Technical method

The isolation and identification of *Vibrio cholerae*

A. L. FURNISS AND T. J. DONOVAN *From the Public Health Laboratory, Maidstone*

Cholera is not endemic in Britain but cases have been occurring amongst people returning from places abroad where the disease is present. A diagnosis of cholera is therefore unlikely unless the patient has been abroad within the last two weeks or has been in very close contact with a known vibrio excreter.

The watery stool of a severe case of cholera may give an almost pure growth of vibrios, and enrichment and selective techniques are hardly necessary. In most cases, however, vibrios are present in small numbers together with other organisms; this means that enrichment and selective media are essential.

Collection of Sample for the Laboratory

A specimen of faeces should be sent to the laboratory as soon as possible. It should not be sent by post because of the delay. Transport medium is not necessary in Britain where laboratories are so accessible.

Direct Examination of Stool

The microscopic appearance of even a severe cholera stool is not diagnostic; isolation of the organisms is of fundamental importance. Treatment, if necessary, will depend on the degree of dehydration and will not await a laboratory report.

Enrichment

Vibrios will multiply more rapidly in alkaline peptone water than other organisms, but the selective effect is lost if subculture is delayed until other organisms have multiplied or if the peptone water is too heavily inoculated. This period of selective advantage for the vibrios may be prolonged if the alkaline water is incubated at 20°C.

Selective Media

Monsur's medium (Monsur, 1963) is an excellent

Received for publication 30 May 1974.

selective medium for vibrios, but has the disadvantage that it is not commercially available.

The base can be prepared in the laboratory and stored until required; the potassium tellurite can then be added just before pouring to give a final concentration of 1 in 200 000. Familiarity with the medium is necessary to gain full advantage from it.

Thiosulphate-citrate-bile-salt-agar (TCBS) is commercially available and easy to use (Kobayashi, Enomoto, Sakazaki, and Kuwahara, 1963). It is recommended at present for routine use in Britain for the isolation of vibrios.

In the Laboratory

Inoculate the following: (1) About 2 ml faeces into 20 ml alkaline peptone water, pH 8 (first peptone water), and incubate for five to eight hours. (2) Thiosulphate-citrate-bile-salt-agar plate with a heavy inoculum of faeces (first TCBS plate). (3) The usual media for the isolation of shigellas, salmonellas, etc.

After five to eight hours' incubation from the first alkaline peptone water inoculate the following: (4) a new peptone water (second peptone water) with about 1 ml of fluid from the top of the first peptone water. This second peptone water is to be incubated for a further five hours or overnight. (5) A new TCBS plate (second TCBS plate) with a heavy inoculum. (6) Nutrient agar which may be found useful as a source of colonies for agglutination.

SECOND DAY

If the first and second TCBS plates are negative, inoculate a third TCBS plate from the top of the second peptone water.

EXAMINATION OF TCBS PLATES

Cholera vibrios appear as yellow colonies because of the fermentation of sucrose and the surrounding

<i>V. cholerae</i>	Medium sized (2-3 mm diameter after 18 hours' incubation), yellow colonies
<i>V. alginolyticus</i>	Large, yellow colonies
<i>V. parahaemolyticus</i>	Large, deep blue-green colonies
<i>Pseudomonas</i>	No growth or small, colourless or pale green colonies
'Coliforms'	No growth
<i>Proteus</i>	No growth or small, yellow or greenish colonies
<i>Aeromonas</i> (most strains)	No growth
Enterococci	Small, yellowish-white colonies

Table Colonial appearance of organisms on TCBS agar after overnight incubation at 37°C

medium may also be turned yellow. It should be noted that the yellow colour may fade. (Some freeze-dried strains do not grow on TCBS agar and some have lost their ability to ferment sucrose overnight. Typical strains that have been recently isolated are available from the authors.)

RECOMMENDED IDENTIFICATION PROCEDURE

Typical yellow colonies should be subcultured to a nutrient agar plate which is incubated at 37°C for five to eight hours. After this time there is normally sufficient growth for the following tests.

1 OXIDASE TEST (KOVACS, 1956)

This should be done on colonies on nutrient agar and not on growth from a medium which contains a fermentable carbohydrate, as does TCBS.

2 AGGLUTINATION WITH TYPE 'O' 1 CHOLERA ANTISERUM ('POLYVALENT')

It should be possible to use fresh colonies on TCBS agar for slide agglutination but after some time the colonies become so sticky as to make agglutination impossible. It is, therefore, preferable to check the agglutination from the growth on nutrient agar.

3 GRAM STAIN TO CHECK THE PURITY OF THE CULTURE

Curvature of the bacterial axis may or may not be recognizable; this is not a diagnostic feature.

The following biochemical media are then heavily inoculated from the growth on the nutrient agar plate:

4 DECARBOXYLASE MEDIA (MØLLER, 1955)

Modified by the addition of 1% sodium chloride, ie, lysine, arginine, ornithine, and a blank. Such media with added sodium chloride are still satisfactory for use with enterobacteria.

5 PEPTONE WATER SUGARS WITH ANDRADE'S INDICATOR

Glucose, sucrose, arabinose, mannose, and simple peptone water.

6 TUBES OF HUGH & LEIFSON'S OXIDATION-FERMENTATION MEDIUM (HUGH & LEIFSON, 1953)

In addition, TCBS, blood agar (for purity check), a nutrient agar slope (to be sent for typing), and electrolyte-deficient medium (see below) should be inoculated and incubated at 37°C. Most cholera vibrios grow sufficiently for the results to be read after overnight incubation.

Vibrios are characteristically actively motile, oxidase-positive, Gram-negative bacilli. Other

characters are: acid with no gas in glucose, acid in sucrose, acid in mannose, arabinose negative, indole positive, arginine negative, lysine positive, ornithine positive.

OTHER TESTS

Vibrios are sensitive to the vibriostatic agent O129 (2,4-diamino-6, 7 di-isopropyl pteridine) (Shewan *et al*, 1954). This sensitivity can be tested by means of a disc placed on a lawn of the culture. One hundred and fifty mg O129 compound is dissolved in a 1:1 alcohol-ether mixture; discs are impregnated with a drop of this solution and dried (Caselitz, 1966).

We have ourselves little experience of the so-called 'string test' (Smith, 1970), but think it may be of help in identifying *Vibrio cholerae* colonies. A colony is emulsified in a drop of 0.2% desoxycholate in distilled water on a slide. Cholera and allied vibrios will characteristically become viscid so that when the point of the wire is withdrawn from the slide the drop is adherent enough to be elongated momentarily as a 'string'.

USE OF ELECTROLYTE-DEFICIENT MEDIUM IN THE IDENTIFICATION OF VIBRIOS

This medium, CLED, was originally designed to prevent the swarming of proteus (Mackey and Sandys, 1966, modified by Bevis, 1968). It has been found to be of great practical value in differentiating vibrios. *V. cholerae* will grow on CLED. The salt-requiring vibrios, which include *V. parahaemolyticus* and *V. alginolyticus*, will not grow on CLED because they will not grow in the absence of sodium chloride. *V. cholerae* can multiply in the absence of sodium chloride, although like all vibrios its growth is enhanced by the addition of sodium chloride.

TYPING OF VIBRIOS

Although identification of cholera vibrios is practicable in the general diagnostic laboratory, all strains of vibrios isolated, whether agglutinating or not, should be sent to a Reference Laboratory as soon as possible; serotyping and phage typing can be undertaken. Biotyping of strains from the present pandemic shows them as El tor biotype, but the occurrence of variants makes it clear that the idea of two polar forms of immutable biotypes is false. There is much still to be done before it can be said that vibrios can be adequately typed.

References

- Bevis, T. D. (1968). A modified electrolyte deficient culture medium. *J. med. Lab. Technol.*, 25, 38-41.
- Caselitz, F-H. (1966). *Pseudomonas-Aeromonas und ihre human-medizinische Bedeutung*, p. 32. Fischer, Jena.
- Hugh, R., and Leifson, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by

- various gram-negative bacteria. *J. Bact.*, 66, 24-26.
- Kobayashi, T., Enomoto, S., Sakazaki, R., and Kuwahara, S. (1963). A new selective medium for pathogenic vibrios: TCBS (modified Nakanishi's agar). (Japanese) *Jap. J. Bact.*, 18, 387-392.
- Kovács, N. (1956). Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature (Lond.)*, 178, 703.
- Mackey, J. P., and Sandys, G. H. (1966). Diagnosis of urinary infections. *Brit. med. J.*, 1, 1173.
- Møller, V. (1955). Simplified tests for some amino acid decarboxylases

and for the arginine dihydrolase system. *Acta path. microbiol. scand.*, 36, 158-172.

- Monsur, K. A. (1963). Bacteriological diagnosis of cholera under field conditions. *Bull. Wld Hlth Org.*, 28, 387-389.
- Shewan, J. M., Hodgkiss, W., and Liston, J. (1954). A method for the rapid differentiation of certain non-pathogenic, asporogenous bacilli. *Nature, (Lond.)*, 173, 208-209.
- Smith, H. L. (1970). A presumptive test for vibrios, the 'string' test. *Bull. Wld Hlth Org.*, 42, 817-818.

Book reviews

Laboratory Methods 1. Public Health Laboratory Service Monograph Series No. 5. By Joan R. Davies, E. J. G. Glencross, J. Marks, C. D. Plows, and Mair E. M. Thomas. (Pp. vii + 35; 2 illustrations. 50p.) London: HMSO. 1974. In the now well known Monograph Series published by the Public Health Laboratory Service it is the intention to publish collections of papers on various laboratory techniques that experience has shown to work well. This is the first group of such papers and like other monographs in the series will find its way into most microbiology laboratories. It contains a selection of descriptions of very different techniques.

Readers will find precise instructions for success with Elek plates for the demonstration of diphtheria toxin, and adherence to the suggestions will no doubt help to reduce the number of occasional failures with this technique. Methods for the isolation and characterization of *Listeria monocytogenes* are collected together and are here for easy reference. The way that we should go about the isolation and identification of mycobacteria is carefully and comprehensively described including a host of practical tips. This is an authoritative collection of advice from the Tuberculosis Reference Laboratory. A further two subjects are dealt with: the slide tests for glandular fever and finally there is a note on the isolation and identification of genital tract lactobacilli.

D. M. JONES

Haemophilia and Its Related Conditions: A Brief Guide to Diagnosis and Treatment. By Rosemary Biggs (Pp. v + 42; tables. 34p.) London: HMSO Research Council Memorandum No. 44, April 1974.

The publication of this MRC memorandum is most welcome, especially as the previous edition, which was published in

1955, had been out of date for well over a decade. The present monograph is an ideal introduction for anyone concerned with patients suffering from haemostatic disorders. Brief and always to the point, the author covers the clinical and laboratory diagnosis of hereditary and acquired coagulation defects and their treatment. Stress is laid on the need to treat these patients at special centres and the author succeeds in showing that, although the scene has been revolutionized by increased supplies of concentrate, there is much more to the treatment of haemophilia than the administration of blood products. At 34p the pamphlet is cheap enough and anyone connected with the management of bleeding disorders will benefit from having a copy, whether they be laboratory technician, nurse, physiotherapist, or doctor. I have only one adverse criticism and this refers to Appendix 2 which lists the haemophilia centres in the UK. Although the list, as it stands, is far better than no list at all it is a pity that, even at the time of publication, it was (like so many other printed lists) partly out of date and full of little errors.

KATHARINE M. DORMANDY

Tumors of the Extra-Adrenal Paraganglion System (Including Chemoreceptors). By George G. Glenner and Philip M. Grimley. (Pp. 90; illustrated. \$4.50.) Washington, D.C.: Armed Forces Institute of Pathology. 1974.

The fascicles comprising the Armed Forces Institute of Pathology 'Atlas of tumor pathology' have long since established themselves as essential companions to the practising histopathologist, and this new issue on neoplasms of the extra-adrenal paraganglia is a most useful addition to the series.

The first 38 pages are devoted to the anatomy, histology, cytology, electron microscopy, histochemistry, cytochemistry, and physiology of the normal paraganglia, and a classification of these structures into a number of anatomical groups or 'families' is suggested. Tumours

and tumour-like lesions of the extra-adrenal paraganglia are then described. The text is concise, but contains much useful clinical, as well as pathological, information. The illustrations are of high quality.

This publication maintains the high standards already set by earlier additions to the 'second series' of AFIP fascicles. It represents a most valuable compendium of up-to-date knowledge concerning the paraganglia and their tumours.

N. F. C. GOWIN

Cardiomyopathies (Recent Advances Studies on Cardiac Structure and Metabolism, Volume 2). Edited by E. Bajusz and G. Rona. (Pp. xiii + 842; illustrated. £17.25.) Lancaster: Medical and Technical Publishing Co. Ltd. 1974.

This is the proceedings of an international symposium on cardiomyopathies, and like other reports of its kind, covers the field widely. There is more than the usual quota of pathology, with less consideration of clinical and therapeutic problems; pathologists will find this of value as reference work. It would be a pity if weakening of links between this country and Africa, such a rich source of many types of cardiomyopathy, held back the development and spread of knowledge in this subject.

R. A. B. DRUMMOND