcolony grown overnight on nutrient agar, was inoculated into 3·0 ml of nutrient broth in Bijou bottle. It was incubated for 6 h at 37°C. A sensitivity agar (Wellcome Reagents Ltd, England) plate was flooded with each culture, the excess removed and multodiscs (Oxoid) were placed on the agar plates. The multodiscs contained oxytetracycline (OT, 10 mcg), ampicillin (PN, 10 mcg), erythromycin (E, 10 mcg), streptomycin (S, 10 mcg), furazolidone (F, 15 mcg), triple sulpha (S_3, 300 mcg) nalidix acid (NA, 30 mcg), penicillin (P, 1·5 units) and polymyxin B (PB, 100 mcg). The plates were left at room temperature for 1 h before incubating at 37°C for 24 h. The zone of inhibition around each disc was measured in mm and recorded.

Growth requirements

Davis minimal medium was prepared as follows: KH_2PO_4, 3 g; K_2HPO_4; $3H_2O$, 9·2 g; Na_3-citrate.$2H_2O$, 0·5 g; $MgSO_4.7H_2O$, 0·1 g; $(NH_4)_2SO_4$, 1·0 g and agar (Gibco, U.S.A.), 14·0 g. Each salt was dissolved in the order indicated in a litre of distilled water, waiting until previous salt is dissolved before adding the next. The solution was then filtered into 2-litre flask and the agar added; the mixture was divided into aliquots of 100 ml and autoclaved at 15 lb/in² for

15 min. After autoclaving and cooling to 55°C sterile glucose solution was added to each aliquot so that the final concentration of glucose was 0·5%.

Amino acids

The following amino acids were prepared in five groups so that the final concentration of each amino acid was 20 mcg/ml of the L-forms:

Group A — arginine, proline and glutamic acid;
Group B — glycerine, serine, histidine and cysteine;
Group C — lysine, methionine and aspartic acid;
Group D — valine, leucine, alanine, isoleucine and threonine;
Group E — phenylalanine, tyrosine and trytophan.

Deoxyribonucleic acid (DNA)

Deoxyribonucleic acid from calf thymus (Sigma Chemicals Co., U.S.A.) was prepared so that the final concentration was 10 mcg/ml. The amino acids and DNA were sterilized by filtration using 450 nm millipore filter.

Amino acids in each Group A–E were added to aliquot of 100 ml minimal agar while all the amino acids in the groups were added to another aliquot of minimal agar. To another aliquot was added DNA while another aliquot contained neither amino acids nor DNA. Agar plates were prepared by the conventional methods.

Each strain including the 'wild' strain was streaked on the different media, incubated at 37°C for 24–72 h and the results recorded. If growth occurred on a medium containing a group of amino acids, the particular amino acid or amino acids required for growth were determined by streaking the strain on minimal agar plate and discs (6 mm diam.) impregnated with 20 mcg of the amino acids using 50-dropper pipette were put on the streaked lines. The plates were incubated at 37°C for 24–72 h and growth around the discs if any recorded.

Shelf-life of bacterial discs

The strains were preserved by drying in gelatine ascorbic acid discs according to the method of McCraken (1964). The discs were stored in sterile Bijou bottles and kept on the bench at room temperature (22–28°C). The discs were prepared in June 1976 and subcultures made in nutrient broth at 3-month intervals for over 3 years.

Choleragen or choleragenoid production

The strains were grown in syncase medium which consists of the following (per litre): Na_2HPO_4, 5·0 g; K_2HPO_4, 5·0 g; sucrose, 5·0 g; Na_2SO_4, 0·089 g; NH_4Cl, 1·18 g; $MgCl_2.6H_{20}$, 0·0042 g, $MnCl_2.7H_{20}$, 0·004 g, $Fecl_3.6H_{20}$, 0·005 g and casamino acids (Difco), 10·0 g.

The different constituents were dissolved separately and made up to 1 litre. The pH was usually 7·2–7·4. The medium was autoclaved at 15 lb/in² for 15 min.

The 24-h culture was centrifuged at 4°C at 6000 rev/min for 30 min. The supernatant was removed, cultured on nutrient agar to ascertain that the supernatant was free of bacteria. The supernatant was then concentrated. The concentration was achieved by putting the supernatant in a dialysis tube, both ends tied and polyethylene glycol (Sigma Chemical Co., U.S.A.), approximately 20000 mol. wt., poured over the tube in a plastic container. The container and its contents were kept at 4°C until the final volume was about 10% of the original volume.

Skin permeability factor (PF) using the supernatant was carried out according to the method of Craig (1966) while gut ligation test was done in rabbits according to the method of Ojo and Smith (1979). Single radial immunodiffusion (SRID) was carried out according to the method of Holmgren (pers. comm.). For PF, gut ligation tests and SRID, choleragen obtained from Dr Holmgren was used as the positive control.

Results

Growth on media

The two strains (Ib5 and Ib5S) grew well on nutrient agar, though the colonies of Ib5 were larger than those of Ib5S which were pin point after 24 h and even after 48 h they were not as large as those of the 24-h culture of Ib5 or the 'wild-type' strain of *V. cholerae* (Figs 1a and 1b). Both strains grew very well on chocolate agar after 24 h, but they only produced patchy and

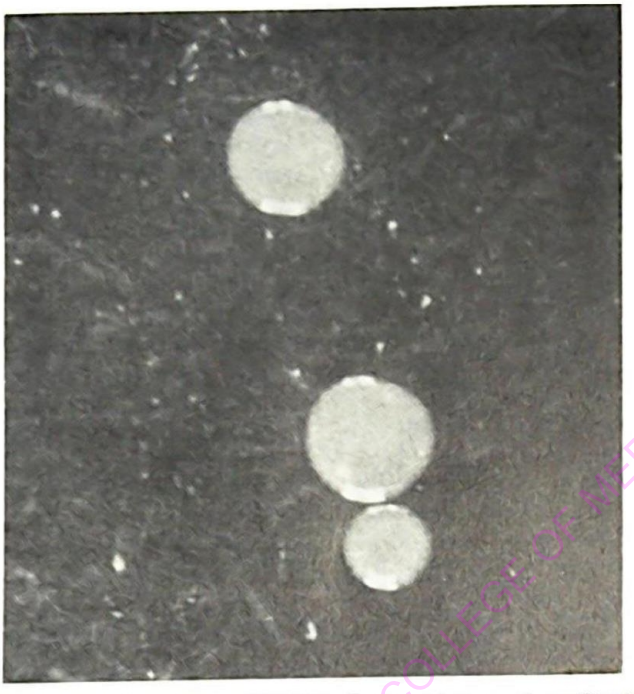

FIG. 1a. Twenty-four hour colonies of Ib5S strain on nutrient agar (magnification × 15).

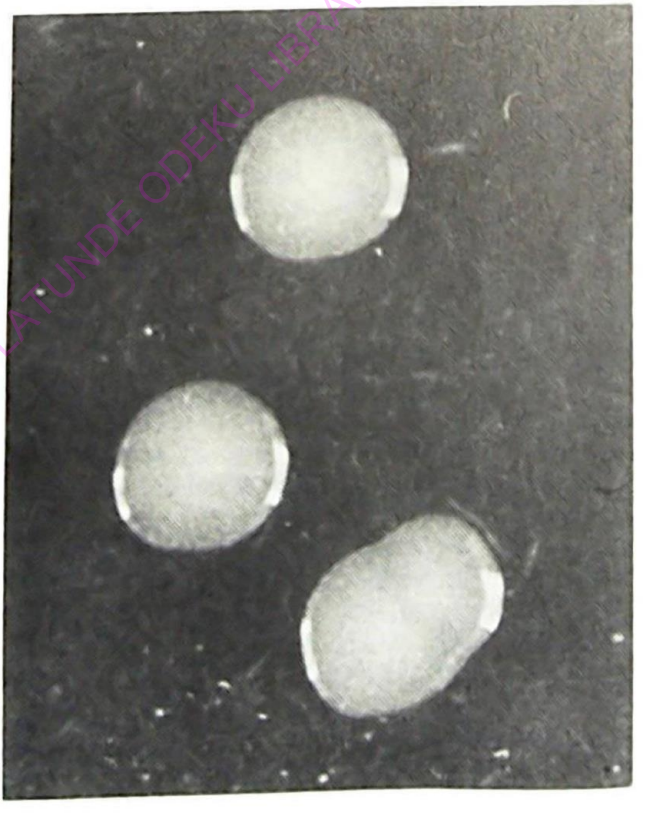

FIG. 1b. Twenty-four hour colonies of 'wild-type' *V. cholerae* on nutrient agar (magnification × 4).

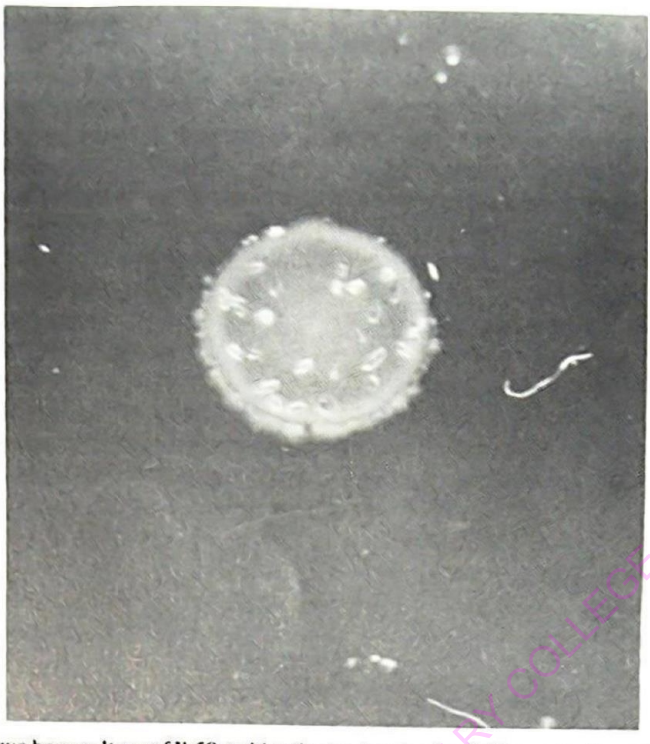

FIG 2a. Seventy-two hour culture of Ib5S on blood agar showing knob-like secondary colonies (magnification × 3·5).

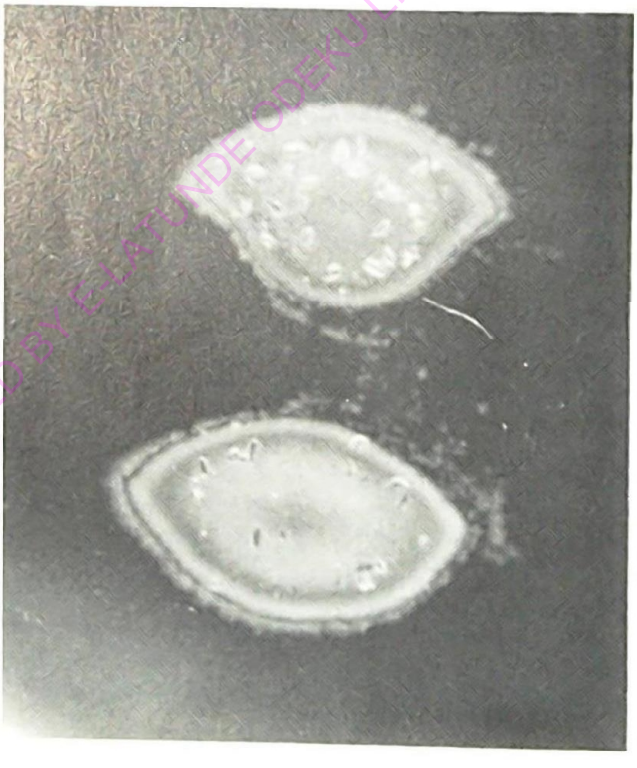

FIG. 2b. Microcolonies between two 72-h colonies of Ib5S on blood agar (magnification × 2·7).

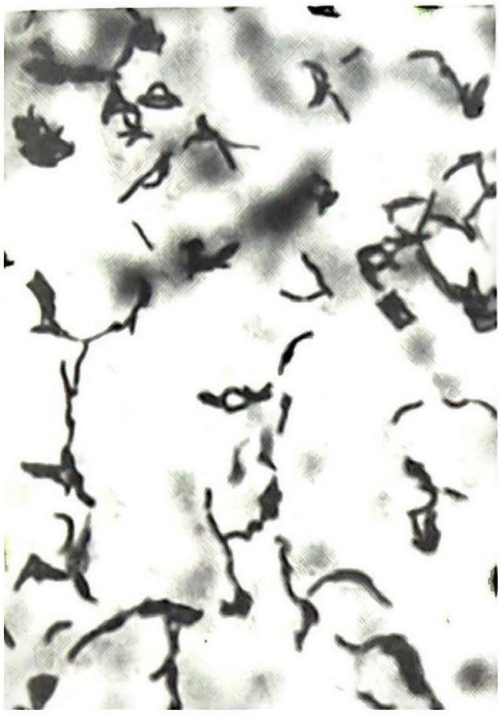

FIG. 3. Gram stain of Ib5 culture on MacConkey agar; comma-shaped organisms are present (magnification × 640).

FIG. 4. Gram stain of Ib5S culture on chocolate agar; filamentous and bacillary forms predominate (magnification × 640).

FIG. 5. Gram stain of 48-h Ib5S culture on nutrient agar; organisms are mainly filamentous (magnification × 640).

irregular growths on blood agar. After about 72–120 h incubation both strains showed small knob-like secondary colonies on the surface of the parent colony (Fig. 2a) and in some cases, microcolonies appeared between two adjacent parent colonies (Fig. 2b). Strain Ib5S only grew at the inoculum on MacConkey agar while Ib5 produced patchy growth on the medium. The two strains did not grow on DCA.

Gram stain

On MacConkey agar, Ib5S was mainly bacillary while some comma-shaped bacilli were seen in Ib5 (Fig. 3). On chocolate agar filamentous, bacillary and a few comma-shaped forms were seen in Ib5S (Fig. 4). Filamentous form predominates in both strains when grown on blood and nutrient agar, but a few comma-shaped organisms were seen in Ib5S, cultured on nutrient agar (Fig. 5). The filamentous form appeared in Ib5S only in 48-h culture on nutrient agar.

Biochemical reactions

They were both oxidase and catalase positive.

Strain Ib5S only produced acid in sucrose and glucose at room temperature and none at 37°C. The other tests were negative even after 21 days' incubation. Where there was no turbidity, subcultures were made on nutrient agar and both strains grew, except from glycerol and Koser's citrate.

Sensitivity test

A strain was considered sensitive when the zone of inhibition was at least 2 mm. Strain Ib5 was sensitive to all the antibacterial agents except triple sulpha, polymyxin and penicillin, while Ib5S was only resistant to triple sulpha and polymyxin. The 'wild' strain was resistant to polymyxin, tetracycline, erythromycin and penicillin. The microcolony on the other hand was only resistant to triple sulpha.

Growth requirements

Strain Ib5 required either amino acid cysteine or methionine for growth but when the two amino acids were present the growth was considerably improved. Strain Ib5S only grew when DNA was added to the minimal medium but the 'wild' strain grew well on minimal medium.

Shelf-life

Both strains grew very well when subcultured in nutrient broth for at least 3 years 8 months.

Tests for choleragen or choleragenoid production

Both the skin and gut ligation tests were negative for both strains, when the supernatants were used. Choleragen was positive in both the

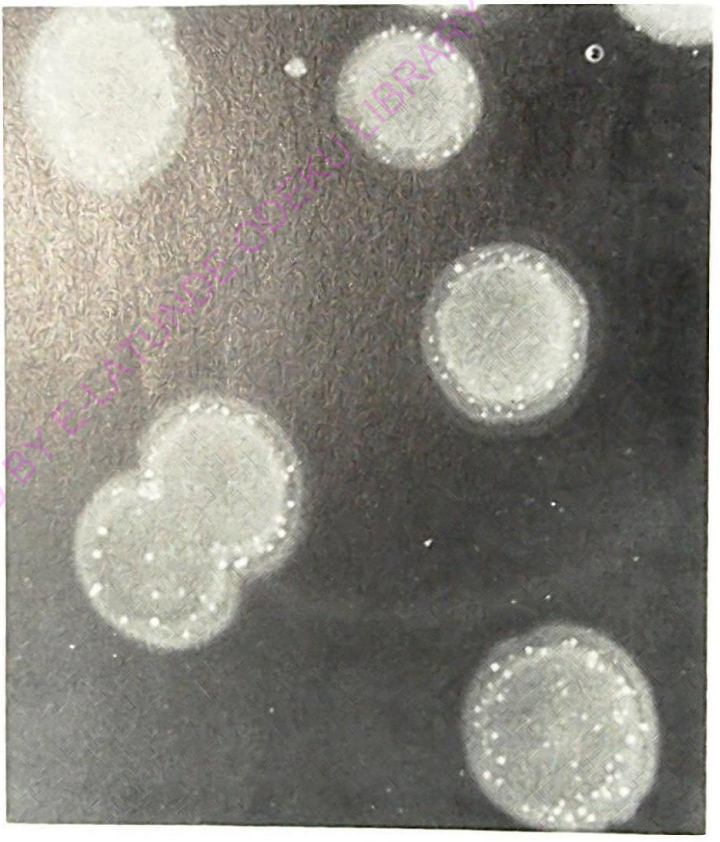

FIG. 6. Seventy-two hour colonies of Ib5 on nutrient agar showing opaque centres and transparent peripheries (magnification × 2).

skin and gut ligation tests. Using the SRID method, the test was positive in fifteen out of eighteen tests for Ib5S supernatant when DNA was added to syncase medium. Strain Ib5 was only positive when the supernatant was concentrated at least ten-fold. The positive reaction was obtained on four out of eight occasions when the test was carried out. Strain Ib5S, without DNA added to it, was only positive on two out of eight occasions even though the supernatant was concentrated at least ten-fold. Generally the rings appeared after 3 days incubation but they became stronger after 7 days incubation.

Discussion

Small, knob-like secondary colonies observed in both strains growing on blood agar are characteristics of *V. cholerae* colonies incubated for about a week (Wilson & Miles, 1966). Balteau (1926), cited by Wilson and Miles (1966), described three colonial variants in culture of cholera and cholera-like vibrios, namely rugose, opaque centre with a transparent periphery and opaque. According to him the first two variants reverted to type when subcultured on agar while the later slowly reverted in broth but remained constant for a long time on agar. Colonies of Ib5 are mainly of the variant with opaque centre and transparent periphery (Fig. 6). Strain Ib5, however, does not revert. Biochemically, Ib5S belongs to group II of Heibert's classification of vibrios. The reactions only took place at room temperature. On the basis of incubation at 37°C, the two strains belong to group VI.

Negative gut ligation skin permeability tests and positive SRID test indicate that the strains produce choleragenoid (Holmgren, pers. comm.). Finkelstein and LoSpalluto (1969) isolated a non-toxic protein choleragenoid that is immunologically similar to the toxin but has a molecular weight of only 56000 daltons. Lonnroth and Holmgren (1973) later showed that this protein contains the same L-subunits as does the toxin but that it lacks the H-subunit. The indication that the two strains produce choleragenoid needs to be confirmed using a more sensitive test, for example, ELISA. The facilities for ELISA test are not available in my Department.

Choleragenoid is more readily produced by Ib5S strain when DNA is added to the growth medium. It is therefore suggested that a tablet form of the organism, grown in DNA-enriched medium may be a good oral vaccine. Ojo and Smith (1979) showed that rabbits given 2 ml of Ib5S broth culture orally for 5 days failed to respond to gut-ligation test when later challenged with virulent *V. cholerae* strain. The volume may be reduced by concentrating the culture by centrifuging at 4°C. On the other hand the toxoid added to lipopolysaccharide (LPS) can be used as an immunogen (Holmgren *et al.*, 1977). Other strains have also been developed but detailed studies on them have not been carried out.

Acknowledgments

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