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CHOLERA STUDIES *

7. Practical Laboratory Diagnosis

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SYNOPSIS

The first portion of this study describes in detail the different aspects of stool examinations, including the collection, preservation, and pooling of specimens, macroscopic and bacterioscopic examination, enrichment methods, and cultivation on a variety of solid media. The author also deals with the examination of vomits and of water. The performance and value of different identification tests (agglutination, haemolysis, and bacteriophage) and confirmatory tests are then considered. An annex is included on bacteriological procedures in the laboratory diagnosis of cholera.

Stool Examination

Collection of specimens

Different procedures have to be used for the collection of stool specimens for cholera laboratory work according to whether faeces of patients or of suspected carriers of *V. cholerae* have to be examined. Moreover, a special technique is required for obtaining the material necessary for such examinations from the intestinal contents of supposed victims of the disease.

The method usually resorted to when handling the fluid stools of cholera patients is to put adequate quantities of freshly voided faeces—preferably with the aid of small pasteboard spoons of the type supplied by ice-cream vendors—into suitable containers, which are then forwarded as rapidly as possible to the laboratory. In place of thick-walled, small Erlenmeyer flasks or glass jars ordinarily used for commercial samples, and recommended early by Dunbar (1896), other containers, particularly the earthenware jars cheaply available in eastern countries, may be utilized. In fact, as long as the laboratory is situated near to the wards, any leak-proof container which is provided with a cover fitting tightly enough to avoid spilling,

* This is the seventh of a series of studies which will be published as a monograph on cholera in separate editions in English and French.—ED.

access of flies, and rapid drying of the samples, and which can be sterilized by autoclaving or boiling, is serviceable. However, it would be more expedient to employ, in place of such containers, the pasteboard boxes recommended originally for quarantine work (McLaughlin, 1916) because these, if taken immediately before use from the original packages, can be utilized without preliminary sterilization and, after completion of the examination, can be discarded easily as well as safely by incinerating them together with their contents.

When collecting the specimens, it is imperative to avoid their coming in contact with disinfectants or with ordinary water, which, besides generally not being sterile, may contain cholera-like vibrios. The greatest possible attention must be paid to the proper labelling of the samples. To use merely the bed number for this purpose is altogether unreliable, because, when the results of the examination are reported to the ward, the bed in question may already be occupied by another patient. Particular care must be taken in this respect when further samples from a previously examined patient are sent to the laboratory.

In order to avoid the difficulties and delays attendant upon the collection of stools from suspected carriers of *V. cholerae*, many of whom may have to be handled simultaneously in quarantine work, several workers, e.g., Creel (1911), Craster (1913), and Ahuja et al. (1950, 1951), recommended obtaining material for laboratory examination from such persons with the aid of rectal swabs. McLaughlin (1916) pointed with great reason to the disadvantages of this method: it was difficult to obtain sufficient material, or even any at all, if the suspected persons were constipated; the material on the swab was apt to be scraped off by the anal sphincter during the process of retraction; in the case of individuals afflicted with haemorrhoids, in particular, the insertion of the swab was quite painful. It is true that some of these drawbacks can be obviated by dipping the swabs before insertion into peptone-water or some other sterile fluid, or, better still, by encasing them in clipped rubber catheters or rubber tubes, as advised for instance by Ahuja et al. A still more satisfactory, but also more tedious, procedure is, according to Creel (1911), the initial insertion into the rectum of short thick-walled glass tubes with rounded edges, which serve as specula for the subsequent introduction of the swabs. Nevertheless, on the whole the method of obtaining faecal specimens with swabs seems not an advantageous one; all the less so because, according to a small series of observations recorded in the 1949 report of the Indian Research Fund Association, the percentage of positive results obtained through their examination alone was below that obtainable with stool samples.

The usual procedure of obtaining stool specimens from suspected cholera carriers is to issue them with wide-mouthed bottles or jars of a capacity of about ½-1 fluid once (about 15-30 ml), provided with corks or preferably with metal covers, or, best, with screw-caps. Small wooden,

tin, or pasteboard spoons wrapped in paper may be issued together with these containers to facilitate insertion of the stool specimens into the latter, but it is better to affix the spoons to the inside of the covers. The persons to be examined must be instructed to put a piece of stools of about hazel-nut-size into the containers and to close them carefully.

A definite, though often not realized, drawback to this otherwise convenient method is that one can be never quite sure whether the collected samples are actually derived from the individuals named on the labels of the containers. The present writer knows of instances where ships' stewards obligingly filled the jars supposed to have been issued to the passengers with their own stools.

An alternative method of stool collection used by Müller (1915) was to issue to each of the numerous soldiers he had to examine a piece of thick packing paper, measuring 15 × 15 cm, for deposition of their faeces. Wooden sticks were then used to collect pea-sized portions of the stools. A similar procedure was successfully used by the present writer to study the incidence of cholera and cholera-like vibrios in the population of Shanghai through collection of numerous samples in the public privies.

In the official instructions for the collection and transport of cholera-suspect objects for examination, compiled by Koch and co-authors and promulgated by the Prussian authorities in 1902, it was prescribed that " if no voluntary voiding of stools can be obtained, the same must be effected through the introduction of glycerin ".

Recommendations for inducing the passing of stools artificially for the purpose of laboratory examinations were also made by several subsequent cholera workers. While Pottevin (1915) suggested the use for this purpose of either (?glycerol) suppositories or enemas with boiled water or purgatives, most authorities advocated the use of the latter. McLaughlin (1916) recommended in this respect that passengers who had to undergo quarantine be given magnesium sulfate routinely before breakfast so as to ensure the collection of stool samples. However, for persons suffering from diarhhoea as well as for children he used large catheters or rectal tubes with several openings at the inner end to obtain specimens, which, like those from the naturally voided stools, were put into pasteboard " cuspidors " or similar containers.

It is important to note that the administration of magnesium sulfate to cholera convalescents whose stools had proved bacteriologically negative on two or three previous examinations was found by some workers, such as Tanda (1911), Zirolia (1911), and Piras (1913), to lead more or less frequently to a reappearance of *V. cholerae* in the faecal specimens. Gohar & Makkawi (1948a) were unable to obtain such results when administering magnesium sulfate to a group of cholera convalescents examined about one month after an epidemic in the village in question had terminated. They added that

" in a few cases duodenal intubation and administration of magnesium sulphate were carried out but this did not alter the result. Apart from the trouble this procedure entailed, it was found that the concentrated magnesium sulphate solution when added to the peptone water medium caused inhibition of the cholera vibrio, in a dilution of 1.5 per cent., though it was not bactericidal in as much as 25 per cent. concentration ".

However, in contrast to these findings, the actual experiences of the earlier workers did not indicate an unfavourable influence of magnesium sulfate on the appearance of *V. cholerae* in the stools. Their method therefore perhaps deserves more attention than it has recently received.

The routine administration of laxatives in quarantine practice, as used by McLaughlin, is in the opinion of the present writer not recommendable. If such mass examinations have to be undertaken, there is, with but few exceptions, no difficulty in obtaining stool specimens. If constipated individuals are met with, faecal specimens can be quite easily obtained from them with the aid of glycerol suppositories or small enemas.

Amending the rather elaborate original recommendations of Koch and co-authors (1902) for the collection of material for bacteriological examination from cholera-suspect dead bodies, instructions promulgated by the German authorities in 1916 and reprinted by Kolle & Prigge (1928) prescribed the removal of a loop about 10-cm long of the lowest part of the small intestine after double ligature. This piece of intestine was to be enclosed in a thick-walled, wide-mouthed glass jar provided with a ground-in glass stopper or with a well-fitting and freshly boiled cork. The jars had to be safely packed before transport to the laboratory.

Adequate though this method is, modern workers will prefer to remove material from the small intestine for dispatch to the laboratory at autopsy under aseptic conditions; this is best done with a sterile pipette provided with a rubber bulb. For the reasons discussed in the preceding study,[1] it is essential, when dissecting the dead bodies of cholera-suspect individuals, also to obtain material from the gall-bladder for bacteriological examination.

Preservation of specimens

Discussing the chemical affinities of *V. cholerae*, Nicholls (1917) recommended preserving cholera-suspect stools by the addition of an adequate amount of sodium carbonate.

Panganiban & Schoebl (1918) studied possibilities of preserving cholera-suspect faecal specimens through experiments conducted at room temperature (32° C) with artificially contaminated stools. They recorded the following results:

(*a*) Glycerol exerted no preserving action, the cholera vibrios surviving in 20% or 25% concentrations of this carbohydrate for 4 days only, while

[1] See *Bull. Wld Hlth Org.* 1955, 13, 1171.

at a concentration of 30% the organisms were no longer cultivable on the fourth day.

(*b*) Sodium chloride in concentrations of from 0.5% to 5% preserved the cholera faeces throughout the observation period of 5 weeks, but in concentrations of more than 5% the organisms were no longer demonstrable after 4-5 days.

(*c*) Ox bile used in a concentration of 50%, 75%, or pure, gave identically good results and proved more apt than sodium chloride at a 1% concentration to maintain the viability of cholera vibrios added to the faecal emulsions in small numbers.

The problem of the admissible delay in the examination of stools for *V. cholerae* was again studied by Soda et al. (1936), who were able to experiment with the faeces of three cholera patients, keeping the specimens respectively at 37° C, at an unspecified room temperature, and in the refrigerator. As was to be expected, survival of the organisms was shortest at incubator temperature (37° C)—sometimes not longer than 3 hours—and as long as 8 days in the refrigerator. Survival of the cholera vibrios was also found to be longer in diarrhoeic than in solid stools. On account of these experiences and of further tests made with artificially contaminated faecal specimens, Soda et al. recommended that in actual work with the faeces of suspected cholera carriers not less than 5-g quantities of stools should be used for examination and that these should be collected not more than 24 hours beforehand and kept at a low temperature. If, in sea-borne traffic, a ship's doctor was available, he should immediately make peptone water cultures with the travellers' faeces collected within 24 hours before arrival. Otherwise, it was recommended that the faecal specimens be preserved at a low temperature after addition of 10 ml of peptone water with a pH of 8.4.

Seal (1939), making a comparative study of enrichment methods to which further reference will be made below, preserved his stool specimens by adding them in quantities of 2 teaspoonfuls (= 8-10 g) to 20 ml of 2% NaCl solution filled into 100-ml, wide-mouthed and glass-stoppered sterile bottles, afterwards adding 2-3 drops of a N/1 NaOH solution. In the latter part of his study, however, he increased the quantity of the NaCl solution to 40 ml with a corresponding increase in the inoculum.

A similar technique was used by Read & Pandit (1941), when studying the incidence of cholera and El Tor vibrios in rural areas of India.

> Sufficient amounts of stool samples, quite freshly collected in earthenware pots with lids, were put with the aid of bamboo sticks into 50-ml amounts of a 2% solution of common sea-salt, contained in wide-mouthed, glass-stoppered bottles, to obtain thick emulsions. As a rule, 3-4 drops of a normal NaOH solution were added to the sea-salt solution before inoculation, so as to counteract the usually acid reaction of the stools.

Venkatraman & Ramakrishnan (1941) found that: (*a*) a sea-salt concentration of 2% and a pH of 9.2 were most suitable for a prolonged

survival of *V. cholerae* in the presence of other bacteria; and (*b*) cholera vibrios survived in boric acid solution at a concentration of up to 1.5%, whereas *E. coli* and *Aerobacter aerogenes* were completely inhibited by a concentration of 0.6% and, to a considerable extent, even of 0.3%. Accordingly, these two workers devised a preserving fluid for the transmission of cholera-suspect specimens prepared as follows:

" 12.405 g. boric acid (H_3BO_3) and 14.912 g. potassium chloride (KCl) are dissolved in about 800 c.c. of hot distilled water, the solution cooled and made up to 1 litre. From this stock solution 250 c.c. are taken, mixed with 133.5 c.c. of M/5 NaOH and the whole made up to a litre. Twenty grammes dried sea-salt (common salt from the bazaar serves equally well) are dissolved and the buffered saline filtered through paper, dispensed in 10-c.c. quantities in 1-oz. [28 ml] screw-capped bottles and sterilized in the autoclave."

The two workers added that:

" The sterilized buffer has a pH of 9.2 and is found to maintain the same pH for months. The collecting outfit includes, for convenience, a small aluminium spoon which will hold about 1 g. to 3 g. of stool depending on the consistency. In use, a spoonful of the stool specimen is well mixed in the buffer which is then mailed."

Reporting on the use of their preserving fluid, Venkatraman & Ramakrishnan stated that in the case of artificially contaminated faeces only a slight initial multiplication of the cholera vibrios took place, but that the organisms remained viable in the buffer solution for as long as 62 days. From stool samples collected from a cholera patient on the first day of illness and kept in the preserving fluid, *V. cholerae* could be isolated up to the 92nd day, i.e., until the specimens were exhausted. Referring to a field trial of their preserving fluid, Venkatraman & Ramakrishnan recorded that during an epidemic:

" Two sets of specimens of stools were collected in the preservative from clinically typical cases of cholera. One set was examined immediately on the spot, while the other was mailed to the laboratories at Tanjore, taking 4 to 7 days in transit, and occasionally longer. Both in the field and in the laboratory, platings were made after preliminary enrichment in mannose-bismuth-sulphite medium. Sixty-six specimens, including a specimen of vomit, were taken from 60 cases. *V. cholerae* was isolated from 64 specimens in the field, and from 60 in the laboratory. No case was missed. In two instances, *V. cholerae* was isolated from mailed specimens, while the immediate examination of specimens taken in the field proved negative. We had the impression that failure to isolate *V. cholerae* occurred in those instances where an excess of stool had been added to the preservative causing a drop in the pH."

Venkatraman & Ramakrishnan also obtained apparently satisfactory results when using their preserving fluid for the examination of stools of cholera contacts.

The use of this method in the case of suspect stools which had to be examined after some delay was recommended in instructions for cholera laboratory work published by the Public Health Laboratory Service under the auspices of the British Medical Research Council in 1947. It is also noteworthy that Felsenfeld et al. (1951), in the course of a laboratory

study to which further reference will be made below, found the average survival time of *V. cholerae* in the preserving fluid at present under review to be satisfactorily long, regardless of whether tests were made with pure cultures or with mixtures containing both cholera vibrios and other organisms, including *E. coli*, *A. aerogenes*, and *Proteus vulgaris*.

Pooling methods

As far as can be ascertained, Müller (1915) was the first worker to report on the successful use of pooling methods for the detection of *V. cholerae* in the stools of large groups of individuals, in his case of battalions of soldiers among whom cholera cases had occurred and who therefore had to be quarantined. In order to examine them in a simplified manner, he successively emulsified pea-sized pieces of the stools of 10 individuals in 50 ml, or sometimes even in only 30 ml, of peptone water and then continued with examination of these initial cultures in the usual way. Whenever positive results had been obtained by this method, the groups in question were isolated and treated with animal charcoal and tincture of iodine; the individuals actually found to harbour cholera vibrios were discharged only after further examination of their stools had proved negative three times.

The pooling method was again used by Shahin (1933) for the mass examination of Mecca pilgrims. However, though he once succeeded in finding *V. cholerae* in a pool of ten stools and isolated on three further occasions cholera-like vibrios from such pooled specimens, he was unable to ascertain who among the members of these groups were responsible for the positive results. It is possible that he omitted the now universally adopted procedure of keeping the individual stools which remain after specimens have been taken for pooling until the examination of the pooled material had been completed. To rely instead upon repeated collection and examination of individual specimens is rather unsatisfactory in view of the often markedly intermittent character of vibrio excretion by cholera carriers.

The method of examining pooled stool specimens for the presence of *V. cholerae* has also been utilized more recently by Gohar & Makkawi (1948a) and in a large-scale investigation in Madras State in India carried out under the direction of Venkatraman (1949). The first two observers, working in an Egyptian village where an epidemic had ended about a month previously, were unable to detect carriers with the aid of pooling tests in a group of people who had not been in direct contact with patients. A search for subclinical cholera cases undertaken with the same technique by Venkatraman and his staff also gave entirely negative results, even though Venkatraman & Ramakrishnan's preserving fluid was used in 5-ml quantities for the collection of individual stool samples, and although highly specific media, which will be described below, were utilized for enrichment and cultivation of the pooled specimens. That, however, these negative results

were due merely to an absence of cholera in the investigated villages and not to any inadequacy of the pooling technique was proved by the examination of 2240 stool specimens from 245 staff members of a hospital where cholera patients were accommodated at the time. Cholera vibrios could be detected in the pooled stool specimens of these 245 persons upon five occasions and in all but one of these instances it was possible to identify the carriers in question by referring back to the remnants of emulsified stools left in the collecting bottles. Continued daily examination of their stools, on the other hand, failed to give positive results.

These findings as well as the results originally recorded by Müller support the contention that it is legitimate to resort to pooling tests when trying to detect cholera carriers in the course of mass examinations.

Macroscopic inspection of stools

General agreement exists that, though as a rule severely attacked cholera patients void rice-watery stools during the acute stage of the disease, this feature is of no diagnostic importance, because it is also quite often met in gastro-intestinal affections due to other causes. Moreover, it is frequently possible to isolate *V. cholerae* from quite uncharacteristic stools not only of carriers but also (*a*) of patients suffering merely from choleraic diarrhoea; (*b*) of individuals who, though afterwards showing symptoms and signs of a severe cholera attack, are first seen quite early in the disease; and (*c*) of sufferers past the acute stage of cholera or convalescing from it. The marked variance in the aspect of bacteriologically positive stools met with in laboratories to which faecal specimens of contacts as well as of cholera patients are sent is well illustrated by a series of observations recorded by Dunbar (1896), who succeeded in isolating *V. cholerae* from faecal specimens showing the following macroscopic appearances:

Typically rice-watery	10	Watery and bile-coloured	14
Like gruel (" mehlsuppenartig ")	6	Thin " faecal " fluid	49
Clear, watery, yellow	21	Thin, pap-like, brown	11
		Solid, brown	24

Hence, while, particularly during an epidemic, clinicians may with some reason pay attention to the evacuation of rice-watery stools by patients showing other features usually met with during the acute stage of severe cholera attacks, laboratory workers should beware of being influenced by the macroscopic aspect of the faecal specimens submitted to them for examination.

Bacterioscopic examination

In view of the rather limited importance accorded by modern cholera workers to bacterioscopic examinations, it is interesting to note that even

the opinions held by the early observers regarding the value of this diagnostic method were divergent. The following of these early statements deserve attention:

In a report presented at the 1884 cholera conference Koch stated that he had resorted to an examination of stool smears stained with watery solutions of fuchsin or methylene blue, but that in only comparatively few cases could a diagnosis be arrived at without resorting to cultivation. Occasionally, however, it was possible to find microscopically a number of vibrios sufficient to permit a diagnosis.

Some other early workers, of whom the first was Escherich (1884), warned against placing reliance in cholera-diagnostic work solely upon bacterioscopic examinations, particularly in view of the occurrence of spirilla which, more or less resembling *V. cholerae*, were specially apt to abound in the mucoid diarrhoeic stools of cholera nostras patients.

Schottelius (1885), admitted that the bacterioscopic examination of specimens taken directly from the stools of cholera-suspect individuals was apt to give doubtful or even frankly negative results. At the same time, however, he stressed that hanging-drop or stained preparations made after the stools had been enriched in broth [1] proved fully satisfactory and that, therefore, their microscopic examination sufficed to establish the diagnosis of cholera.

The statements made by Gruber (1887) regarding the problem at present under review, though interesting, have to be interpreted with caution, not only because he examined less cholera stools than intestinal contents of supposed victims of the disease, but mainly because most of these materials had reached him with considerable delay. As far as his findings go, they showed that bacterioscopic examinations alone were not of decisive diagnostic importance: on the one hand, several times no positive cultures could be obtained from specimens showing the presence of vibrios under the microscope, while, on the other hand, in two instances *V. cholerae* could be cultivated from microscopically negative specimens. However, Gruber found that reliable results could be obtained if hanging-drop preparations, protected against evaporation, were kept for half an hour. On account of the tendency of the vibrios to accumulate at the periphery of the drops, the re-examination of these preparations or of stained smears made from them was apt to give clear-cut results.[2]

Judging from observations made during the 1892 Hamburg outbreak, Fraenkel (1892) considered cultivation a far more reliable means of diagnosing cholera than smear examination. Nevertheless, the latter was apt to give frankly positive results as long as the stools showed the rice-watery appearance with mucus flocculi usually met with during the acute stage of the disease.

Revising his previous rather sceptical views, Koch (1893), in an important article on the current state of bacteriological cholera diagnosis, stated that according to the experiences recently made in his institute it had been possible to arrive at a positive diagnosis through mere smear examination of almost 50% of the stool specimens afterwards confirmed to contain *V. cholerae*, whereas not a single instance was met with in which subsequent tests necessitated a revision of the bacterioscopically established diagnosis. Koch stressed, however, that in order to get such reliable results, adequate experience on the part of the observers was an indispensable prerequisite.

The simple technique recommended by Koch for the bacterioscopic examination of cholera-suspect stools or intestinal contents consisted of the preparation of smears on

[1] See *Bull. Wld Hlth Org.* 1955, **12**, 796.

[2] It was presumably on account of this early observation by Gruber that the method of examining hanging-drop preparations of cholera-suspect materials not only immediately but also after ½ hour's incubation at 37°C was prescribed in the successive editions of the official German instructions for cholera-diagnostic work (see Kolle, 1904; Kolle & Schürmann, 1912; Kolle & Prigge, 1928). It was only in the last (1916) edition of these instructions, quoted by Kolle & Prigge, that the use of this method as well as examination of stained smears prepared directly from the suspect materials was made optional in all but the first cholera cases in a given locality.

cover glasses which, after they had become air-dry, were fixed by heat and stained with diluted carbol fuchsin.[1]

Dunbar (1896), reporting on the observations he had made during the 1892 Hamburg cholera outbreak, endorsed the value of bacterioscopic examinations. He recorded that he had demonstrated the presence of cholera vibrios with this method in 60.3% of 68 patients showing manifest clinical signs of the disease and in 53.2% of 47 persons suffering from choleraic diarrhoea but only in 14.8% of 27 individuals found to be healthy carriers.

Another claim made by Dunbar was that he succeeded in arriving at a speedy diagnosis of cholera by the examination of smears made from the initially inoculated peptone water cultures after short incubation. He stated that, using this method, he was capable in 12 out of 14 instances of arriving at a positive diagnosis with the aid of peptone water cultures incubated for only 3 hours. It deserves attention, however, that Babes (1914) as well as Verzár & Weszeczky (1916), who were able to confirm their bacterioscopic findings with agglutination tests, obtained disappointing results with this rapid method.

Generally speaking, the modern experts are agreed that bacterioscopic examinations do not suffice to establish the laboratory diagnosis of cholera and that even the presumptive value of this method is limited. The main objections made to it may be summarized as follows:

1. As has been stressed by several observers, recently, for instance, by Ahuja et al. (1950, 1951), the use of bacterioscopic examinations is rather redundant in that they prove most often and most frankly positive in those cases in which a presumptive diagnosis can be made on clinical grounds.

2. However, even in clinically suspicious and afterwards confirmed cholera cases bacterioscopic examination may give a negative result (see, for instance, Straus & Roux, 1884; Babes, 1914; Verzár & Weszeczky, 1916; Maitra & Basu, 1924; Pollitzer, 1926).

3. In countries like India and China, where cholera-like vibrios abound in the surface waters, the presence of these organisms in the stools not only of healthy persons, but also of individuals suffering from gastro-intestinal disturbances may prove quite misleading.

4. While the cholera vibrios in stool smears do not invariably show a typical morphology, other intestinal bacteria may assume an aspect more or less closely resembling that of vibrios. As shown by Baerthlein (1912) and some other workers (see summary by Kolle & Prigge, 1928), this is particularly true of the organism commonly known under the name of *B. faecalis alcaligenes* (Petruschky, 1896), met with quite frequently in work with cholera-suspect stools.

Though, in spite of these limitations and drawbacks, the time-honoured method of bacterioscopic examination is still recommended in some of the

[1] Since dilutions of carbol fuchsin are not stable, a concentrated solution must be kept in stock, prepared according to Ziehl (1882) by admixing 10 ml of a saturated solution of basic fuchsin in 95% ethanol to 90 ml of a 5% aqueous solution of carbolic acid (phenol). While Koch (1893) gave no specifications in this respect, most workers resorted for the purposes of cholera laboratory diagnosis to a dilution of one part of concentrated carbol fuchsin with 9 parts of distilled water, as prescribed for instance in the official German instructions (see Koch and co-authors, 1902). However, some experts preferred more concentrated solutions, Gallut (1954), for example, recently advocating the use of a carbol fuchsin dilution of 1: 5 for the purposes of cholera-diagnostic work.

modern guides for the laboratory diagnosis of cholera (see, for instance, Ahuja et al., 1950, 1951; Gallut, 1954), the present writer cannot help wondering whether it still deserves such attention. Working before and during the Second World War under rather difficult conditions in unoccupied China, he was mostly unable to use smear examination of cholera-suspect stools and never had reason to assume that this omission exerted an unfavourable influence on the results of cholera laboratory diagnosis, which almost always became available in less than 24 hours.

Flagellar staining

The differential diagnostic importance of the methods of flagellar staining, already referred to in the third of these studies,[1] is limited in that, though some of the cholera-like vibrios are distinct from *V. cholerae* in possessing more than one flagellum, this is by no means an invariable rule.

Various methods of flagellar staining have been recommended by the different observers who have given their attention to this method (see summaries of Gruber, 1894; Kolle & Gotschlich, 1903; Kolle & Prigge, 1928; Mackie, 1929a, and Pollitzer, 1934). Since these tedious and delicate procedures can be used only for research purposes but not for the routine laboratory diagnosis of cholera, it would be superfluous to enter here into a detailed description of their techniques.

Peptone water enrichment

While, as has been stated in the third of these studies,[2] the idea of using fluid media for the enrichment of cholera vibrios in faecal specimens was originated by Schottelius (1885), Bujwid (1888) was the first to recommend the use of peptone water for this purpose. However, it was only on account of the excellent results which Dunbar obtained during the 1892 Hamburg outbreak and which he afterwards recorded in 1896, that the value of the latter, eminently useful method was generally recognized. As stated by Koch (1893) in a preliminary account on Dunbar's work, the latter used for enrichment the weakly alkaline sterilized solution of 1% peptone and 0.5% sodium chloride originally recommended for the rapid growth of *V. cholerae* by Dunham (1887). In addition to originally using this medium in test-tubes for the inoculation of mucus flocculi or other small particles of the stools to be examined, Dunbar afterwards also resorted to the inoculation of larger amounts of the faecal specimens (1 ml or more) in correspondingly increased quantities of the enriching fluid in Erlenmeyer flasks. This last procedure was again recommended by Abel & Claussen (1895), who advocated, side by side with the tube method, inoculation of 10-20-ml amounts of the faecal specimens in 5-10 times the quantity of peptone

[1] See *Bull. Wld Hlth Org.* 1955, 12, 783.
[2] See *Bull. Wld Hlth. Org.* 1955, 12, 796.

water. After these fluids had been incubated for 20 hours, subcultures were made from their surface in peptone water tubes and examination of the latter was continued in the usual manner.

The utilization both of tubes filled with 10-ml amounts of peptone water and of flasks containing 50 ml each of this medium was also prescribed in the official instructions for cholera-diagnostic work compiled by Koch and co-authors in 1902 and promulgated in the same year. However, the use of the latter was restricted to the examination of specimens obtained from convalescents or supposed carriers, while in the case of the stools of cholera patients the inoculation of several (3-6) peptone water tubes was recommended. All these cultures were bacterioscopically examined after 6 hours' and 12 hours' incubation at 37° C, and each time subcultures in peptone water tubes as well as on solid media were made from the most promising growths.

In a further classical contribution to this subject, Hetsch (1903), while praising the value of peptone water enrichment, which had recently been confirmed through the large-scale investigations of Kolle & Gotschlich (1903), stressed that

" the peptone solution is by no means a medium offering selective growth conditions exclusively for the cholera vibrios but, according to their oxygen requirements and their motility, all vibrio species become more less enriched on its surface ". [Trans.]

Hence, Hetsch continued,

" it is essential for practical diagnostic work to determine the species of the enriched vibrios through reliable and easily applicable methods of identification and for this purpose their even distribution on agar plates and the testing of the colonies thus isolated with highly specific immune sera will serve ". [Trans.]

It is indeed the mode of procedure described by Hetsch' and not any rash attempt to use the primary peptone water cultures for the direct identification of *V. cholerae*, which constitutes the outstanding value of this and other methods of enrichment for cholera-diagnostic work.

The method of preparing peptone water for enrichment prescribed by Koch and co-authors (1902) was to make stock solutions by dissolving by heat, in 1-litre quantities of sterile distilled water, 100 g of peptonum siccum Witte, 100 g of sodium chloride, 1 g of potassium nitrate, and 2 g of crystalline sodium carbonate, to filter these solutions, and to fill them in 100-ml quantities into flasks which were then sterilized. Peptone water could then be easily prepared by mixing 1 part of this stock solution with 9 parts of water. The mixtures were then filled in 10-ml quantities into tubes as well as in 50-ml amounts into flasks, and sterilization was repeated.

The following comment must be made on this method, which was also prescribed in the subsequent editions of the official German instructions with the exception that the one published in 1916 and reprinted by Kolle & Prigge (1928) rendered it obligatory to use not less than 50-ml quantities of peptone water in flasks for all cholera-diagnostic work:

(*a*) While Koch and co-authors specified one particular kind of peptone, many other brands have been found equally suitable, and some even more suitable, for cholera-diagnostic work. However, as maintained by Kabeshima (1922) and by Bengston (1924), some kinds of peptone cannot be used for this purpose because they have a strongly acid reaction. As pointed out by Bengston, this untoward feature could be counteracted by a corresponding adjustment of the reaction of the media. Nevertheless, when one has to use a brand of peptone hitherto untried for cholera laboratory work, it is well to ascertain its suitability through tests with known strains of *V. cholerae*.

(*b*) It will be noted that, in contrast to the original recommendation of Dunham (1887), the official German instructions prescribed the use of peptone water containing 1% sodium chloride. While, as stated in the third of these studies,[1] some authorities advised the use of a salt concentration of 3%, many workers preferred to adhere to Dunham's formula, which is also given for peptone water manufacture in the standard textbooks on laboratory methods. Gallut (1954) recommended either following this practice or utilizing peptone solutions containing 30 g of sodium chloride per litre.

(*c*) The advice of Koch and co-authors (1902) to bring the peptone solutions to a suitable reaction by the incorporation of standard doses of sodium carbonate cannot be considered invariably satisfactory—not merely because this produces only a moderate alkalinity, but mainly because the reaction not only of different brands of peptone but even of the water used in the various laboratories may vary considerably. Modern workers therefore prefer to bring each lot of their peptone water media to a suitable pH by the easily applicable methods now generally used for the standardization of media. However, so far no uniform pH standard has been adopted for this purpose. Thus Mackie (1929b) recommended a pH of 8.0-9.0 for peptone water manufacture and Gallut (1954) recently one of 8.6, while the investigations of Read et al. (1939), referred to in the third study,[2] as well as those of Venkatraman & Ramakrishnan (1941), indicated the desirability of using a pH somewhat in excess of 9.0. As far as the present writer can judge, the figure of 9.2, suggested by the two last-mentioned groups of workers, might to advantage be adopted as standard for peptone water manufacture.

(*d*) Ample experiences have shown that, when dealing with the faeces of cholera patients in the acute stage of the disease, it is quite sufficient to implant loopfuls of the specimens in tubes containing 10-ml quantities of peptone water, as was originally prescribed by Koch and co-authors (1902).

[1] See *Bull. Wld Hlth Org.* 1955, 12, 790.
[2] See *Bull. Wld Hlth Org.* 1955, 12, 788.

Certainly, however, when examinations have to be made of the stools of convalescents and contacts, i.e., of specimens the vibrio contents of which are presumably scanty, it is indicated to inoculate correspondingly larger amounts of the faeces (Koch and co-authors proposed 1-ml quantities) into flasks containing 50 ml of peptone water. It is true that, if numerous examinations of this kind have to be made at the same time, the use of these larger amounts of peptone water might heavily tax the resources of the laboratories. However, as has been indicated earlier in this study, this difficulty can easily be overcome, and much labour can be saved at the same time, by using instead of individual faecal specimens the pooled stools of suspected cholera carriers for enrichment in peptone water or other suitable media.

As been discussed in the third of these studies,[1] the question for how long the peptone water cultures and subcultures used for enrichment should be incubated has also not been settled. It was stated that, while as a rule incubation periods of 6 hours or even more were recommended, the present writer found it sufficient, when dealing with the stools of patients during outbreaks, to incubate his peptone water cultures and subcultures for periods of 3 hours only. The great advantage of this procedure was that, without it being necessary to arrange for a night-shift of workers, even specimens received late in the afternoon could be plated on the same day and that thus a diagnosis could as a rule be arrived at on the following morning. As noted, the precaution was taken of keeping the peptone water cultures and subcultures (from the latter of which the platings had been made) so as to make it possible to re-examine them should the original platings give an indefinite result. Actually, however, instances where this was necessary were exceptional.

There can be no doubt, however, that in the case of specimens collected from cholera convalescents or from suspected healthy carriers the peptone water cultures and subcultures must be incubated for at least 4-5 hours, and preferably for 6 hours in the case of the primarily inoculated cultures. This prolongation of the incubation time entails hardly any inconvenience, because arrangements can as a rule be made for collecting such specimens early in the day.

Other enrichment methods

Several proposals have been made for using other fluid media for the enrichment of *V. cholerae* in suspect materials in place of the time-honoured peptone water method. Though the procedures recommended for this purpose in the past are not utilized any more, the following among them, because they attracted considerable attention for some time, deserve discussion :

[1] See *Bull. Wld. Hlth Org.* 1955, 12, 796.

Ottolenghi (1911) found that ox bile, after alkalinization with sodium carbonate, favoured the growth of *V. cholerae* while inhibiting that of *E. coli* and the other organisms usually met with in human faeces. He therefore prepared an enrichment medium as follows:

Fresh ox bile was filtered through paper, and 3% of a 10% solution of crystalline sodium carbonate and 0.1% potassium nitrate were added to the filtrate. The mixture was distributed in 5-ml quantities into tubes and these were sterilized in the autoclave at a pressure of ½ atmosphere.

Ottolenghi recommended that for each faecal specimen 3 of these tubes should be inoculated with 1 loop, 3 loops, and 0.1 ml of the material under test, respectively. After incubation, material for bacterioscopic examination and implantation of agar plates was taken from the surface of the bile cultures.

Experimenting with pure cultures and artificially contaminated faecal specimens, Ottolenghi found that his medium, while as suitable for enrichment as peptone water, sometimes promoted the multiplication of *V. cholerae* more slowly than the latter. However, owing to its inhibitory action on the other organisms, the bile medium facilitated the isolation of cholera vibrios on the plates even if platings were made from the primarily inoculated tubes with considerable delay. It therefore seemed particularly suitable for the examination of specimens from convalescents and suspected carriers, the vibrio content of which was apt to be scanty. The bile medium, like peptone water, facilitated the growth of cholera-like as well as of cholera vibrios.

While a few workers, including Hach (1924), who worked with actual cholera stools, confirmed the good experiences of Ottolenghi, others did not share these favourable opinions. Thus, as summarized by Kolle & Prigge (1928), Dieudonné & Baerthlein (1912) and Haendel & Baerthlein (1912) noted that Ottolenghi's method "sometimes gave better results than peptone water enrichment, but that the latter was often successful where the bile method failed". Schürmann & Abelin-Rosenblat (1913) maintained in this connexion that if small inocula were used the bile medium could exert an inhibitory action on *V. cholerae* as well as on other organisms. In their opinion peptone water, which could be easily prepared and was stable, was preferable to Ottolenghi's medium.

Krombholz & Kulka (1912), while admitting that this medium exerted an inhibitory action on certain competitors of *V. cholerae* occasionally met with in the stools of the patients, considered it a far less suitable substrate for the growth of cholera vibrios than peptone water. These two workers stressed, moreover, that:

"In general, the solution of the problem of a maximally certain and accelerated diagnosis in cholera-suspect cases lies less in the inhibition of the competing organisms than in the provision of optimal conditions of growth for the cholera vibrios." [Trans.]

Schoebl (1915) who, using dry instead of fresh bile, obtained unsatis-

factory results with Ottolenghi's method, also maintained that bile could exert an inhibitory action on the growth of *V. cholerae*.

All in all it is difficult to contradict the opinion of Kolle & Prigge that there was no necessity for the adoption of Ottolenghi's method in practical cholera laboratory work.

In an article which appeared in the same year as that of Ottolenghi, Kraus and co-authors (1911) reported on the suitability of a blood-alkali broth for the enrichment of *V. cholerae*. For practical purposes they recommended the preparation of this new medium as follows:

" To 100 ml of neutral broth are added 25 ml of blood alkali [prepared according to the method of Dieudonné (1909) described below]. The open flasks are kept for 3 hours at 50° and then for 24 hours at 37°, and are then tubed in 5-ml quantities and inoculated." [Trans.]

Kraus & co-workers claimed that this medium was superior to peptone water as far as both the inhibition of other organisms and the enrichment of *V. cholerae* were concerned.

The medium of Kraus & co-workers shared the fate of that of Ottolenghi, being considered suitable by some workers and unsatisfactory by others. Among the former observers were Dieudonné & Baerthlein (1912), Haendel & Baerthlein (1912), and Schoebl (1915). Sgalitzer & Loewy (1913) even claimed that this medium, while promoting the growth of *V. cholerae*, exerted an inhibitory action on the cholera-like vibrios. However, Schürmann & Abelin-Rosenblat (1913) disapproved of the method, and their opinion was evidently shared by Kolle & Prigge. Certainly account has to be taken of the tedious process of manufacturing the medium and of the inconstant results different lots may give.

Goldberger (1913) made the interesting proposal to use an alkaline-egg-peptone medium for the selective enrichment of *V. cholerae*. As summarized by Mackie (1929b), this medium

" is composed of whole egg mixed with an equal volume of water, to which mixture is added an equal volume of 5 per cent sodium carbonate; the alkaline-egg preparation is finally mixed with nine parts of peptone water ".

It was found that in this medium, the manufacture of which was again carefully studied by Bengston (1924), *V. cholerae* multiplied less rapidly than in peptone water but continued to multiply for a longer period.

According to Mackie, Goldberger's medium was " recommended as an alternative to alkaline peptone water in the Medical Research Council Special Report (1920) on the 'laboratory diagnosis of intestinal infections' ".

A further enrichment method to be referred to at the present juncture was that of Yen (1933), who prepared for this purpose a starch-containing medium in the following manner: 2 g Witte peptone, 1 g maltose, 0.5 g potassium nitrate, 0.5 g crystalline sodium carbonate, 10 g sodium chloride, and 0.5 g crystalline magnesium chloride are dissolved in 900 ml of distilled

water; the solution is boiled for 3 minutes and then filtered. Next 100 ml of a 5% solution of soluble starch, which has been previously boiled for 2 minutes, are thoroughly mixed with the filtrate. The mixture is filtered through cotton and then through asbestos. After readjustment of the volume the clear filtrate is sterilized by boiling for 3 minutes and is then kept in the refrigerator. Immediately before use the reaction of the medium is adjusted to pH 9.0-9.2, 5 ml of a saturated solution of phenolphthalein in 50% ethanol per litre serving as indicator. The ready-made medium is distributed into sterile 50-ml flasks.

To utilize this medium, 0.1-0.2 ml amounts of fluid, or loopfuls of solid stools, were inoculated in 50-ml quantities and the flasks were incubated for 5-8 hours. If a decolourization of the fluids indicated the presence of cholera or cholera-like vibrios, direct agglutination tests were made with material taken from the surface of the growths. If, however, decolourization was incomplete or the growths were too scanty for agglutination tests, 1-ml quantities were transferred to new flasks for a further incubation for 5-8 hours.

Making comparative tests with his medium and with peptone water, Yen found that the former better promoted the growth of *V. cholerae* and restricted the growth of *E. coli* than the latter. However, as far as these experiences, made with artificially contaminated faecal specimens, went, even the degree of inhibition produced by the starch media was by no means spectacular.

The modern phase of this problem may be said to have started in 1939, when Read recommended the use of a modification of the bismuth-sulfite medium originally devised by Wilson & Blair (1931), for work with typhoid bacilli, for the selective enrichment of *V. cholerae*. The prescriptions given for the preparation of this modified medium, as brought up to date in a WHO technical report published in 1950, were as follows:

Formula of the medium :	*ml*
2% peptone solution	8.8
Sea-salt mixture	1.2
Distilled water or stool suspension in distilled water	10.0
10% mannose solution	1.0
Liquor bismuthi	0.12
20% sodium sulfite solution	1.2
Absolute alcohol	0.2
Mercury perchloride ($HgCl_2$), 1/10 000 solution	0.8

adjusted to a pH of 9.2 with N caustic soda, with thymol blue as indicator.

Formula of the sea-salt mixture :	*Parts*
Sodium chloride (NaCl)	27.00
Potassium chloride (KCl)	1.00
Magnesium chloride ($MgCl_2$, $6H_2O$)	3.00

Magnesium sulfate ($MgSO_4$, $7H_2O$)	1.75
Distilled water	100.00

Formula and preparation of liquor bismuthi :

Bismuth citrate	60 g
Ammonia (12.5%)	20 ml
Distilled water to make up to	500 ml

Liquor bismuthi is prepared as follows: a bottle with a ground-glass stopper is filled almost completely with 500 ml of distilled water and the liquid level is marked on the side of the bottle. The water is poured out, and 60 g of bismuth citrate are introduced through a wide funnel, followed by 50 ml of distilled water. The citrate is mixed with the water into a smooth paste, using a glass stirrer. Next, 20 ml of 12.5% ammonia are added. The mixture is stirred with a glass rod and a chemical reaction takes place with evolution of heat. The glass stopper is inserted, the bottle shaken, and as soon as the bismuth citrate has almost entirely dissolved distilled water is added up to the 500-ml mark.

Note. It should be noted that Read & Pandit (1941), while otherwise following the formula for preparing the medium, reduced the amount of liquor bismuthi to 0.04 ml.

Preparation of the medium :

All ingredients are made up in separate solutions and kept in stoppered bottles. According to Read (1939) the 2% peptone solution and the distilled water alone were autoclaved before use. The sea-salt mixture, if made up with sterile water, remained sterile for practical purposes. The mannose was made up in 10% solution for the needs of the day and was sterilized by boiling, while the sodium sulfite was merely exposed to slight heat to dissolve it.[1]

Some modifications of Read's formula were proposed by Wilson & Reilly (1940), who recommended in particular the following method for preparing a sulfite-bismuth mixture:

" . . . to avoid any variation and for convenience we have found it an advantage to use bismuth ammonio-citrate scales instead of liquor bismuthi. In the sulphite-bismuth solution there is put glucose (if we had had a supply we would have used mannose) which not only serves for the nutrition of the vibrios but also prevents oxidation of the sulphite.

" The stock solution is prepared by dissolving 20 g. sodium sulphite anhydrous in 100 c.c. of boiling water and adding to it 0.1 g. bismuth ammonio-citrate scales dissolved in 10 c.c. of water. A precipitate of bismuth hydrate separates out on boiling. A solution of 20 g. of commercial glucose in 100 c.c. of boiling water is made and when cool both solutions are mixed. In place of glucose, saccharose, mannitol or mannose may be employed. The stock solution keeps for months and is added to the saline peptone water just before use: the *p*H of the stock solution is 9.4.

" To 100 c.c. of saline peptone water *p*H 9.1, 10 c.c. of stock glucose sulphite bismuth mixture are added and then 1 c.c. of absolute alcohol. We have found little advantage in the addition of 4 c.c. of 1/10,000 $HgCl_2$. Wilson & Blair employed $HgCl_2$ with a view to the suppression of *Proteus* strains, but recently we have found it disappointing

[1] Indications for the preparation of a cheap substitute for chemically pure mannose from ivory-nut shavings (a by-product in button manufacture) have been given by Bose (1939) and in an improved form by Narayanan (1941). These substitutes were found to be suitable not only for the manufacture of the above medium but also for the preparation of solutions for fermentation tests. However, as pointed out by Read & Pandit (1941), " allowances in the media formula had to be made owing to the reduced concentration of mannose obtained in the rough extracts (4 per cent to 6 per cent) ".

for this purpose. In the tubes enterococci often develop, and we have an impression that $HgCl_2$ tends to suppress them.

" In our work tubes containing 10 c.c. of the enrichment medium were inoculated with a drop of peptone water culture of the vibrio. In field work it would probably be found more convenient to employ a medium of double strength and to add to it an equal volume of a saline faecal emulsion."

Concerning the last paragraph of these instructions it should be noted that, for the purpose of stool examinations, Read (1939) originally recommended placing the portions of his medium, prepared according to the formula on page 721, into 50-ml screw-capped medicine bottles, but, in order to accomodate the Seitz discs or filter-papers used for an improved method of water examination (see below), he recommended substituting 100-ml wide-mouthed, glass-stoppered flasks. According to him stool emulsions were prepared

" by measuring out the required volume of stool in the barrel of a Roux syringe and mixing with an equal quantity of normal saline and filtering through a single layer of lint. The mixture could then be diluted as required so that the volume added to the media bottle was 10 c.c."

Commenting on the results of his laboratory observations, Read maintained that:

" By the use of this medium mannose-fermenting vibrios can be successfully differentiated from non-mannose-fermenting vibrios and from coliform types. Other common water and stool organisms except streptococci are suppressed but no method of facilitating *V. cholerae* against mannose-fermenting inagglutinable vibrios was discovered. The value of the method will depend on whether the mannose-fermenting vibrios found in natural sources can outgrow *V. cholerae* or not. The difficulty due to the growth of total organisms when ordinary peptone-water enrichment is employed is overcome."

Parallel with the laboratory investigations discussed above, a field trial of the new medium was undertaken by Seal (1939), who, besides using the faecal samples, collected by him according to the method described earlier in this study (see page 709), in 10-ml quantities for inoculation in Read's fluid, for the sake of comparison also inoculated 2-ml quantities into 20 ml of 1 % peptone water. In both cases the pH of the fluids after inoculation was readjusted to 9.2. Plates were implanted after the flasks had been incubated at 37° C overnight.

Evaluating the results obtained with a total of 309 faecal specimens, Seal stated that :

" Of the cases of clinical cholera 64.7 per cent yielded *V. cholerae* in the new medium as compared to 43.1 per cent in alkaline peptone water. Only 4 of 21 recent contacts gave positive results with both media."

A further important finding was that :

" Compared with the alkaline peptone water there was some restriction of growth of the inagglutinable vibrios in the new medium and the majority (87.4 per cent) were mannose fermenters."

The value of this method for the selective enrichment of *V. cholerae* was confirmed through the laboratory studies of Wilson & Reilly (1941) and through further field work of Read & Pandit (1941).

Wilson & Reilly (1941) found that—in contrast to 31 strains of cholera vibrios, which developed rapidly and profusely in their medium—only 6 out of 25 cholera-like and " para-cholera " strains grew well, and 19 but scantily or not at all. Out of 11 El Tor strains 5 only grew in the fluid bismuth-sulfite medium.

Recording the results of their field investigations, Read & Pandit (1941) stated that they had found

" the non-haemolytic agglutinable vibrio ... in all except one of the clinical cases in areas where the presence of cholera could be established, provided the examination was carried out sufficiently early in the disease ".

It is further noteworthy that Venkatraman (1949) used the enrichment method described above with evident success for the detection of *V. cholerae* in pooled stool samples which had been collected from the staff members of a cholera hospital (see page 711 above).

In a series of articles which began to appear two years after Read's initial publication, Gohar (1941, 1951) and Gohar & Makkawi (1947, 1948a, 1948b) recommended a medium containing potassium tellurite for the selective enrichment of *V. cholerae*. The technique finally adopted for this purpose was thus described by Gohar & Makkawi (1948b) and by Gohar (1951):

Whenever the medium was required, an aqueous solution containing 1% peptone and 0.5%NaCl was made, and enough sodium carbonate (usually 0.2%) was added to obtain a pH of 9.0. 0.5% sodium taurocholate was added and the medium was distributed into flasks, preferably 25-ml conical flasks, which were filled to the bottom of the neck so as to obtain a comparatively small surface and thus to concentrate the cholera vibrios growing in the medium.

If possible 3 such flasks were provided for each faecal specimen, to which enough potassium tellurite was added to obtain concentrations of 1/100 000, 1/120 000, and 1/400 000, respectively. However, if economy was essential, the medium was distributed in 10-ml quantities into tubes, and 0.002% of potassium tellurite was inserted to produce a concentration of 1/200 000. This usually sufficed to inhibit the growth of *E. coli*, while the sodium taurocholate suppressed that of coccal forms and most of the anthracoids met with in the stool specimens.

The flasks or tubes were heavily inoculated with the stools and incubated at 37° C, loopfuls being taken from the surface after 8 and again after 24 hours with the help of a loop bent at right angles, so that it was level with the surface when touching the medium. The material thus taken was implanted into a special semi-solid agar containing 1% mannite and 0.1% glucose with Andrade's indicator (see Gohar, 1947, 1948).

Testing several other chemicals and dyes besides potassium tellurite for their suitability in inhibiting the growth of the usual intestinal organisms without interfering with that of *V. cholerae*, Gohar & Makkawi (1947) found

potassium and sodium selenite in dilutions of 1: 500 to 1: 1000 of limited usefulness in this respect. Neutral red and other dyes, on the other hand, were found to exert an inimical action on the vibrios rather than on *E. coli* and *B. faecalis alcaligenes.*

Before dealing with a further potassium tellurite medium recommended by Ch'i & Zia (1949), attention has to be drawn to a peculiar method devised for the examination of cholera-suspect faecal specimens by Panja (1942), who found

" that when a sample of cholera stool is put into an L_3 candle fitted into a wider test-tube containing peptone-water in such a position that the unglazed part of the candle is covered by the surrounding peptone-water and the whole is then incubated, vibrios from the stool grow through the candle into the peptone-water in 24 to 48 hours, and sometimes a pure culture is obtained by this procedure in 18-20 hours. *Bact. faecalis alkaligenes,* motile *coliform* organisms and late lactose-fermenters also grow through the candle but not so readily as the vibrios ... If a small amount of the stool is mixed with peptone-water and partly aspirated through the candle into the surrounding peptone-water by vacuum action, growth of the vibrios occurs earlier (18 to 20 hours) ".

Panja further established that the value of this method for the isolation of *V. cholerae* was greatly enhanced if, in order to counteract the growth of coliform organisms, boric acid in a strength of 0.08% was added to the peptone water and the final pH was adjusted to 9.0.

The great value of Panja's " candle-boric-peptone-water " method was demonstrated by tests with 45 stool samples collected during the declining period of an epidemic from patients showing clinical signs of cholera: while direct plating of these specimens on bile-salt agar gave only 44% positive results, *V. cholerae* was isolated with the aid of Panja's method in 87%. Further, as will be recorded later in this study (see page 749), Panja's method of cultivation also yielded considerably more positive results than direct plating of cholera-suspect faecal specimens on a bismuth-sulfite medium.

However, impressive though these results are, account has to be taken of the great difficulties which would arise with the large-scale use of Panja's method under field conditions. He himself pointed out in this connexion that :

" To ensure success candles should be tested before sterilization for patent porosity and leakage by forcing air under pressure of 15 lb. to 20 lb. [1.05 to 1.40 kg per cm^2] above the atmospheric pressure, while the candles are immersed in water. If no air passes through, blockage is indicated and if large bubbles come out there is leakage."

It is well-nigh impossible to see how in places where no first-class laboratory facilities are available, this desideratum could be fulfilled and how under such circumstances the candles could be adequately sterilized without damaging them. It is, therefore, not surprising to find that, in spite of its excellent record, Panja's method was not mentioned in the instruc-

tions for cholera laboratory work compiled by a group of the leading Indian cholera experts (see Ahuja et al., 1950, 1951).

Ch'i & Zia (1949) tried to combine the enriching properties of the fluid medium of Yen (1933—see page 720) and of potassium tellurite in the following way:

A broth base was prepared by adding 5 g of beef extract, 10 g of peptone, 8 g of sodium chloride, and 1 g each of potassium nitrate and of magnesium chloride ($MgCl_2$, $6H_2O$) to 900 ml of distilled water. After this mixture had been heated to solution, it was added to a solution of 5 g of soluble starch in 100 ml of water, which had been boiled for 2 minutes.

The pH of the fluid was next adjusted to 9.2 with 10 N caustic soda, using 0.04% thymol blue as indicator, or with the aid of a potentiometer. The medium was then distributed into flasks in 100-ml quantities and sterilized in the autoclave at 12 pounds per square inch (0.84 kg per cm^2) for 20 minutes.

Immediately before use 1 ml each of a sterilized 0.2% aqueous solution of potassium tellurite and of a 0.5% solution of rosolic acid in 90% ethanol were added to each flask. After thorough mixing, the medium was then distributed in 10-ml amounts into sterilized tubes.

To test the medium, the tubes were inoculated with drops of artificially cholera-contaminated stool emulsions. After an incubation of from 10 to 24 hours, the tubes were inspected and those showing decolouration were tested with Gram's iodine solution. The growths giving a negative starch reaction were then used for slide agglutination tests and for plating.

While the results obtained with this new medium in the laboratory were most satisfactory, Ch'i & Zia stressed that

" while absence of decolorization and positive starch test have invariably indicated the absence of *V. cholerae* in the specimens, simple decolorization and negative test did not always indicate growth [of this organism]. It appeared to us that certain as yet unknown enzymes probably acted on the starch to produce this false effect. For that reason the medium must be either freshly prepared before use, or it must be stored in flasks with rubber stoppers before its final preparation . . . "

The two workers added that :

" In view of the inhibitory action of rosolic acid and potassium tellurite, it may be possible to plant larger inoculum with feces from suspected carriers or with infected water. In that case, a double strength liquid medium may be employed and equal volume of inoculum could be used. The possibility of recovering the infecting organism may thus be greatly increased."

However, one cannot help feeling that the rather complicated procedure involved would limit the usefulness of this method, even if it should be found as satisfactory in the field as in laboratory tests.

While trying to devise a solid medium suitable for the identification of *V. cholerae*, Dishon (1951) also established that a fluid medium prepared on similar lines was useful for the selective enrichment of this organism. The fluid medium was obtained by adding the following ingredients to

100-ml quantities of a broth base, containing 0.6% meat extract, 0.5% bacto-peptone, and 2% NaCl:

10% sodium sulfite solution, 1.5 ml; 20% sodium carbonate solution, 4.0 ml; 20% saccharose solution, 2.5 ml; saturated alcoholic solution of acid fuchsin, 0.2 ml; as well as gentian violet and brilliant green to a concentration of 1/200 000.

Dishon stated that his fluid medium

" inhibits the growth of Coliforms, spore formers and Pseudomonas for 12 hours, and of Cocci for 24 hours, whilst the pellicle of the *Vibrio cholerae* appears after the first 6 hours ".

In comparative tests made with artificially contaminated faecal specimens, it was found that cholera vibrios could be isolated with the aid of Dishon's fluid medium when present in the stool samples in a dilution of 10^{-9}, whereas in the case of alkaline peptone water the highest dilution from which positive results could be obtained was 10^{-7}. It deserves attention, however, that the peptone water used for these tests had a pH of only 8.5-8.7.

For practical purposes Dishon recommended adding 1 ml of the test specimens to 10 ml of the fluid medium, incubating for 6 hours at 37° C, and then taking material for plating besides making stained smears from the pellicle.

A comparative study of the suitability of various fluid media for the growth of *V. cholerae* in the presence of other organisms with which it has to compete under normal conditions was made by Felsenfeld et al. (1951). Details of the technique they used for this purpose were as follows:

The media used in 100-ml quantities were (1) the preserving fluid of Venkatraman & Ramakrishnan (1941—see page 709); (2) Wilson & Reilly's (1940) modification of the bismuth-sulfite medium, prepared by dissolving 20 g anhydrous sodium sulfite in 100 ml boiling distilled water, adding 10 ml of a 1% solution of iron ammonium sulfate " green scales " and 100 ml of a 20% glucose solution and mixing 10 ml of this stock solution with 100 ml of peptone water,[1] containing 1% peptone and 2% NaCl, pH 9.2; (3) Gohar's (1948) medium, made up by adding 0.2 ml of a 1% aqueous solution of potassium tellurite to 100 ml of peptone water, pH 7.8-8.0; (4) alkaline peptone water, containing 1% peptone and 0.5% NaCl; (5) alkaline selenite-F ' broth ' " prepared by adding enough 10 per cent aqueous sodium carbonate solution to Selenite-F . . . to bring the pH of the fluid to the desired alkalinity " (see tabulation below).[2]

The inocula used for these tests consisted of (*a*) 500-1000 cholera vibrios per ml of the media and (*b*) 10 000-15 000 organisms each of *E. coli*, *Aerobacter aerogenes*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and an enterococcus strain.

The results of the tests made according to this technique with 53 cholera strains were summarized by Felsenfeld et al. in the form of the following

[1] Though Felsenfeld et al. spoke at this juncture and also elsewhere in their article of " peptone broth ", they obviously referred to what is called peptone water in the present study.

[2] The commercial selenite-F product used by Felsenfeld et al. was undoubtedly similar to, if not identical with, the " selenite F broth", prepared according to the handbook, *Diagnostic Procedures and Reagents*, issued by the American Public Health Association (1950), by dissolving 5 g peptone, 4 g lactose, 10 g anhydrous sodium phosphates and 4 g sodium acid selenite in 1 litre of water.

table, showing the average multiplication rate of cholera vibrios in fluid media inoculated with bacterial mixtures (as specified above):

Medium	*Vibrio count in millions per ml medium after incubation*			
	At 37° C		*At 22°-26° C*	
	24 hours	*48 hours*	*24 hours*	*48 hours*
Venkatraman & Ramakrishnan	100	420	30	250
Wilson & Reilly	70	90	25	30
Gohar	380	800	250	370
Alkaline peptone " broth " (water)	400	880	220	680
Selenite broth pH 6.9 to 7.1	150	75	40	45
Selenite broth pH 7.4 to 7.6	270	310	130	160
Selenite broth pH 7.8 to 8.0	330	570	180	240

It will be noted that in these laboratory tests the modified bismuth-sulfite medium gave results far below those obtained by enrichment in the other media. However, as has been discussed above, the efficacy of the bismuth-sulfite medium for the selective enrichment of *V. cholerae* under actual conditions has been amply proven, so that there is no reason to doubt its full adequacy for practical cholera laboratory work. At the same time, however, it would seem indicated to compare its efficacy in diagnostic work with that of the potassium tellurite medium, because the latter, if equally reliable, would be preferable on account of the simplicity of its preparation.

Cultivation on solid media

The method of cultivating cholera-suspect stools on solid media originally recommended by Koch (1884) was as follows:

" A very small mucus floccule is put into 10 cc nutrient gelatin [meat-infusion peptone gelatin with a gelatin content of 10% and a weakly alkaline reaction] and is distributed by moving the fluid. One then pours the fluid gelatin on a horizontal glass-plate, which is kept cooled by putting ice under it. If spread out with a sterile glass rod, the gelatin very rapidly solidifies. The plate is kept moist under a glass bell until the bacterial colonies develop and ... is then examined with a suitable magnification of the microscope." [Trans.]

As Koch described in great detail, the growth appearances of *V. cholerae* on the gelatin plates, and more still those becoming manifest when gelatin stab subcultures were made from the cholera-suspect colonies developing on the plates, seemed to be so characteristic as to permit a distinction of this organism from all other bacterial species. On the other hand, though briefly mentioning that the cholera vibrios could be grown also on " agar-agar " plates, Koch evidently placed no confidence in this method of cultivation.

Again dealing with the methods of cholera laboratory diagnosis in 1893, Koch no longer upheld the diagnostic importance of gelatin stab-cultures, but still stressed that of gelatin plates, particularly if this mode of cultivation was used in combination with peptone water enrichment. He empha-

sized, however, that in order to obtain reliable results, the gelatin plates (made by then with the aid of dishes devised by Petri, 1887) had to be kept at or quite near 22° C, because only under these circumstances did the cholera colonies, after an incubation of 15-20 hours, assume their characteristic aspect. If, he continued,

" the cultures are kept at too high a temperature, or if the gelatin is unsuitable and becomes too soft at 22°, then the cholera colonies liquefy the gelatin to a greater extent, and thus assume an aspect closely resembling that of Finkler's bacteria ". [Trans.]

It will be noted that Koch thus still stuck to the belief that the growth appearances of *V. cholerae* on gelatin plates were different from those of the cholera-like vibrios—a belief which not long afterwards was definitely disproved (see, for instance, Kolle, 1903; Kolle & Gotschlich, 1903; Hetsch, 1903).

While admitting that a practised observer could also distinguish the cholera vibrios developing on agar plates from the usual faecal and water bacteria, Koch considered it necessary to verify the vibrio nature of such suspect colonies through bacterioscopic examinations. However, the great advantage of agar cultures was that they could be incubated at 37° C and therefore, in contrast to the gelatin plates, yielded colonies of a size suitable for identification tests after as little as 8-10 hours. Koch pointed out, however, that in order to obtain such satisfactory results, it was necessary (*a*) to resort to preliminary enrichment in peptone water; (*b*) to spread out the material to be examined on the surface of the plates instead of admixing it to the liquefied agar; and (*c*) to use agar plates with a dry surface, obtained by keeping them for a few days in the incubator before inoculation.[1]

As has already been described in the fourth of these studies,[2] the wellnigh ritualistic scheme for cholera diagnosis painstakingly built up by Koch was soon swept aside by the tide of epochal discoveries in the field of immunology which led to the introduction of far more reliable serological methods for the identification of *V. cholerae*. This new orientation of cholera-diagnostic work was fully recognized in the regulations framed by Koch and co-authors (1902) for official use. While cultivation on gelatin as well as on agar plates was still made obligatory in these instructions, it was recommended that the pure subcultures necessary for identification tests be obtained from agar plates after an incubation ranging from 12 to 18 hours.

The method of preparing the gelatin media was thus described in these instructions:

(1) A meat-peptone broth was prepared by (*a*) digesting fat-free minced beef in water for 24 hours in the cold (or for 1 hour at 37° C) at a ratio of ½ kg of the meat to 1 litre of water; (*b*) squeezing out the juice of the beef through a cloth; (*c*) adding

[1] Koch and co-authors (1902) stated that incubation of the plates for ½ hour was sufficient, provided that the dishes were kept open during this time in an inverted position.

[2] See *Bull. Wld Hlth Org.* 1955, 12, 945.

per litre of the fluid thus obtained 10 g of peptone and 5 g NaCl; (*d*) boiling for ½ hour; (*e*) rendering the reaction alkaline with sodium carbonate solution; (*f*) again boiling for ¾ hour; and (*g*) filtering.

(2) To prepare the medium, 1 litre of this broth was added to 100 g of gelatin, the latter dissolved by gentle heating, and the fluid rendered neutral to litmus and then alkaline by the addition of 3 ml of a 10% solution of crystalline sodium carbonate per 100 ml. The medium was then heated in the steam sterilizer for ¾ hour and filtered.

Similarly, the method of agar preparation prescribed in the German instructions was to add 30 g of agar per litre of the neutral broth described above, to boil until this was dissolved, to neutralize again, and to add 3 ml of a 10% solution of crystalline sodium carbonate to each 100 ml. The alkalinized medium was again boiled for ¾ hour, filtered, distributed in flasks or tubes, and sterilized repeatedly in the steam sterilizer (or, according to the present practice, once in the autoclave).

As in the case of peptone water manufacture, modern workers, instead of using standard doses of sodium carbonate solutions to bring the solid media for cholera-diagnostic work to an adequate degree of alkalinity, will prefer to determine the pH and to bring this to a proper standard by the addition of the amount of a suitable alkali required for each lot of the media in preparation. A pH above 9.0—e.g., one of 9.2—is desirable in the case of the solid plain media as well as in that of peptone water (see page 717).

Though the problem of preparing solid media selectively suitable for the isolation of *V. cholerae* began to attract much attention only after Dieudonné had proposed such a medium in 1909, this question had already been considered by several earlier workers. Some of them, for instance, Dahmen (1892) and Fraenkel (1892), stressed in this connexion the importance of using, instead of the weakly alkaline gelatin originally resorted to by Koch (1884), a gelatin with a stronger alkalinity. Deycke (1893) recommended for the same purpose an alkali-albuminate gelatin, while Dunbar (1896) worked not only with the usual gelatin but also with soda-gelatin plates.

In view of the ample use afterwards made of blood and blood derivatives for the manufacture of cholera-selective media, it is interesting to note that Heim (1901) devised " blood-decoction " (" Blutdekokt ") media for the isolation of *V. cholerae*. To prepare these in solid form he added gelatin or agar to the fluids obtained by (*a*) boiling the coagulum of cattle, horse, or pig blood, mixed with equal parts of water, in the steam sterilizer; (*b*) squeezing out the juice through a cloth; and (*c*) filtering. The resulting liquid was weakly alkaline in reaction.

The use of the medium originally introduced by Drigalski & Conradi (1902) for the isolation of *S. typhosa* for cholera-diagnostic work was recommended by Hirschbruch & Schwer (1903). The modified method utilized by the last-mentioned two workers for preparing this medium was as follows:

20 g agar, 10 g Liebig's meat extract, 10 g peptone, and 5 g NaCl are boiled in 1 litre of tap-water for 1½ hours and, after filtration, for a further 30 minutes. After addition

of 15 g lactose and boiling for 15 minutes, the reaction is rendered alkaline with the aid of a sterilized aqueous solution of sodium carbonate. One adds then 130 ml litmus solution (Kubel-Tiemann), which has been boiled for ½ hour as well as 10 ml of a solution of crystal-violet B in 100 ml hot distilled and sterilized water. After thorough shaking, 8-ml amounts of the medium are used to pour plates, which are kept uncovered for about ½ hour until they have cooled and solidified.

Hirschbruch & Schwer stated that, in contrast to the colonies of *E. coli*, which, when grown on this medium, were red and surrounded by a red zone, cholera colonies, becoming well developed after an incubation of 10-20 hours, were blue and surrounded by a blue zone.

Commenting upon ample tests they had made with different pure cultures and also with artificially cholera-contaminated specimens of diarrhoeic stools, Hirschbruch & Schwer pointed out that many cholera-like vibrios reacted on this medium like *V. cholerae*. Identification tests were therefore indispensable. For the latter purpose they recommended the use of hanging-drop preparations for preliminary agglutination tests and confirmation of positive results thus obtained by Pfeiffer's reaction.

In a subsequent paper, Hirschbruch & Schwer (1904) advocated the use, instead of litmus solution, of azolitmin in a proportion of 0.4 g per litre of agar for the preparation of their medium. The suitability of modified Drigalski-Conradi media for the isolation of cholera and cholera-like vibrios was confirmed by Klein (1905). Subsequently Rivas & Smith (1912) advised direct cultivation of cholera-suspect faecal specimens on lactose-litmus agar, while Stokes & Hachtel (1913) recommended the use of an azolitmin-containing lactose-glycerol agar for the isolation of *V. cholerae*. Aronson (1915) asserted in contrast to these workers that, owing to the fermentation of lactose produced by this organism, it grew on Drigalski-Conradi plates in the form of reddish instead of blue colonies.

Further solid media for the selective cultivation of *V. cholerae* devised from 1909 onwards by a considerable number of workers may be classified as follows:

1. Media prepared with blood or blood derivatives

As already mentioned, Dieudonné in 1909 introduced a blood-alkali agar for the selective cultivation of *V. cholerae*, describing the manufacture of this medium as follows:

" If one adds to defibrinated cattle blood equal parts of normal caustic potash, a laked blood-alkali solution is formed which can be sterilized in the steam-sterilizer. If one adds 30 parts of this solution to 70 parts of ordinary litmus-neutral agar, [one obtains a medium] on which the cholera vibrios grow abundantly, whereas *B. coli* develops not at all or only very scantily." [Trans.]

Dieudonné pointed out that it was necessary to dry the plates poured with this medium for several days at 37° C or for 5 minutes at 60° C. While plates implanted with normal stools showed no bacterial growth at all,

V. cholerae could be isolated from those inoculated with artificially cholera-contaminated faecal specimens. The agglutinability of the cholera vibrios thus cultivated was not impaired in comparison with that of agar-grown vibrios—an observation which, in spite of some statements to the contrary, was confirmed by the majority of the subsequent workers.

While Dieudonné in his short note gave no description of the growth appearances of the cholera vibrios on his medium, Huntemüller (1909) stated in an article published at the same time that the organisms developed on this medium in the form of large, circular colonies with entire edges, showing a glassy transparency in transmitted light, but appearing greyish in reflected light.

Huntemüller also gave the following more detailed indications for the preparation of the new medium:

> The cattle blood was collected in sterile glass jars containing glass beads, was defibrinated by shaking for ½ hour, and, after the addition of equal amounts of normal caustic potash, steam-sterilized for ½ hour. This blood-alkali solution, which kept indefinitely, was added, whenever needed, to neutral agar in a proportion of 3: 7 and plates were poured forthwith and dried for ½ hour at 60° C.

Huntemüller added that it was indispensable to keep the plates for at least 24 hours at room temperature before they were used so that the considerable amounts of ammonia which were at first given off by the medium and which inhibited the growth of *V. cholerae* could evaporate.

While confirming that Dieudonné's medium inhibited the growth of *E. coli*, Huntemüller found that it promoted the growth of cholera-like as well as of cholera vibrios and also noted in one instance that the medium, though considerably restricting the growth of *Ps. pyocyanea*, did not totally inhibit it. Nevertheless, the experiences made by Huntemüller in ample tests with a large number of cholera strains were so satisfactory that it was decided to use Dieudonné's medium side by side with alkaline agar for the examination of cholera-suspect stools in the Berlin Institute for Infectious Diseases headed by Gaffky.

The initial experiences of Dieudonné and of Huntemüller stimulated many further observations recorded in numerous publications. While admitting the great progress made in cholera-diagnostic work through the introduction of Dieudonné's medium, a considerable number of the subsequent observers pointed out that it was by no means fully satisfactory—mainly on account of the necessity of procuring fresh cattle blood for its manufacture and of the delay of at least 24 hours caused by the need for letting the plates mature. It thus appeared that it was impossible to take advantage of the new medium under the conditions where its use would have been most desirable—namely, when dealing with cholera-suspect stools in localities hitherto free from the infection.

It is important to note that neither of these main criticisms levelled against Dieudonné's method was fully justified. As Huntemüller had noted, the

blood-alkali mixture was rather stable and thus could quite easily be kept in stock to serve in emergencies in places threatened by cholera. Further, as shown by observations recorded by Haendel & Baerthlein (1912), it was even possible to exsiccate Dieudonné's medium after plates had been poured, the resulting coarse brown powder keeping well if protected against moisture and being reconditionable at short notice. Moreover, as found, in 1909, by Hachla & Holobut, it was not indispensable to use cattle blood for manufacturing the medium, pig or horse blood proving even more satisfactory for this purpose. Finally, as will be further discussed below, several methods were recommended for rendering the Dieudonné plates usable within a short time of being made.

Nevertheless, on account of the objections mentioned above and of others, during the years immediately following Dieudonné's publication several modifications of his mrthod and several substitute selective media were recommended. The following among these alternative procedures deserve particular mention.

(*a*) *Modifications of Dieudonné's method.* In a valuable study on cholera-selective media, Neufeld & Woithe (1910) recommended that, immediately before the plates were poured, 2 ml of 10% lactic acid should be added to each 100 ml of Dieudonné's blood-alkali agar medium in order to counteract ammonia formation and to neutralize any excess of alkali that might be present. The two workers found that plates made with this modified medium became ready for use as soon as they had been dried for ½ hour at 60° C. However, in contrast to ordinary Dieudonné plates, which remained fit for use for a week (see Weisskopf, 1911), those prepared according to Neufeld & Woithe's method remained selective for 24-28 hours only, because their alkalinity rapidly decreased.

Pilon (1911) claimed that freshly poured Dieudonné plates could be made fit for use by keeping them for about one hour in a carbon-dioxide atmosphere. He explained this result by the assumption that the unsuitability of such plates immediately after pouring was due to the presence of an excess of free caustic potash and not to that of ammonia, as maintained by most observers. In a CO_2 atmosphere the caustic potash was rapidly converted into potassium carbonate, whereas in the case of the plates exposed to air it took about 24 hours before this conversion reached a sufficient degree to render the medium fit for use.

Another way to hasten the maturing of Dieudonné plates was according to Moldovan (1912) to admix, when preparing the medium, only one part of the blood-alkali mixture to 4 parts of agar. He claimed that plates made from this modified medium could be used after only 6 hours, i.e., at the time when the peptone water cultures inoculated with cholera-suspect stools had become ready for subcultivation. However, Haendel & Baerthlein (1912) found that the media prepared in this manner as well as those made according to Neufeld & Woithe's method were less reliable than the

Dieudonné plates, occasionally inhibiting the growth of *V. cholerae*.

According to Hall (1916) it was essential to store the blood-alkali mixtures for 6-8 weeks in flasks plugged with cotton-wool, because plates prepared with such stock material were immediately ready for use. More important still was the assertion of Mackie (1929b)

" that by repeated steaming of the blood-alkali until the ammoniacal odour is removed, the completed [Dieudonné] medium prepared from it can be used immediately and the blood-alkali can be kept for considerable periods without loosing its selective properties ".

Studying possibilities of improving Dieudonné's medium, Hofer & Hovorka (1913) paid particular attention to the fact that this, while inhibiting the growth of most intestinal bacteria, exerted no such action on some organisms, especially the *B. faecalis alcaligenes* (see Glaser & Hachla, 1911). To enhance this incomplete selectivity, Hofer & Hovorka prepared a modified medium by (*a*) adding 4 ml of cattle blood to 16 ml of normal caustic potash; (*b*) incorporating this blood-alkali mixture into 80 ml of freshly prepared 3% neutral agar; and (*c*) then adding per 10 ml of the medium 0.5 ml of a 0.1% solution of crystal violet in distilled water. To become ready for use, the poured plates had to be kept partly open in the incubator for 24 hours and, after closing, for a further 12 hours at room temperature.

Experimenting with pure cultures of cholera and cholera-like vibrios as well as of *B. faecalis alcaligenes*, *Proteus*, etc., and with mixed peptone water cultures of these organisms, Hofer & Hovorka found that their modified medium effectively inhibited not only the growth of the bacillary species but also that of a number of cholera-like strains.

These favourable experiences were confirmed by Fügner (1914). However, Baerthlein & Gildemeister (1915), while admitting the high selectivity of Hofer & Hovorka's medium, found that it very often impeded the growth of *V. cholerae* as well.

Lentz (1915), obtaining unsatisfactory results in field work with Dieudonné media which had been kept in stock in dry form (but had apparently not been stored in a proper manner), recommended drying the blood-alkali mixture separately and admixing the resulting powder after solution in distilled water to the agar immediately before plates had to be prepared. He found that such media could be used at once and continued to give fully satisfactory results for 8-10 days.

While confirming the value of Lentz's method, Fürst (1916) found it advantageous to use, instead of distilled water, a 0.3% solution of sodium carbonate for dissolving the blood-alkali powder and to employ an agar containing 2% of cane sugar.

Finally, reference has to be made at the present juncture to the recommendation by Ghedini (1916) to enhance the selectivity of Dieudonné media by (*a*) titrating the blood-alkali solution and adding further alkali if necessary; and (*b*) using an alkaline, instead of a neutral, agar.

(*b*) *Substitute selective media.* Esch (1910) recommended that, instead of fresh cattle blood, dry haemoglobin, as commonly prepared from horse blood, should be used for the manufacture of blood-alkali media. A 5-g quantity of this substance was dissolved in 15 ml of normal caustic soda + 15 ml of distilled water, and, after the solution had been sterilized in the steam sterilizer for 1 hour, 15-ml amounts of it were added to 85-ml quantities of neutral agar. Esch (1910, 1912) stated that plates poured with this medium, which could be used after they had been dried at room temperature for 1 hour, were as satisfactory for the cultivation of *V. cholerae* as Dieudonné's original medium. Further, though not exerting such marked inhibitory action on other organisms, Esch's medium retarded the growth of even the alkali-tolerant *Ps. pyocyanea* to a degree sufficient for the practical purposes of cholera diagnosis.

According to Takano and co-workers (1926) a similar medium was also prepared by Tokunaga (1911).

Pilon (1911) stressed that, as had been shown by Deeleman (1897), the cholera vibrios were endowed not so much with a tolerance to caustic potash or sodium, as with a tolerance to carbonates. Pilon accordingly recommended preparing a medium for the selective cultivation of *V. cholerae* as follows: defibrinated blood of pigs, goats, or rabbits is mixed with an equal amount of a 12% solution of crystalline sodium carbonate and 3 parts of this mixture are added to 7 parts of neutral 4% agar; after thorough mixing the blood-soda-agar is poured into Petri dishes which are left open until solidification has taken place; the plates become fit for use after 30-45 minutes.

Following Esch's method, but using a different haemoglobin product, Kabeshima (1913) prepared a selective medium for cholera-diagnostic work by (*a*) adding to 80 ml of 3% neutral agar, which had been liquefied in the steam-sterilizer, 10 ml of an 18% solution of sodium carbonate; (*b*) boiling the mixture for about 10 minutes; (*c*) adding, after the agar had been cooled to about 50° C, 3 g of a commercial haemoglobin extract; and (*d*), after thorough mixing, using this material for pouring 7 plates, which were left uncovered until the agar had solidified. The plates were immediately fit for use, but it was well to dry them first by keeping them for 20-30 minutes open and in an inverted position in the incubator.

A medium similar to that of Kabeshima was recommended by Baerthlein & Gildemeister (1915).

An exhaustive study on the comparative value of Dieudonné's and the above-mentioned substitute media—with the exception of that of Esch, which in the experience of Haendel & Baerthlein (1912) showed an unsatisfactory degree of selectivity [1]—conducted by Baerthlein & Gildemeister

[1] It is important to note that, no doubt in view of the favourable experiences recorded by some other observers, the 1916 German instructions for cholera diagnostic work (see Kolle & Prigge, 1928, p. 81) recommended the use of Esch's medium if no suitable Dieudonné plates were available.

(1915) with over 100 stools of cholera patients or carriers, led to the following main conclusions:

(1) Pure cultures of *V. cholerae* grew most abundantly (" üppig ") within 16-18 hours on Kabeshima's medium, and vigourously on Dieudonné's and Pilon's media.

(2) Pure cultures of cholera-like vibrios invariably grew on these three media though less well than cholera vibrios.

(3) Pure cultures of *B. faecalis alcaligenes* usually grew abundantly on Dieudonné's and Pilon's media, but scantily on Kabeshima's medium. *Ps. pyocyanea* grew almost invariably on these three media, but developed scantily, while pure cultures of *Proteus* grew but exceptionally.

(4) Inoculation of faecal specimens of cholera patients or carriers invariably gave a positive result in the case of Dieudonné's and Kabeshima's media, while Pilon's medium failed in some instances.

(5) When such faecal specimens were used, pure cultures of *V. cholerae* resulted in 54.7% of plates with Kabeshima's medium, in 55.6% of Dieudonné and 57.5% of Pilon plates. Among the contaminants *B. faecalis alcaligenes* was met with in 25.7% of the Dieudonné plates, in 26.6% of Pilon plates, but in only 4.75% of the Kabeshima plates. While poorly developed colonies of enterococci were invariably present on all three media, colonies of *Proteus*, *Ps. pyocyanea*, and dysentery bacilli were met with but exceptionally.

(6) While the value of Dieudonné's original method for the isolation of *V. cholerae* had thus been demonstrated once more, it had to be stressed that this medium could not be used immediately and often did not sufficiently suppress the growth of *B. faecalis alcaligenes.*

(7) Pilon's medium, while immediately fit for use, was in general not inferior to that of Dieudonné but did fail occasionally.

(8) Apart from being highly selective and suitable for the cultivation of *V. cholerae* within 12-16 hours, Kabeshima's medium also had the advantages of (*a*) the commercial availability of its ingredients; (*b*) a simple method of preparation; (*c*) immediate readiness; and (*d*) marked inhibition of *B. faecalis alcaligenes.* However, this medium did not keep well and its alkalinity was apt to vary with the result that the growth of cholera vibrios was sometimes markedly impeded or even inhibited.

(9) To overcome these drawbacks, Baerthlein & Gildemeister advocated (*a*) sterilizing the haemoglobin extract through boiling in caustic potash; and (*b*) resorting, as far as necessary, to further alkalinization with a 5.5% solution of anhydrous sodium carbonate (" Sodamehl "). This modified medium kept for two weeks and ensured a constantly good growth of *V. cholerae.* Its selectivity was also satisfactory, an occasionally more

marked growth of *B. faecalis alcaligenes* never interfering with the rapid isolation of the cholera vibrios.

Whatever the merits of the substitute media were, both before and during the First World War Dieudonné's method was by far the most frequently used in actual laboratory practice by Bürgers (1910) and a considerable number of subsequent workers in Europe as well as by Greig (1913, 1917) in India, with equally satisfactory results. A large-scale study undertaken during the 1930 cholera outbreak in co-operation with the present writer by the Shanghai Public Health Laboratory (see Pollitzer, 1934) also confirmed the value of Dieudonné's method. Still, while parallel tests made with alkaline agar almost invariably gave positive results, absence of growth on the Dieudonné plates inoculated with the same cholera stools or peptone water subcultures, though rare, was not exceptional. The practical recommendations made on account of these experiences were that in laboratory work with actual cholera stools it was indispensable (*a*) to use cultivation on plain alkaline agar side by side with that on Dieudonné plates, and (*b*) to ascertain the proper ripeness of the latter through continuous controls with known *V. cholerae* strains.

Interest in the use of blood-alkali media in cholera laboratory work was revived through profound investigations undertaken by Vedder & van Dam (1932a, 1932b). Studying the reasons why the Dieudonné plates at first inhibited all bacterial growth and, after they had selectively promoted for some time the growth of *V. cholerae*, they lost their selectivity, Vedder & van Dam (1932a) came to the conclusion that in the case of this medium

" ripeness and selectivity are connected with a definite pH range which lies between about 9.0 to 9.6. At lower values other organisms also grow and therefore the plate is no longer selective. At higher values the cholera vibrio also does not grow. The ripening of the Dieudonné medium is due therefore to a decrease of the pH value, caused mainly by an entry of CO_2 from the air, but partly also by an elimination [' Austritt '] of NH_3 from the plate ... Differences in ripeness and selectivity of different Dieudonné fluids and series of plates as well as the variations in the ripeness and selectivity of one and the same fluid at different times are connected with differences in the pH values ". [Trans.]

On the basis of these and further studies Vedder & van Dam (1932b) recommended preparing the following two media for cholera diagnostic work:

(1) 1 g haemoglobin is boiled for a few minutes in 20 ml of 0.2 N caustic potash. After the fluid has been rapidly cooled, one adds 120 mg glycocoll and, after a few minutes, 80 ml of liquefied but not too hot agar, made from 1 g peptone, 0.5 g sodium chloride, and 3 g agar per 100 ml of water. The 6 plates poured from this material are immediately fit for use without preliminary drying in the incubator, but lose their selectivity if kept in storage.

(2) 1 g haemoglobin is triturated in a mortar with 4 ml K_2CO_3 (prepared by dissolving 6.9 g anhydrous potassium carbonate in 100 ml boiled distilled water). One then adds (*a*) 1 ml of $KHCO_3$ solution (prepared by dissolving 5 g of hydrogen potassium carbonate in 100 ml of boiled distilled water); (*b*) 10 ml of distilled water; and finally (*c*) 35 ml of

liquefied and somewhat cooled peptone-agar (see above), i.e., enough to pour 3 plates, which can be used immediately and, in contrast to those made with medium 1, retain their selectivity upon storage.

In laboratory tests, including those with cholera-contaminated faeces, both media proved most satisfactory: while cholera and El Tor vibrios invariably grew well or, particularly in the case of medium 2, even abundantly, other organisms, including *Ps. pyocyanea*, *B. faecalis alcaligenes*, *E. coli*, and enterococci failed to grow. Unfortunately Vedder & van Dam had no cholera-like vibrios at their disposal.

Wahbi (1938), who had the opportunity of using Vedder & van Dam's media for the stool examination of 61 pilgrims at a quarantine station in Iraq, stated in a short note that he had isolated El Tor vibrios on plates prepared with these media but not on Dieudonné's medium. Since, however, some of the previous workers—e.g., Neufeld & Woithe (1910) and Pilon (1911)—had observed growth of *V. El Tor* on the latter medium, it is difficult to believe that the unfavourable results obtained by Wahbi were due to other than accidental causes.

Lefebvre & Gallut (1937) recommended a cholera-selective medium similar to those of Vedder & van Dam which, as summarized in the *Tropical Diseases Bulletin* (1938), was made up as follows:

" (1) Prepare ordinary nutrient bouillon of *p*H 8. (2) Add 3 per cent. agar. (3) Sterilize 20 min. at 120° C. (4) Filter and add 40 cc. normal soda per litre. (5) Remove precipitate by filtration through cotton wool. (6) Distribute in large tubes in amounts of 14 cc. (7) Sterilize 15 minutes at 115° C. (8) Prepare: 10 per cent. solution of commercial crystalline haemoglobin in 10 per cent. soda 10; distilled water 90. (9) Prepare a buffer solution (*p*H 9.3) containing 8 volumes of sod. chloride 5.650; glycine 7.505 and 2 volumes of decinormal soda. (10) Mix haemoglobin solution and buffer solution in the proportions respectively of 1 to 2.75 and sterilize for 20 minutes at 120° C. (11) Add 6 cc. of buffered haemoglobin to each 14 cc. of agar medium. (12) Slope. (13) Use immediately, or preferably after drying for 24 hours in the incubator."

Growth of *V. cholerae* on this medium was fairly rapid, the organisms sown from a mixture of different bacterial species appearing in the form of large colonies after an incubation of 10-12 hours, thus anticipating the growth of the concomitant organisms.

Since their medium kept at least six months, Lefebvre & Gallut (see also Gallut, 1954) recommended speeding up the diagnostic work by issuing the tubes to field workers for direct inoculation of suspect stools at the bedside of sufferers. As the two workers stated with great reason, this procedure would be indicated particularly in cholera-affected localities distant from laboratories.

2. Egg-media

Krumwiede, Pratt & Grund (1912) found it possible to prepare solid media for the selective growth of *V. cholerae* by substituting egg-white or

whole eggs for the blood or blood-derivatives used by the workers enumerated above. According to Goldberger (1913), the whole-egg medium of Krumwiede and co-workers, which seemed preferable to a similarly prepared egg-white medium, was made up as follows:

" Shake thoroughly equal volumes of egg (whole) and water. Then mix equal volumes of this egg water and a 12 per cent solution of crystalline sodium carbonate and filter through a thin layer of cotton. Steam the alkaline egg solution for 20 minutes.

" Three volumes of the alkaline egg solution are mixed with 7 volumes of a 3 per cent peptone agar (salt 0.5, peptone 1, agar 3, water 100) and plates are poured. Allow to set and dry with covers off for 20 to 30 minutes. They are then ready for seeding."

Krumwiede, Pratt & Grund stated that on this medium, which in contrast to that of Dieudonné was translucent, cholera vibrios (as well as cholera-like vibrios) grew in the form of distinctive colonies: examined in transmitted light, they had the appearance of being deeply embedded in the agar and had a peculiar hazy look owing to the presence of a halo round the colonies, which—in the case of vigorous growth—was surrounded by a zone of clearing. Hence, though the egg medium was somewhat less selective than that of Dieudonné, this untoward feature was counterbalanced by the ease with which the cholera colonies could be picked out. The simple method of preparation from readily available ingredients likewise rendered this medium attractive. However, in a valuable study to which reference has already been made (see page 720), Goldberger (1913) expressed dissatisfaction with the method of Krumwiede and co-workers, because he found their medium " to restrain not only the ordinary fecal bacteria, but also to markedly inhibit the growth of cholera itself." Goldberger therefore recommended the following modified egg-medium for the selective growth of *V. cholerae*:

"(*a*) *Alkaline-egg solution.* First prepare an egg water by beating up a whole egg (or any multiple) with an equal volume of distilled (or good quality of tap) water. Then mix one volume of this with an equal volume of a 6.5 per cent solution of *anhydrous* sodium carbonate and steam for half to one hour.

" (*b*) *Meat extract glucose agar.* This is prepared as follows: Meat extract . . . 3, peptone . . . 10, sodium chloride (c.p.) 5, glucose 1, agar 30, distilled (or good quality tap) water 1,000. Steam for 3 hours to bring the agar into thorough solution and decant. Distribute in flasks in convenient quantities and sterilize by steaming for an hour and a half. Store and use as needed.

" For use, 1 volume of the alkaline-egg solution (*a*) is well mixed with 5 of the hot, freshly melted meat extract glucose agar (*b*) and plates poured.

" The plates, if poured in a quiet room free from dust, may be left to set and dry with the covers off for half to three-quarters of an hour, when they are ready for seeding. If such a room is not available or if the plates are not for immediate use, it is much better to allow the plates to cool and set with the covers on and to get rid of the condensed moisture and dry the plates in the incubator at 37°. This is conveniently done by sliding the dish partly over the edge of the cover in an inverted position."

Note. Goldberger added that the latter procedure, which prevented contamination, was also advantageous in the case of Dieudonné's and related media.

On Goldberger's medium, which was translucent, cholera and some cholera-like vibrios grew well, the colonies showing the distinctive features described by Krumwiede and co-workers, while the growth of the ordinary faecal bacteria was markedly restrained. The plates, if kept at 15° C, remained fit for use at least for 10 days, whereas the alkaline-egg solution, if stored at the same temperature, was found to remain serviceable for 83 days.

Comparing the medium described above with an alkaline meat-infusion agar he had also devised for selective cultivation of *V. cholerae*, Goldberger reached the conclusion that:

" All things considered, the choice for practical work must fall upon the alkaline-egg glucose agar medium. In comparison with Dieudonné's the alkaline-egg glucose agar permits of a more luxuriant growth of the vibrio colony; the vibrio colony is more distinctive in appearance; it exercises but little if any less restraint for the common fecal bacteria; its ingredients are more generally available, and, most notably, plates may be used at once."

As far as the present writer can judge, it is certainly regrettable that neither Goldberger's alkaline-egg medium nor Vedder & van Dam's media have been tried out on a worth-while scale in laboratory work with actual cholera stools.

3. *Dye-containing media*

The use of the medium devised for the differential isolation of *S. typhosa* by Endo (1904) [1] for cholera-diagnostic work seems to have been recommended first by Creel (1911). While considering the subcultivation of peptone-water-enriched faecal specimens on Dieudonné agar or the egg medium afterwards described by Krumwiede and co-workers instead of on alkaline agar to be an " unnecessary refinement of the technique ", he maintained that:

" All the advantages that Endo's medium possesses for isolation of *B. typhosus* obtain in cholera work if the alkalinity of the medium is increased to double that usually employed, for on this medium vibrio colonies give a very typical, clear, amethystine color."

As quoted by Takano and co-authors (1926), Yoshida (1911), noting that cultivation of *V. cholerae* on Endo's medium led to the appearance of scarlet-coloured colonies, recommended for cholera-diagnostic work a medium as follows:

3% agar base	1000 ml
10% sodium carbonate	30-40 ml
Saturated alcoholic solution of fuchsin	4 ml
Pure glucose	30-40 g
10% sodium sulfite solution	25 ml

[1] To manufacture Endo's medium, Harris (1925) recommended (*a*) the preparation of an agar base with the following ingredients per litre of distilled water: dibasic potassium phosphate (K_2HPO_4) 3.5 g, peptone 10 g, agar 15 g, and lactose 10 g; and (*b*) the addition of 0.25 g of anhydrous sodium sulfite and 3.5 ml of a filtered 10% alcoholic solution of basic fuchsin per 100 ml of the agar base.

On this medium cholera and cholera-like vibrios developed in the form of scarlet-red colonies, while those of the usual faecal organisms were colourless.

To judge from a short quotation by Kolle & Prigge (1928), a medium similar to the one just described was recommended for the isolation of *V. cholerae* by Mitsutake (1912), while Stokes & Hachtel (1913) once more referred to the possibility of using Endo's original medium for this purpose. However, related methods of cultivation began to attract much attention only when Aronson (1915) recommended rendering Endo's medium more selective for cholera-diagnostic work by curbing the growth of *E. coli* through (*a*) an increased alkalinity and (*b*) the use of saccharose (and dextrin) as fermentable carbohydrates. The method of preparing Aronson's medium was as follows:

> One litre of tap water is added to 35 g of agar in a flask. On the following day 10 g of meat extract, 10 g of peptone, and 5 g of NaCl are added, and the mixture is steamed for 4-5 hours. To obviate filtering, the flask is put in a slanting position and, after the sediment has settled, the supernatant is decanted in 100-ml quantities into 200-250 ml Erlenmeyer flasks. One adds then per 100 ml of the still hot agar 6 ml of a 10% solution of sodium carbonicum siccum and steams for 10-15 minutes. Afterwards one incorporates (*a*) 5 ml each of a 20% saccharose and a 20% dextrin solution, which have been sterilized through steaming for ½ hour; (*b*) 0.4 ml of a saturated alcoholic solution of diamond fuchsin; and (*c*) 2 ml of a fairly freshly prepared 10% solution of sodium sulfite, which has been sterilized through short boiling. After the flasks have been kept in a slanting position for the purpose of sedimentation, the contents of each are used for pouring 2 plates, which are dried open and in an inverted position for ½ hour at 50° C or correspondingly longer in the incubator. The transparent medium is then ready for use and, if kept in the dark, remains usable for several days.

As described by Aronson, the cholera colonies, which became visible on his medium after an incubation of only 10 hours, were at first colourless, but after 15-20 hours showed an increase in size and a bright red colour, while *E. coli* colonies did not develop within 15-20 hours. Observations he was able to make with one cholera stool fully confirmed these findings: while growth of *E. coli* was inhibited for 24 hours, the colourless cholera colonies developing after an incubation of 10 hours could be easily identified with bacterioscopic and slide agglutination tests, the characteristic red colour of the growths becoming manifest 17 hours after inoculation of the plates.

The validity of Aronson's claims soon became the subject of considerable debate. The great usefulness of his method was confirmed by the observations of several workers, among which those made by Schürmann & Fellmer (1915) and Stern (1915), partly with actual cholera stools, are particularly noteworthy. However, a number of other observers (see the summaries by Baumgarten & Langer-Zuckerkandl, 1917; Hesse, 1920; Kolle & Prigge, 1928) were less favourably impressed by the value of Aronson's medium and tried in part to improve it by lowering its alkalinity

or using substitute dyes or other ingredients. Particularly noteworthy in this respect is that Taylor & Ahuja (1938) and Read (1939), continuing to make successful use of Aronson's medium in actual cholera work, while otherwise following the original formula of this author reduced the amount of sodium carbonate solution incorporated into the medium by one-sixth.

It was afterwards pointed out by Venkatraman (1949), that a precipitation of rosaniline from the alcoholic solution of fuchsin by alkali was apt to render certain batches of Aronson's medium inhibitory for the growth of *V. cholerae* and that the isolated colonies developing after massive implantation of such plates with cholera cultures tended to be rough in character. Bhaskaran (1953), besides confirming these observations through an elaborate investigation, also established that the rough variants developing under these conditions differed markedly from their smooth parent strains in O antigenic structure and were avirulent for mice. However, notwithstanding these shortcomings, Ahuja et al. (1950, 1951) considered Aronson's medium, the use of which had been recommended in the 1947 instructions for cholera work issued by the British Ministry of Health, to be " generally reliable."

It has to be added that a method of subcultivating cholera-suspect colonies on and in a semi-sloped agar medium, which contained mannite and Andrade's indicator,[1] has been described by Gohar (1947, 1948). It would seem, however, that this procedure, ingenious though it was, has been superseded by the more expedient methods now available for the isolation and identification of *V. cholerae*.

4. Starch-containing media

Though Eijkman recorded as early as 1901 that cholera and cholera-like vibrios, in contrast to *E. coli*, produced a halo round their colonies when grown on agar plates containing rice- or arrowroot-starch, Gordon (1906) seems to have been the first worker who proposed to take differential-diagnostic advantage of the marked ability of *V. cholerae* to decompose starch. Using for this purpose a litmus-tinted fluid medium which contained 0.5% starch besides 1% meat extract, 1% peptone, and 0.1% sodium bicarbonate, he found that in this the cholera vibrio alone produced a strongly acid reaction within 24 hours, whereas (*a*) Finkler & Prior's vibrio produced only a feeble acidity by the third day of incubation, and (*b*) other organisms including *E. coli*, *S. enteritidis*, and *Proteus* failed to acidify the medium.

Stokes & Hachtel (1913), apparently the first to work with similar solid media, stated that they had been unable to distinguish between cholera and *E. coli* colonies when growing the organisms on 3% starch-litmus agar plates. They found, however, that, when inoculated in 0.55% semi-solid

[1] The indicator of Andrade (1906) is prepared by dissolving 0.1-0.5 g of acid fuchsin in 16 ml of N sodium hydroxide and 100 ml of water.

agar containing 1% starch and litmus, cholera and cholera-like vibrios had a characteristic appearance, growing in the form of pink colonies, whereas *E. coli* and other organisms like *B. faecalis alcaligenes*, *Proteus*, and typhoid bacilli formed blue colonies.

As summarized by Takano and co-authors (1926):

" Ito (1914), taking advantage of the fact that the cholera vibrio changes starch into sugar and then produces acid, while colon organisms do not have any such action, devised the following starch medium:

Water	1,000 c.c.	Saturated alcoholic solution	
Starch	10 g.	of fuchsin	5 c.c.
Peptone	30 g.	10% sodium sulphite solution	25 c.c.
Salt	5 g.	10% crystal sodium carbonate	
Agar	30 g.	solution	30 c.c.

" On this medium the colonies of cholera become visible in 12 hours and are bright scarlet; in 20 hours the colonies have a diameter of about 2 mm. and are surrounded with a clear zone. The colon bacilli do not thrive very well and the colonies are greyish white, without any clear zone around them. Differentiation between cholera and the other vibrios on this medium is not possible ... Cane sugar may be substituted for starch and litmus for fuchsin."

As claimed by Lange (1915, 1916), a suitable medium for cholera-diagnostic work could be prepared by mixing 6 parts of hot highly alkaline agar (containing 40 ml of 10% sodium carbonate solution per litre) with 1 part of 5% rice-starch size (" Kleister "), obtained by gluing up the starch with boiling hot water and afterwards autoclaving. In distinction from other organisms, cholera and cholera-like vibrios, after an incubation for 14-20 hours on this medium, showed a peculiar growth, characterized by the presence of dew-drop-like colonies surrounded by a distinct halo—features which greatly facilitated the identification of *V. cholerae* with slide agglutination tests and subcultivation of the organisms.

Discussing the value of his medium, Lange admitted that, if used for the direct cultivation of cholera-suspect faecal specimens, it was not quite as reliable as other selective media. He asserted, however, that in combination with peptone water enrichment " it was definitely superior to the hitherto known cholera media."

Bötticher (1915), who had an early opportunity of working with Lange's medium, was not in agreement with the claims made by the latter, stating that:

" Lange's agar does not show the selectivity for cholera vibrios possessed by Dieudonné agar. For less experienced workers it may come into consideration as a substitute for Koch [i.e., alkaline] agar. Those familiar with cholera diagnosis will hardly be willing to prefer the halo formation to the characteristic appearance of *V. cholera* colonies on Koch agar." [Trans.]

Returning to the method suggested by Stokes & Hachtel and used by Ito, Gibson (1916) recommended the use of a medium containing per litre of

water 30 g agar, 10 g each of peptone and starch, as well as 1.5 g sodium bicarbonate to which, after fractionate sterilization, a sufficient amount of a sterile aqueous litmus solution was added to produce a blue colour.

As stated by Gibson, the *V. cholerae* colonies becoming visible on this medium after an incubation of 18 hours, already showed a faint pink colour, whereas those of other organisms, including even the cholera-like vibrios, still exhibited a blue or whitish colour at this stage. After an incubation of 36 hours the colonies of the cholera-like vibrios also showed a pink tinge, but this colouration was less marked than that of the *V. cholerae* colonies, which after an incubation of about 24 hours were already seen to be surrounded by a faint pink halo. With the exception of some gram-positive diphtheroids, no other faecal organisms grew in the form of pink colonies.

Considering his findings, Gibson was of the opinion that his medium was suitable for the direct cultivation of broth-emulsified faecal specimens, including even specimens from suspected cholera carriers—a claim which the present writer for one would not be prepared to endorse.

Starch-containing media were also recommended for the selective cultivation of *V. cholerae* by Kodama (1921, 1922a, 1922b) and Kiribayashi (1933), as well as more recently by Yen (1947), Ch'i & Zia (1949), and Dishon (1951).

Yen (1947), noting that the fluid medium he had devised for the same purpose (see above, page 720) rarely yielded pure growths of *V. cholerae*, recommended hastening the isolation of this organism by the use of an analogous solid medium, prepared as follows:

" To 40 ml. egg white [are] added 280 ml. distilled water and 20 ml. *N*.NaOH. The mixture is beaten thoroughly, gently boiled for 30 minutes in an evaporating basin and cooled to room temperature. 100 ml. of a 10% aqueous solution of soluble starch, previously warmed to 40° C and thoroughly shaken for 2 minutes, are then added and the volume made up to 1 liter with distilled water. To this are added:

Peptone	10 gm.	Maltose	1 gm.
Meat extract	3 gm.	Magnesium chloride	1 mg.
Potassium nitrate	3 gm.	Agar agar	20 gm.
Sodium chloride	3 gm.		

" The whole mixture is heated in a water bath for one hour with frequent shaking, then filtered through cotton, and the reaction of the medium adjusted to pH 8.0. The filtrate is then distributed into flasks in 100 ml. lots and autoclaved at 15 lbs. [1.05 kg per cm^2] pressure for 15 minutes. Just before pouring into plates, 1 ml. of 1 : 10,000 dilution of Malachite Green in 95% ethanol, and 0.5 ml. of 1 : 100 dilution of Rosolic Acid in 95% ethanol are added to each 100 ml. of the medium, and thoroughly shaken until the indicators are evenly distributed throughout the mixture. It is then poured into sterile plates to a depth of about 0.3 cm. During inoculation, the surface of the cooled medium should not contain excessive moisture."

Cultivation of cholera vibrios on this medium for 18-24 hours at 37° C gave rise to transparent colonies surrounded by greenish-yellow haloes, whereas other organisms, including *E. coli*, *Proteus*, and *B. faecalis alcaligenes* failed to produce such zones of clearing and enterococci failed

to grow. Yen claimed that his medium had " been found to be of great practical use for the primary isolation of *V. cholerae* ".

Ch'i & Zia (1949), finding Yen's media unsatisfactory, tried to improve them by the incorporation of potassium tellurite. While the fluid medium they thus manufactured has already been dealt with above (see page 726), their method of preparing an analogous solid medium may be described as follows:

To a broth made with 5 g meat extract, 10 g peptone, 1 g KNO_3, 1 g $MgCl_2$, $6H_2O$, 8 g NaCl, and 900 ml distilled water one adds 20 g of agar. After this mixture has been dissolved by heating in a double boiler and the water lost by evaporation has been restituted, the pH is adjusted to 9.2. Then, after the medium has been cleared by sedimentation and the sediment discarded, 100 ml of a 5% soluble starch solution, which has been sterilized by boiling for 2 minutes, are added. After thorough mixing the medium is distributed in 100-ml lots into flasks and these are sterilized at 12 pounds per square inch (0.8 kg per cm^2) for 20 minutes. Just before plates are poured from the medium cooled down to 60° C, one adds to each lot (*a*) 1 ml sterile 0.2% potassium tellurite solution (aqueous), and (*b*) 1 ml of a 0.5% rosolic acid solution in 90% ethanol.

According to Ch'i & Zia, growth of *V. cholerae* on this medium was manifested after an incubation of 12-24 hours by the appearance of black colonies. These, if found to consist of vibrios, were used for slide agglutination tests with cholera immune serum diluted 1:80. Apart from the occasional development of bluish colonies of coliform organisms the medium inhibited the growth of the normal intestinal flora. However, while finding their solid medium reliable, Ch'i & Zia were able to arrive at a more rapid diagnosis of cholera with the aid of their fluid medium.

The solid medium of Dishon (1951) was prepared with a 2%-2.5% agar base containing 0.6% meat extract, 0.5% peptone, 2% NaCl, and 1% starch. After this medium had been autoclaved, the following ingredients were added per 100 ml: 1.5 ml of a 10% sodium sulfite solution; 4 ml of a 20% sodium carbonate solution; 2.5 ml of a 20% saccharose solution; 0.2 ml of a saturated alcoholic solution of acid fuchsin, and 1/200 000 each of gentian violet and brilliant green (final pH 8.5-8.7).

Cholera colonies developing on this medium were transparent and slightly pinkish; they had a diameter of 2-5 mm and were surrounded by a clear zone, which already began to become manifest after an incubation of 6-8 hours. Nevertheless, as has been indicated above (see page 726), Dishon recommended inoculating cholera-suspect stools first into his fluid medium and transferring material from this to plates of the solid medium after an incubation of 6-8 hours. After the plates had been kept in the incubator overnight, isolated colonies were picked out for identification tests. Examination of the plates was greatly facilitated by the fact that growth of contaminants was inhibited for 48 hours.

As far as could be ascertained, Dishon's media as well as those of Ch'i & Zia have not yet been tried out in laboratory work under actual cholera conditions.

5. Casein-containing media

In the course of his classical investigations on bacterial enzymes, Eijkman (1901) found that some species, including *V. cholerae*, had the property of producing haloes round their colonies on milk agar plates as well as on agar media to which, instead of milk, a mixture of (*a*) a solution of casein carbonate and (*b*) calcium chloride had been added. There was no doubt, therefore, that the halo formation on milk agar plates was the result of an action of the bacterial enzymes on the casein and not on the milk fat. Further establishing that the property of halo production on milk or casein agar plates was possessed solely by gelatin-liquefying species, Eijkman concluded that one and the same bacterial enzyme was responsible for both these phenomena. He pointed out that for diagnostic work it would be more convenient to use milk agar instead of gelatine plates in view of the low melting point of the latter and their progressive liquefaction during incubation. It has to be noted, however, that the property of halo production on milk or casein agar plates was possessed also by many cholera-like vibrios as well as by some other organisms, such as *Ps. pyocyanea*, apt to be met with in cholera-suspect stools. It was possibly for this reason that no immediate diagnostic advantage seems to have been taken of Eijkman's method of cultivation. However, in later years casein-containing media were again recommended for the cultivation of *V. cholerae* by Boccolari & Olivi (1916), Ko-Ran (1922a, 1922b), Vardon & Datta Roy (1938) and Koch & Kaplan (1952, 1953).

Boccolari & Olivi (1916) found it preferable to use 6 g of trypsinized casein per litre instead of 10 g of peptone for the preparation of Aronson's medium.

The medium recommended by Ko-Ran (1922a, 1922b) was prepared by adding to 100-ml amounts of a 3% agar base, made up with peptone and sodium chloride only, (*a*) a mixture of 0.5 g casein or nutrose and of 2 ml of a 10% solution of anhydrous sodium carbonate in 10 ml distilled water; and (*b*) 0.1 ml of a saturated solution of fuchsin in ethanol.[1]

On plates poured with this medium cholera vibrios grew in the form of light-red, round colonies surrounded by a clear zone.

Papain-casein digest broth and agar media devised by Vardon & Datta Roy (1938) were found to be satisfactory for the growth of various bacteria including *V. cholerae*. However, since these media would be useful for vaccine manufacture and bacteriophage work rather than for diagnostic purposes, it does not seem indicated to deal here with the details of their manufacture, which are clearly set forth in the well-documented publication of these two authors.

As a result of investigations made with the aim of finding a simple medium for the cultivation of cholera vibrios with an increased yield, Koch & Kaplan (1952, 1953) arrived at the following formula:

Peptone 0.5%, casein hydrolysate 0.5%, sodium chloride 0.5%, disodium hydrogen phosphate 0.25%, Bovril 0.15%, Marmite, 0.15% glycerol 2.2%, and agar 2.5%.

The two workers found that the yield of *V. cholerae* on this medium was more than twelve times that on plain peptone agar and that the organisms grown according to

[1] The statement made in the review of Ko-Ran's article by Takano and co-authors (1926) that the ingredients enumerated above were added to one litre amounts of 0.3% agar is obviously due to a misprint.

their method possessed satisfactory immunizing properties. It would seem, therefore, that, like the fluid casein hydrolysate medium utilized by Sokhey, Habbu & Bharucha (1950),[1] Koch & Kaplan's medium would be useful principally for the manufacture of cholera vaccine.

6. *Bile-containing media*

As summarized by Takano and co-authors (1926):

" Toyoshima (1914) took advantage of the fact that cholera grows well in ox bile and substituted it for broth in preparing strongly alkaline agar medium. Cholera grows in large colonies greyish white in colour, with a moist surface. Around the colonies are discoloured zones. The growth of other organisms than the vibrio is inhibited."

An attempt made by Maitra & Basu (1924) to utilize the bile-salt/lactose medium, which was devised by MacConkey (1905) for the cultivation of *S. typhosa* and allied organisms, for the cultivation of *V. cholerae* as well proved disappointing: while cultivation of about 200 cholera stools on plain alkaline agar was invariably successful, parallel inoculation of these specimens on MacConkey's medium gave a negative result in 80%. However, it deserves attention that, as Gohar & Makkawi (1947) afterwards claimed, the ingredient in MacConkey's medium responsible for this untoward result was the neutral red, which, if added in concentrations above 1/20 000, was apt to inhibit the growth of *V. cholerae*. It is certain that, as proved by ample experiences in India (see, for instance, Pasricha and co-authors, 1932a; Asheshov et al., 1933a; and Panja, 1942), agar media containing 0.5% sodium choleate (commonly, but not quite correctly, called sodium taurocholate) were fully suitable not only for the subcultivation of *V. cholerae*, but even for its direct cultivation from cholera-suspect stools, and could therefore be used to advantage for practical purposes. Similarly, direct cultivation of cholera-suspect faecal specimens on desoxycholate citrate agar (a medium described in the standard works on laboratory technique) was recommended in the 1947 instructions for stool examination issued by the British Ministry of Health.

Panja & Ghosh (1943), who were unable to prepare desoxycholate citrate agar during the Second World War, recommended in its place a medium of the following composition:

Meat extract	0.5%	Sodium phosphate	0.75%
Peptone	0.5%	Ferric citrate	0.30%
Sodium taurocholate	0.85%	Lactose	1.25%
Sodium citrate	0.80%	Agar	2.50%
Sodium thiosulfate	0.85%	Neutral red (0.25% solution)	1.5 ml/100 ml

Compared with other bile-salt-containing media, including that of MacConkey, Panja & Ghosh's agar gave better results not only with artificially contaminated faecal specimens but also with actual cholera stools. The

[1] See *Bull. Wld Hlth Org.* 1955, 12, 1047.

plates of the new medium, which was transparent, remained fit for use for about a week.

7. Bismuth-sulfite agar

In their important article on bismuth-sulfite media for the isolation of *V. cholerae* already referred to earlier in this study (see page 722 et seq.) Wilson & Reilly (1940) drew attention to the fact that the solid medium devised by Wilson & Blair (1931) for work with the typhoid-paratyphoid group of bacteria, if slightly modified, was also eminently useful for cholera-diagnostic work. The method of preparing such a modified medium was as follows:

" Peptone 40 g., NaCl 20 g., agar 80 g., water 4000 c.c., sodium carb. sol. (53 g. to 400 c.c. water) 40 c.c. The medium is autoclaved and without being filtered is adjusted to a reaction of *p*H 8.6.

" To 100 c.c. of this medium melted and cooled to 50° C are added 20 c.c. stock mannitol saccharose sulphite bismuth solution, 2 c.c. phenol red 1/1000 watery solution, and 2 c.c. absolute alcohol . . . Plates are poured and the surface inoculated.

" The stock mannitol saccharose bismuth sulphite solution is prepared as follows:

(*a*) 100 g. sodium sulphite anhydrous dissolved in 500 c.c. boiling distilled water.
(*b*) 30 g. bismuth ammonio-citrate scales dissolved in 250 c.c. of boiling water.
(*a*) and (*b*) are mixed and boiled for two minutes, cooled and then added to (*c*) which consists of 50 g. saccharose and 5 g. mannitol dissolved in 250 c.c. of water. To the mixture are added 15 g. sodium bicarbonate dissolved in 50 c.c. cold water."

As established by Wilson & Reilly, their modified medium, while promoting the rapid and profuse growth of *V. cholerae*, not only suppressed that of *E. coli* and *B. lactis aerogenes (Bact. aerogenes)*, but also proved to be unfavourable for the development of many cholera-like vibrios: of 25 such strains tested six only grew well, while the growth of nineteen was scanty or even nil. More important still, the medium was also not favourable for *V. El Tor*, the colonies of which were much smaller than those resulting from the implantation of " epidemic " strains of classical non-haemolytic cholera vibrios.

Describing the growth appearances of the latter, Wilson & Reilly stated that:

" On the mannitol saccharose sulphite bismuth phenol red alcohol agar plates colonies of the cholera vibrio appeared after one night's incubation and were yellowish brown in colour. In the case of some strains after two days the colonies exhibited a dark metallic lustre. In general the characteristic feature was a yellowish brown growth resulting from the action of the acids produced from fermentation of the mannitol and saccharose on the phenol red."

Wilson & Reilly admitted that somewhat similar colonies were formed on their medium by various strains of the genus *Proteus*, which, however, were unable to form spreading films of growth. For this reason and also because it was easy to differentiate cholera vibrios not only with slide agglu-

tination tests but even by mere smear examination, the ability of *Proteus* strains to grow on the bismuth-sulfite medium did not cause difficulties.

On the basis of their findings, Wilson & Reilly felt entitled to postulate that:

" Rich growth on our sulphite bismuth agar medium will be another differential test supplementing serological tests and the finding of Taylor (1937) and others that the true cholera vibrio is (*a*) non-haemolytic of washed goat erythrocytes, (*b*) ferments saccharose and mannose but not arabinose, (*c*) gives a positive cholera red and a negative Voges-Proskauer reaction."

A modified bismuth-sulfite medium afterwards recommended for cholera-diagnostic work in India (see Pandit, 1941; Ahuja et al., 1951) had the following formula:

2.5% agar base (pH 8.8) *	100 ml
20% sodium-sulfite solution	4.8 ml
Liquor bismuthi **	0.16 ml
Absolute ethanol	0.2 ml
10% mannose solution	1.0 ml

* Pandit (1941) recommended preparing the agar base with papain-digest broth, whereas Ahuja et al. advised the use of a tryptic digest broth (Douglas, 1914) for this purpose.

** Liquor bismuthi was prepared according to the formula given above on page 722.

Reporting in 1942 to the Scientific Advisory Board of the Indian Research Fund Association on the examination of 233 cholera-suspect stools, the Director of the School of Tropical Medicine in Calcutta stated that direct plating of these specimens gave 58% positive results in the case of Wilson & Reilly's medium as against 59% positives on bile-salt agar plates. Both these results were thus inferior to those obtained with the enrichment method devised by Panja (1942—see above page 725), which gave 81% positive results.

However, Pandit (1941) as well as Ahuja et al. (1950, 1951) found Wilson & Reilly's modified medium particularly useful for the direct plating of cholera-suspect stool specimens. The last-mentioned observers, while admitting that Aronson's medium also exerted an inhibitory effect on many organisms other than vibrios, stressed that (*a*) certain batches of the latter medium were " poorly supportive of the growth of *V. cholerae* ", and (*b*) in contrast to Aronson's medium that of Wilson & Reilly inhibited the growth of many cholera-like strains and even hampered that of *V. El Tor*.

It has to be noted that Felsenfeld et al. (1951), comparing various solid media by growing on them bacterial mixtures containing, per millilitre, 500-1000 cholera vibrios as well as 10 000-15 000 organisms each of *E. coli*, *B. aerogenes*, *Proteus vulgaris*, *Proteus aeruginosa*, and an enterococcus, arrived at a less favourable estimation of Wilson & Reilly's medium. They

recorded the results of their comparative tests as follows:

Probability of isolation of at least one colony of cholera vibrios when inoculating 0.01 ml vibrio+mixture to each of two identical plates

Medium	*Chemical composition*	*Probability (Poisson distribution)*
Alkaline agar	Peptone agar, pH 7.8-8.0	91.7
Dieudonné (see page 731)	Alkaline blood agar	67.8
Krumwiede (see page 739)	Alkaline egg agar	72.5
Aronson (see page 741)	Alkaline-sucrose-dextrin-agar+Andrade	97.8
Wilson & Reilly (see page 748)	Alkaline bismuth-sulfite agar	88.7
Teague & Travis (1916) *	Alkaline eosin-vesuvin agar	81.2
Panja & Ghosh (see page 747)	Bile-salt agar	90.5

* As given by Gradwohl (1948), this medium was prepared by adding 1 g of sucrose, 2 ml of 3% aqueous solution of eosin B, and 4 ml of a 1 : 1000 aqueous vesuvin (Bismarck brown) solution per 100 ml of 2%-3% beef extract agar (pH 8.0).

It would not seem wise, however, to lay greater stress upon the observations made under rather artificial as well as rigid conditions by Felsenfeld et al. than upon the favourable results obtained with the modified medium of Wilson & Reilly (not tested by Felsenfeld et al.) in the course of laboratory work with actual cholera stools. As far as the present writer can judge, it is the involved method of preparation, and not any lack in reliability, which limits the usefulness of this medium.

While, as discussed above, many investigators were intent upon devising specially selective media for cholera-diagnostic work, some continued to advocate the use of plain alkaline agar for this purpose.

Thus Creel (1911), describing the methods used at the New York Quarantine Station for the detection of cholera carriers, stated that, in order to save time and labour, plain alkaline agar media were used for making the subcultures from the originally inoculated peptone water cultures.

Similarly, Babes (1914; see also Neumann, 1915) recorded that, when having to make mass examinations of cholera-suspect stools during the Balkan wars, he resorted to subcultivation on plain alkaline agar. He first used agar tubes for this purpose, starting inoculation at the bottom of the slants and continuing it upwards in a zigzag course. He claimed that by following this technique it was possible to find isolated colonies of *V. cholerae* at the top of the slants after incubation for only six hours. Later, in order to save glassware and labour, Babes distributed layers of plain alkaline agar on the inside of 1-litre bottles and used these for the inoculation of 20 specimens in the manner just described.

Volpino (1916) considered subcultivation of peptone-water-enriched faecal specimens on plain alkaline agar as satisfactory as that on Aronson's medium, on which the characteristic appearance of the cholera colonies was apt to become manifest with some delay.

Attention has to be drawn also to various procedures devised by some of the earlier workers to increase the selectivity of plain agar media for *V. cholerae*.

Crendiropoulo & Panayotatou (1910) tried to reach this goal by separately preparing (1) a 3% agar base containing 1% peptone and 0.5% sodium chloride, and (2) an alkaline

peptone solution made by (*a*) dissolving 5 g of peptone in 190 ml of tap water; (*b*) adding, according to the kind of peptone used, 8-10 ml of a 10% solution of caustic soda; (*c*) after short heating and subsequent cooling of this mixture, filtering it through paper; and, finally, (*d*) sterilizing it for 1 hour in the steam sterilizer.

Immediately before use 4 parts of this alkaline peptone solution were admixed under aseptic conditions to 6 parts of the agar base, and plates were poured.

Crendiropoulo & Panayotatou claimed that their new medium, besides being easily preparable and transparent, had the advantage of inhibiting the growth of the usual faecal bacteria and—in contrast to Dieudonné's agar—considerably retarding that of *Ps. pyocyanea.* However, while Crendiropoulo (1912) recorded satisfactory experiences with the new medium in actual cholera work, Goldberger (1913) found it insufficiently selective.

Tokunaga (1911) recommended, for the isolation of *V. cholerae*, the use of a serum-containing alkaline agar, which, being transparent—in contrast to Dieudonné's medium—facilitated diagnostic work. For the same reason, Violle (1915) advocated the use of an alkaline agar medium to which 10% of glycerol had been added.

The alkaline meat-agar (" Fleischnatronagar ") devised by Esch (1915) was prepared by heating 500 g of meat (or of fish) in 250 ml of normal soda solution, filtering the product after dissolution through cambric, sterilizing it, and admixing it, when needed, in a proportion of 3 : 7 to a neutral agar base. Like Dieudonné's agar, the plates poured from this medium required a ripening period of 24 hours. However, while otherwise possessing qualities comparable to those of Dieudonné's medium, the alkaline meat-agar plates had the advantage of being transparent.

While none of the modified media just described has been permanently adopted, a study of the newer literature as well as of the recent instructions for cholera laboratory work (Seneca & Henderson, 1949; Ahuja et al., 1950, 1951; Gallut, 1954) shows that notwithstanding the introduction of special selective media the use of plain alkaline agar plates has been continued. This is not surprising if it is considered that under certain conditions the latter media are apt to give as satisfactory results in cholera-diagnostic work as the former. This is particularly true if the solid media are used merely for the subcultivation of preliminarily enriched faecal specimens collected from patients in the acute stage of the disease.

There can be no doubt, however, that if sole reliance is placed upon the rapid method of directly plating the faecal specimens—a procedure already recommended by a few of the earlier workers, e.g., by Rivas & Smith (1912), and increasingly used in recent years—cultivation on highly selective media is indicated. However, even these give reliable results only with the vibrio-rich faecal specimens collected from patients in the acute stage of cholera, but not with the stools of cholera convalescents or carriers, which usually contain only scanty numbers of the causative organisms. It is generally agreed, therefore, that preliminary enrichment of the latter kind of specimens in fluid media is indispensable. If, as is indicated, the highly effective enrichment methods now available are applied for this purpose, it seems a moot point whether it is necessary also to use highly selective solid media for subcultivation.

Examination of Vomits

The contention made in the sixth of these studies [1] that the occurrence of the causative organisms in the vomits of cholera patients is by no means as exceptional as has been claimed by some authors, is well supported by systematic observations made in this respect by Panja, Malik & Paul (1942). Examining single specimens of the vomits of 52 cholera patients both with the aid of peptone water enrichment and by direct plating on bile-salt agar, these workers were able to isolate *V. cholerae* 26 times, i.e., in 50% of their material. As was to be expected, they obtained positive results more frequently if the pH of the vomits was above 6.0 (as was usually the case), whereas at a pH below 5.0 no isolations were made.

However, valuable as these observations are, they render it clear that an examination of the vomits of cholera patients could only supplement but not replace that of faecal specimens.

A unique observation by Lieou (1938) deserving attention at the present juncture concerned an individual who succumbed within less than 24 hours to an illness characterized solely by severe gastric pains, anuria, and collapse. Since food-poisoning was suspected, the stomach contents obtained at autopsy were used for subcutaneous inoculation of a guinea-pig and a rabbit as well as for intraperitoneal injection of a mouse. The first-mentioned animal, which had received a dose of 2 ml, succumbed after about 30 hours, showing at autopsy a sero-purulent infiltration at the site of injection and congestion of the abdominal organs. Cultivation of its heart blood led to the isolation of *V. cholerae*. There can be no doubt that an adequate bacteriological examination of the stomach or intestinal contents of this victim (which was omitted for the not very cogent reason that the material had not been collected under sterile conditions) would have led to an identical result. In fact, material for such tests might have been obtained during the life of the sufferer either through rectal swabbing or with the aid of an enema, or—as was afterwards suggested by Panja, Malik & Paul (1942)—by giving the patient sterile water to drink so as to induce vomiting.

Water Examination

The history of the examination of water samples for the presence of *V. cholerae* goes back to Koch who stated at the 1884 cholera conference in Berlin that by cultivating small quantities of such specimens directly on gelatin plates he had

" succeeded in finding comma bacilli with all their characteristic properties in a tank which supplied the drinking and otherwise used water for all people living in the vicinity and in the immediate environment of which a number of fatal cholera cases had occurred ". [Trans.]

[1] See *Bull. Wld Hlth Org.* 1955, 13, 1168.

As can be gathered from the literature, especially from a valuable summary by Prausnitz (1903), some other early workers, using the same technique as Koch, also claimed to have isolated cholera vibrios from surface waters, or from wells or other sources, e.g., the bilge-water of a steam-tug coming from the infected port of Hamburg (Lubarsch, 1892). However, as maintained by some observers, such as Gruber (1894) and Prausnitz (1903), none of these early isolated strains could be properly identified so that, as the latter author put it, " the question of their cholera nature must remain open ".

However, even if these early claims as to the occurrence of cholera vibrios in surface or other waters could have been accepted at face value, the scantiness of such allegedly positive findings was certainly disproportionate to the apparently paramount role played by contaminated surface waters in the contemporaneous cholera outbreaks. Stressing this discrepancy, Fraenkel (1892) thus wrote in a little noted but interesting article:

" To furnish final and exact proof that the water was not unjustly incriminated, namely, the discovery of the cholera bacteria in the suspected rivers, was not possible. Competent and most experienced observers examined the Elbe water at Hamburg and the Spree water at Berlin most painstakingly, yet no positive findings rewarded their endeavours." [Trans.]

But, Fraenkel aptly continued,

" For the expert this is not surprising. For in view of the extraordinarily small quantities to which bacteriological water analysis is necessarily restricted, and more still on account of the abundance of various saprophytes in surface water, it must be considered a lucky accident of the first order if one nevertheless succeeds in getting hold of the cholera bacteria." [Trans.]

It is not at all surprising that under these circumstances the thought arose of improving the results of water examination by resorting, in place of direct platings with a few drops of the samples, to the enrichment of larger quantities of the suspected waters. In fact, as will be discussed soon, such an improved technique was suggested as early as 1892 by Heim. However, before dealing with this proposal, it seems well first to refer for the convenience of the record to an alternative method devised by Arens (1893) for the discovery of small numbers of cholera vibrios in water samples.

For this purpose, Arens advised the use of 175-ml quantities of the suspect waters, to which 25 ml of a broth, prepared according to the method of Karliński (1890) from cattle pancreas, and—further to promote the growth of *V. cholerae*—1 ml of a 10% caustic potash solution were added. As Arens established through laboratory tests, it became possible in this way regularly to isolate cholera vibrios by subsequent plating from specimens containing not more than two of the organisms per 5 ml of water. Occasionally, positive results were obtained with samples containing one cholera vibrio per 5 ml—once even with a specimen containing one organism per 30 ml.

While in spite of its apparent efficacy Arens' method seems never to have been used in actual practice, the procedure originally suggested by

Heim (1892) soon attracted universal attention. As this worker summarized in 1901, he had pointed out in his original article

" that the detection of cholera vibrios in water is rendered easier, if one takes instead of the usual amount of 1 c.c. a larger quantity of the suspected water and prepares through the addition of substances suitable as pabulum for the bacteria a substrate in which the vibrios, on account of their oxygen requirement, come to the surface and, assembling there, form a membrane from which they can easily be isolated. Having examined several such nutritive substances, I particularly recommended peptone and sodium chloride ". [Trans.]

For work with actual specimens, Heim (1892) advised adding to at least 250-500 ml of the waters to be examined sufficient amounts of these two substances to obtain a peptone concentration of 1%-2% and a NaCl content of 0.5%. The incubated specimens were examined daily by direct platings on gelatin and by broth subcultures, both made with material from the surface membranes of the growths.

The value of the method just described was endorsed by Flügge (1893) as well as by Koch (1893), and accordingly the following procedure for the examination of cholera-suspect water samples was prescribed in the regulations for cholera laboratory work compiled by Koch, Kirchner & Kolle (1902):

" To 1 litre of the water to be examined one adds one flask [100 ml] of the peptone stock solution [1] and shakes thoroughly; the mixture is then distributed in 100-ml quantities into flasks and, after an incubation of 8 and 18 hours respectively at 37° C, these are examined by (*a*) using drops removed from the surface for microscopic examination, and (*b*) making from the flask which shows most vibrios peptone water subcultures as well as inoculating gelatin and agar plates, which are then handled like those made from stool specimens. The pure cultures isolated are identified with the aid of agglutination and Pfeiffer's test." [Trans.]

A similar technique was still recommended in the 1916 German instructions for cholera laboratory work (see Kolle & Prigge, 1928), with the difference that, instead of subcultivation in peptone water tubes and on gelatin- and agar-plates, direct inoculation on Dieudonné- and agar-plates was prescribed. Similarly Gibson (1916) recommended direct cultivation of the peptone-water-enriched water samples on his starch agar medium (see above, page 743). However, in the course of extensive studies made from 1932 to 1936 on the occurrence of cholera and cholera-like vibrios in the Shanghai surface waters, which were as a rule highly contaminated, the present writer found it more adequate to resort before plating to subcultivation of the enriched water samples in peptone water tubes. Plating on plain alkaline agar then gave satisfactory results. He established on the other hand that it was sufficient to collect one specimen of each water to be

[1] The peptone stock solution was prepared by (*a*) disolving by heat in 1 litre of sterile distilled water 100 g peptone, 100 g sodium chloride, 1 g potassium nitrate, and 2 g crystalline sodium carbonate; (*b*) after filtration, distributing the solution in 100-ml quantities into flasks; and (*c*) autoclaving these.

examined in a sterilized, 300-ml, flat, medicinal bottle and to incubate this directly after a corresponding amount of peptone stock solution had been added.

It was gratifying to note that Taylor & Ahuja (1938), in order to study the water vibrios of North India, had independently adopted an analogous, though more refined, technique.

Taylor & Ahuja collected 200-ml amounts of the waters to be examined in 10-ounce (about 280 ml), screw-capped, flat, medicinal bottles and then added 20 ml of a stock solution containing 10% peptone and 5% NaCl. Alternatively, for the collection of water samples in distant localities, bottles containing this stock solution were issued and 200 ml of the water samples to be examined were added in the field. In both cases the reaction of the samples was raised to pH 9.0 by the addition of N/1 NaOH, thymol blue serving as indicator.

After incubation overnight (done at room temperature during the hot season) 2-ml amounts of the enriched specimens were added to 10-ml quantities of peptone water and then, after an incubation for 6 hours, 1 drop of the fluids was used for the inoculation of Aronson plates containing only 5/6th of the originally recommended amount of sodium carbonate (see above, page 742).

A new chapter in the history of the subject presently under review may be said to have commenced in 1939, when Read proposed to replace the hitherto practised enrichment method of water samples to be examined for the presence of *V. cholerae* by filtration of large amounts of the waters, the vibrio-rich residue collected on the filters then being used for cultivation.

Read resorted to filtration either through Seitz filters or through kieselguhr-impregnated filter papers. In regard to the first procedure he stated:

" Using the bismuth-sulphite original modification and passing one litre of water through a Seitz filter and inoculating the disc better results were obtained, especially when 2 per cent NaCl were added to the water and the reaction was raised to [pH] 9.2 immediately after inoculation...

" The samples were run through the Seitz filters by gravity through the pressure type filters using about six feet [2 m] head of pressure."

To carry out the second method,

" Three hundred c.c. of 0.5% Kieselguhr [porous diatomite] were run through a 6-inch [15-cm] filter-paper. The water was held in large 4-litre flasks closed with a rubber cork containing a moderate-sized glass tube. These were inverted in the funnel in such a manner that the outer end of the tube just reached below the surface of the fluid in the funnel. Filtration then proceeded automatically until the flask was emptied. About 15 litres could be passed through two filter-papers in 5 to 6 hours. The filter-papers were then folded and placed in 60 c.c. or so of bismuth-sulphite enrichment medium and incubated overnight."

Actually using the filtration method in field tests, to which reference has been made already earlier in this study (see page 723), Seal (1939) thus described his technique:

" Samples of water were collected in sterile quart [litre] whisky bottles by dipping them directly into the source. To each of these bottles two teaspoonfuls of common salt were

added to make the concentration between 1 and 2 per cent, each bottle accommodating about 750 c.c. of water if filled to the brim. For purposes of comparison water samples were also collected in 250-c.c. screw-capped medical bottles containing 20 c.c. of 10 per cent peptone and 10 per cent NaCl. This medium was directly inoculated with water at its source, the total volume being made up to 200 c.c. Eight drops of N/1 NaOH were then added to increase the alkalinity of the specimen."

Continuing the description of his technique, Seal stated that

" The salted samples were passed through Seitz filters fitted with 6-cm. discs in quantities varying from 300 c.c. to 1,000 c.c. The discs were inoculated each into 20 c.c. of the selected medium freshly prepared in 100-c.c. wide-mouthed glass-stoppered bottles. The pH was adjusted to 9.2 ... and the bottles [were] incubated overnight at 37°C. Subsequent treatment was the same as in stool culture."

Summarizing his findings, Seal stated that whereas with the aid of peptone water enrichment only 5 out of 117 tank-water specimens proved positive for *V. cholerae*, the number of successful isolations obtained with filtration and subsequent enrichment in the bismuth-sulfite medium (modification of Read, 1939) rose to 8.

Read & Pandit (1941), studying the distribution of the cholera and El Tor types of vibrio in rural areas of India, resorted to the kieselguhr method instead of Seitz-filtration of their water samples. Describing the handling of these specimens, the two workers stated that:

" 1,500 c.c. of water from each source were collected in sterilized whisky bottles. To 1,000 c.c. was added by means of a scoop, common bazaar salt so that the salt concentration was about 1 per cent and sufficient alkali to raise the pH to 9.2. This was then filtered through Kieselguhr impregnated filter-papers... the water being poured through funnels by hand. In the Bihar and Sind investigations an improved method consisting of the use of funnel covers and automatic levelling apparatus similar to that described for 'phage filtration by Pandit (1934) was used... After filtration, the filter-papers were folded in small packets with due regard to asepsis and covered consecutively with (*a*) cellophane, (*b*) vaseline paper, (*c*) cellophane and (*d*) ordinary brown paper... The packet was then ready for despatch to the central laboratory for inoculation into the bismuth sulphite medium."

A study of the results recorded by Pandit & Read, to which due attention will be paid in a later study, certainly shows the outstanding value of their method of water examination.

Kieselguhr-impregnated filters were also used by Venkatraman, Krishnaswami & Ramakrishnan (1941) in order to study the occurrence of El Tor vibrios in natural sources of water in the absence of cholera. Details of the technique applied for this purpose were as follows:

" 2,500 c.c. quantities of water were collected from each source, sufficient sea-salt added to make a 1 per cent concentration and the pH brought up to 9.2 by the addition of sufficient N/1 NaOH solution, at the spot of collection. These were then transported to the Laboratory generally within 3 to 4 hours (often within one hour) and filtered through Kieselguhr impregnated filter-paper, which, with the deposit, was then taken in 100-c.c. stoppered bottles containing 60 c.c. of the mannose-bismuth-sulphite medium and incu-

bated overnight. Platings were made on Aronson and agar media from the enriched cultures."

Examining a total of 878 samples of water collected from 237 different sources, Venkatraman and co-workers were able to isolate El Tor vibrios with the above method in 15 instances and classical non-haemolytic vibrios two times.

Panja & Ghosh (1947), in order to utilize the method of isolating *V. cholerae* devised by Panja (1942—see above, page 725) for stool examination for the examination of water samples as well, adopted the following technique:

" Three to five c.c. of unconcentrated river water from each sample were put into the porcelain candle and incubated for one or two days. A few drops of the surrounding boric-peptone water were then placed on bile-salt agar."

The two workers stated that, examining in this way 524 samples of water collected from the Hooghly River at Calcutta, they had isolated cholera vibrios on 16 occasions. They added that they had compared the efficacy of their method with that " of filtration through filter-paper adopted by Dr. S. R. Pandit " (? the kieselguhr method) and claimed that the latter had given less satisfactory results. However, to judge from their protocols, this difference was marked only as far as the isolation of *V. cholerae* in pure culture was concerned. As was to be expected, the candle method gave incomparably better results than direct plating of the water samples on bile-salt agar.

As has been stated already when dealing above (page 725) with the original recommendation of Panja (1942), there can be no doubt that the method devised by this worker is efficacious, and this was again confirmed by the experiences with water samples just recorded. However, as has been pointed out by the present writer when commenting upon Panja's observations, practical difficulties militate against the large-scale use of his method when handling cholera-suspect stools. The same objection holds true if more than occasional water samples have to be tested for the presence of *V. cholerae*.

It deserves attention that Gohar & Makkawi (1947) claimed to have obtained good results when enriching such water samples with peptone stock solutions containing potassium tellurite. However, their findings, made solely with artificially cholera-contaminated specimens, need confirmation through field trials. As matters stand at present, the method of choice for dealing with cholera-suspect water samples is to pass them through kieselguhr-impregnated filters and then to enrich these together with the deposits on them in a highly selective fluid medium.

Identification Tests

Introductory remarks

Ever since the discovery of *V. cholerae* it has been generally agreed that the demonstration of this organism in the stools of the patients is not merely the best, but practically the only means of differentiating between true cholera and other morbid conditions in which merely signs of a choleraic affection were present. However, for more than a decade after the causative organisms of cholera had been found, no permanent agreement could be reached regarding the choice of the methods suitable for their identification. It was soon realized that the problem at issue was not simply that of demonstrating the presence of vibrios with the aid of the bacterioscopic and culture methods devised by Koch but that the main stress had to be laid upon a differentiation of the cholera from the cholera-like vibrios detected by successive workers in ever-increasing numbers. In spite of the assertions of some of the early observers, it was soon shown that none of Koch's original methods sufficed for the latter purpose. As will be further discussed below, the hope that the cholera-red reaction recommended in 1886-87 [1] would fill this gap was likewise soon dispelled. For reasons which have been set forth partly in the sixth of these studies and will be further referred to below, experiments with various laboratory animals also failed to give results permitting an invariably clear-cut differentiation between cholera and cholera-like vibrios.

Firm ground was thus reached only after the introduction of serological methods for cholera laboratory work by Pfeiffer (1895) and Gruber & Durham (1896). Though the reliability of Pfeiffer's test was far greater than that of the early agglutination methods and has retained its outstanding value in spite of improvements in the latter and the introduction of alternative serological procedures, practical difficulties militated from the first against its use in routine cholera laboratory work. It is not surprising, therefore, that Pfeiffer's method fell into disuse even before there was valid justification for abandoning it. This is well illustrated by the fact that this test, which had been still referred to in detail in the 1907 German instructions for cholera laboratory work quoted by Kolle & Schürmann (1912), was no longer recommended in the 1916 revision of these instructions (see Kolle & Prigge, 1928). Since, however, other reliable methods, particularly improved agglutination tests, are now available for practical cholera laboratory work, there really does not seem to be any indication for continuing the use of Pfeiffer's reaction for routine purposes. It appears to be unnecessary, therefore, at the present juncture, to deal once more with this method, the principles of which have already been discussed in the fourth of these studies.[2]

[1] See *Bull. Wld Hlth Org.* 1955, 12, 818.
[2] See *Bull. Wld Hlth Org.* 1955, 12, 981.

As will be gathered from the study just mentioned, a number of alternative serological methods have successively been recommended for use in cholera laboratory work side by side with, or even in place of, the agglutination tests. In view of the fact, however, that none of these substitute procedures surpasses the agglutination method in practical value and that they are almost invariably less expedient, it also seems unnecessary to deal further with them in the course of the present disquisition. On the other hand, it is important to pay additional attention to the problem of haemolysis tests, which are indispensable for a differentiation of the true cholera vibrios from El Tor strains in the strict sense. The attempts made by some workers to use bacteriophage tests for diagnostic purposes also require consideration. The cholera-red test and the problem of animal experimentation will be dealt with together with other methods of confirmatory value in the concluding section of this study.

Agglutination tests

Inasmuch as the subject of agglutination received full general consideration when the problems of cholera immunology were discussed in the fourth of these studies, all that needs to be done now is to deal with (*a*) the methods of manufacturing agglutinating sera; (*b*) the technique of performing the agglutination tests; and (*c*) the problem of making such tests with rough or otherwise dissociated cholera vibrios. In considering these points, attention will be paid solely to the technique of O-agglutination, because, as has been proved by ample recent experiences quoted in the fourth study, this alones gives diagnostically valid results in cholera laboratory work.

(a) Manufacture of O-agglutinating sera. Theoretically the necessity of separately preparing O-agglutinating sera for the purposes of cholera diagnosis could be obviated by observing the action of the formerly used H+O sera, prepared with living or formolized vibrios, on boiled suspensions of the organisms under test. In actual practice, however, this alternative procedure would be not only inexpedient but also rather undesirable because, as was established through the pioneer observations of Gardner & Venkatraman (1935), the boiling of the organisms about to be tested is apt to decrease their O-agglutinability. Accordingly, the principle of working with specific O-agglutinating sera, manufactured with boiled suspensions of suitable *V. cholerae* strains (or otherwise prepared O antigens), has been generally adopted.

It also seems to be the consensus of opinion that—in place of the larger animals (horses or, preferably, donkeys) that were sometimes formerly used—rabbits ought to serve for the manufacture of O-agglutinating sera. However, no full agreement has been reached regarding the details of preparing the O antigens, the route of their administration, or the size and number of the antigen doses it is advisable to utilize in order to obtain sera

with a satisfactory titre. The following rather divergent recommendations made in these respects deserve attention:

Describing their techniques, Gardner & Venkatraman (1935) stated that:

" Pure O sera were made with saline suspensions from agar [cultures] boiled for 2 hours. Two doses of 0.5 and 1.0 c.c. at a week's interval generally gave sera of 1000-2000 O titre and no method of dosage was discovered that would consistently improve on this, though three doses of a fivefold denser suspension appeared sometimes to be a better stimulus."

Gardner & White (1937) briefly referred to the manufacture of O-agglutinating sera by various institutes in India with a dry antigen prepared by White through alcohol extraction of cholera vibrios followed by steaming and washing with ether (see White, 1948). They noted that in Kasauli 4 doses of this O antigen were administered to rabbits at 4-5 days' interval, starting with a dose of not more than 0.25 mg. At Shillong a longer course of immunization was resorted to, an initial dose of only 0.1 mg being followed at weekly intervals by gradually increased doses up to 1 mg or, if no satisfactory titre had been attained in this manner, even up to 1.5 mg. However, Gardner & White, probably influenced by the experience of Gardner & Venkatraman to the effect that such a prolonged course of immunization was of questionable value, were in favour of the method adopted at Kasauli.

Gallut & Grabar (1943) prepared O-agglutinating sera with alcohol-killed antigens, giving during a period of 20 days 5 injections, increasing from 0.25 mg to 5 mg of the dry weight of the organisms, and bleeding the animals 7 days after the last injection. It has to be noted in this connexion that Gardner & Venkatraman strongly objected to the method of using alcohol-killed antigens for the manufacture of O-agglutinating sera. However, as will be recorded below, Gallut & Grabar's procedure has been used again with apparent success by Gallut (1949).

The simple and apparently satisfactory technique adopted by Tang, Chu & Wong (1944) for the manufacture of O-agglutinating sera was (*a*) to wash off *V. cholerae* growths on agar with normal saline and to boil the resulting suspensions for 2 hours in the water-bath; (*b*) to dilute the suspensions to a standard of 10 milliards of organisms per ml; (*c*) to inoculate rabbits first at one day's interval with 0.5 and 1.0 ml amounts of the suspensions, respectively, by the subcutaneous route and then to administer, after a rest period of 4 days, the same doses at one day's interval intravenously; and (*d*) to bleed the animals on the 5th day after the last injection.

In the course of their studies on the antigenic structure of cholera and related vibrios Burrows et al. (1946) found that the method of boiling the vibrio suspensions for two hours according to Gardner & Venkatraman's method " was not sufficient to destroy all effective trace of H antigen but that boiling the suspension for 2 to 3 hours under a reflux condenser gave an antigen which did not stimulate the formation of agglutinins to the H antigen ".

In order to prepare O sera, Burrows et al. resorted to a process of hyper-immunization adapted to the reactions of the individual animals. They found that:

" The best immune response was given by young animals weighing about 2.5 to 3 kg and subjected to a course of inoculation sufficiently rigorous to prevent gains of more than 100 g in body weight per week. This ordinarily consisted of a series of 5 inoculations at 3-4 day intervals, the first 2 intraperitoneal and followed by 3 intravenous inoculations. The first inoculation was 1 ml of a suspension containing 10-20 thousand million vibrios per ml and the dose doubled with succeeding inoculations, except that in the transition from the intraperitoneal to the intravenous route the dose was not increased, and when loss in weight occurred the dose was not increased. Occasional animals did not tolerate the rapid acceleration and gave an inferior immune response. In most instances, however, the immunization was tolerated with maintenance or slight gain in body weight, and peak titer was reached after the third intravenous inoculation as indicated by trial bleeding. If the titer at this time was less than 1 : 20,000, 2 more intravenous inoculations were given without increase in dosage and the animal bled out 3 or 4 days after the last inoculation."

When trying to prepare O-agglutinating sera, Gohar & Makkawi (1948a) used one of the following two methods to reduce the high mortality of the rabbits undergoing immunization: they either injected their animals repeatedly at short intervals intramuscularly, completing the course of immunization with the aid of one intraperitonal and one intravenous injection of boiled *V. cholerae* suspensions; or resorted to intravenous injections of sensitized suspensions prepared thus:

" To a thick suspension boiled for two hours, one-tenth its volume of a high-titre O-serum was added; the mixture was then incubated at 37°C. for two hours and centrifuged; and finally the deposit [was] washed with saline and resuspended in saline to the required density."

Three injections of this sensitized antigen resulted in the production of sera with the quite satisfactory titre of 1 : 1250, with practically no mortality among the rabbits undergoing immunization.

For his studies on cholera immunology, to which reference has been made in the fourth of these studies,[1] Gallut (1949) worked with antigen suspensions prepared according to the method of Burrows et al. (see above) containing 10 milliards of cholera vibrios per ml. He used rabbits of an average weight of 3 kg to which a series of 5-7 inoculations gradually increased from 1 to 6 ml was given at 4-day intervals, the first 2 being administered intraperitoneally, the following intravenously. Since, particularly if recently isolated strains were used, the mortality among the animals undergoing immunization was high, Gallut resorted concurrently to the inoculation procedure of Gallut & Grabar (1943), administering in this manner a total of 20 milliards of cholera vibrios. Most of the O-agglutinating sera produced by Gallut had a titre of not less than 1 : 20 000.

[1] See *Bull. Wld Hlth Org.* 1955, 12, 969.

Kauffmann (1950), in the course of an investigation also referred to in the fourth study,[1] used for the preparation of O-agglutinating sera a method analogous to that of Bruce White (1948—see also Gardner & White, 1937, quoted above), the details of which were as follows:

"A 20-hour agar culture was suspended in saline and boiled for 2½ hours in flowing steam. After centrifuging, the sediment was incubated with 96% alcohol at 37° for 4 hours, then centrifuged again, and washed twice with acetone. Then the sediment was collected together with a few ml. acetone and kept overnight at 37°. Of the powder obtained in this way, a small portion was ground in [a] mortar, stirred with saline and then injected intravenously into the rabbits in increasing doses. 4 injections were given at intervals of 4-5 days. Ten days after the last injection the animals were bled totally."

As Kauffmann added, the O-titre of the sera he was able to produce varied between 1 : 640 to 1 : 2560 with an average of 1 : 1280.

Singh & Ahuja (1950), briefly referring to the technique of manufacturing O-agglutinating sera in a study devoted to a re-evaluation of the findings recorded by Burrows et al. (1946) and by Gallut (1949), stated that they prepared the O-antigens necessary for this purpose by keeping the washings of agar cultures of *V. cholerae*, enclosed in sealed glass ampoules, for 2 hours in boiling water containing salt so as to raise the temperature of the ambient to 101° C. In a further note on the serological analysis of *V. cholerae*,[2] Ahuja (1951) insisted with great reason upon the indispensability of using exclusively strains reliably tested for the absence of roughness for the manufacture of O-agglutinating sera for cholera-diagnostic work.

While the correctness of the specification just mentioned is unquestionable, it is, in view of the rather discrepant and partly incomplete recommendations quoted above, impossible at present to outline a standard method for the preparation of cholera-diagnostic O sera. However, it seems legitimate to maintain that for routine purposes it is probably sufficient (*a*) to heat the antigen suspensions without reflux condensation—a method not universally applicable—but preferably according to the technique of Singh & Ahuja (1950) just quoted, perhaps prolonging the period of exposure of the antigen suspensions in salt-containing boiling water to 2½-3 hours; and (*b*) to use a comparatively short course of immunization (4-6 injections), resorting initially, not to the intravenous, but to the intramuscular or intraperitoneal route of inoculation. Further deliberations definitely to settle these points and to determine in general a uniform procedure for the manufacture of cholera-diagnostic O-sera are urgently required.

In the course of their pioneer studies Gardner & Venkatraman (1935) were able to confirm the claim originally made by Japanese workers [3] as to the occurrence of *V. cholerae* in three serological types. Making

[1] *See Bull. Wld Hlth Org.* 1955, 12, 970.
[2] See *Bull. Wld Hlth Org.* 1955, 12, 985.
[3] See *Bull. Wld Hlth Org.* 1955, 12, 962.

absorption tests with sera raised against Inaba and Ogawa strains respectively the two observers found that

" from each type serum the heterologous type vibrio removes all the subgroup agglutinin for itself but leaves a large residue of agglutinins for the other types ".

However, while these results testified to the presence of a different subsidiary O antigen in the original (Inaba) and variant (Ogawa) types of *V. cholerae* and to the possibility of producing type-specific sera with the aid of the absorption method, numerous observations by Gardner & Venkatraman left no room for doubt that these two types as well as the intermediary (Hikojima) type fell into the cholera subgroup I of vibrios, since (*a*) they were agglutinated by O sera of that subgroup, if not to full titre, at least to a large fraction of the titre; and (*b*) O sera raised against representatives of the three types similarly agglutinated unheated suspensions of various strains belonging to cholera subgroup I of the vibrios. In view of these findings Gardner & Venkatraman recommended that for practical laboratory work a " standard subgroup I O serum " should be manufactured which contained both the main and the subsidiary antigens of that subgroup.

Analysing field experiences made in India with O sera which had been manufactured with a dry antigen furnished by White (see above), Gardner & White (1937) also came to the conclusion that

" for the purpose of routine diagnosis it is recommendable to use an O serum of the bivalent or mixed type, while the use of separate [i.e., type-specific] O sera ought to be restricted to scientific or epidemiological observations ". [Trans.]

In agreement with this advice Tang, Chu & Wong (1944) relied, for the purpose of determining the incidence of the serological types of *V. cholerae* during the 1942 cholera outbreak at Kunming, China, upon the use of type-specific Inaba and Ogawa sera, the preparation of which they described as follows:

" For preparation of specific type sera, the Inaba ('original') serum was absorbed with the Ogawa ('variant') suspension and vice versa... The absorption test was done by diluting the serum ten times with saline and mixing the diluted serum with 1/10 of its volume of packed bacterial cells which had previously been boiled for two hours. The mixture was then incubated at 37°C. in a water-bath for four hours and placed in the refrigerator overnight. Next morning the mixture was centrifuged for one hour at high speed and the supernatant removed and used as the specific ' O ' serum after having the titre for the homologous and the heterologous strains controlled."

However, while using this elaborate method of preparing type-specific sera and tube-agglutination tests for the purposes of their scientific investigations, Tang and co-workers resorted for routine purposes to the preliminary identification of cholera-suspect strains through rapid slide tests made with an unabsorbed serum raised against a stock strain of *V. cholerae*.

As recorded by Ahuja (1951), it remained

" in most laboratories in India the routine procedure for identification and typing of vibrios... to test them against sub-group I ' O ' anti-sera designated ' cholera non-differential O sera '. Such anti-sera contain cholera group-specific agglutinins and a proportion of Inaba and Ogawa type specific agglutinins depending on the antigen employed in raising these sera. If the organism reacts against this serum further tests with ' mono-specific ' Inaba or Ogawa serum are done to find out their appropriate types."

Similarly, as has already been stated in the fourth of these studies,[1] Kauffmann (1950) recommended using for cholera-diagnostic purposes a polyvalent O serum produced by simultaneous immunization of guinea-pigs with both Inaba and Ogawa antigens and resorting for differential diagnosis between the two types to a serum obtained by absorption of a polyvalent or an Ogawa serum by an Inaba strain.

As has been discussed in the fourth study, these proposals were not in agreement with the conclusions reached by Burrows et al. (1946) and by Gallut (1949), who, considering their A antigen the group-specific antigen of the cholera subgroup, advocated that exclusive use be made for the serological identification of *V. cholerae* of monospecific anti-A sera. Since, however, as further discussed in the fourth study, the validity of these recommendations has been vigorously opposed by Kauffmann (1950), Ahuja & Singh (1950), and Ahuja (1951), it would be unwise, pending further investigations, to deviate from the hitherto adopted methods for the serological identification of *V. cholerae*. It is reassuring to note in this connexion that in his 1954 article on cholera laboratory diagnosis, Gallut, while insisting upon the use of monovalent O sera for tube-agglutination tests, considered it legitimate to use for rapid slide tests a serum manufactured by immunization of rabbits with cholera vibrios of either the Ogawa or the Inaba type, which had been heated at 100° C for 3 hours.

It has to be noted in connexion with Gallut's work that he evolved a reliable and comparatively simple method of absorbing immune sera for cholera-diagnostic work similar to that used by Venkatraman & Pandit (1938). As stated in his 1949 article, Gallut proceeded as follows:

" An agar culture of the vibrios in a Roux bottle is harvested after an incubation at 37° for 18 hours and suspended in 19 ml of normal saline; one adds 1 ml of the immune serum and shakes the mixture mechanically for one hour. After the mixture has been kept in the refrigerator for 24 hours, it is centrifuged. One verifies that the serum thus diluted to 1 : 20 is effectively absorbed: it should no longer agglutinate the strain used for absorption at a titre of 1 : 100. The agglutination titre for the homologous strain invariably becomes reduced by the absorption, but this reduction takes place at varying proportions according to the antigenic relationships of the strains used... The absorbed sera diluted to 1 : 20, if kept in the refrigerator, retain a sufficiently high agglutinating titre for several weeks." [Trans.]

[1] See *Bull. Wld Hlth Org.* 1955, **12**, 970.

(*b*) *Technique of agglutination tests.* As has been discussed in the fourth of these studies,[1] Bandi (1910) recommended the direct use of immune-serum-containing fluid media into which the suspect stools were inoculated for the purposes of cholera diagnosis, and similar methods have also been proposed by some other workers, recently, for instance, by Seneca & Henderson (1949). However, either these methods have been found unsatisfactory or their usefulness, which, *a priori*, is rather problematic, has not been confirmed through field experiences. It therefore remained the generally adopted practice to use the growths of cholera-suspect materials on solid media or subcultures made from such originally inoculated media for the purpose of preparing suspensions for agglutination tests. Gardner & Venkatraman (1935) alone recommended the alternative method of obtaining antigens for such tests by growing the suspect organisms for 24 hours in veal broth (pH 8.0) and then killing them by adding, respectively, 0.2% of formol and of chloroform. It deserves attention, however, that in the experience of Burrows et al. (1946) formol-saline suspensions of vibrios made from solid media were not inferior in agglutinability to the formolized broth antigen of Gardner & Venkatraman and were preferable in view of the not infrequent sediment formation in the latter type of antigen.

As far as the usual method of obtaining material for agglutination tests from solid media is concerned, it is generally agreed that after inoculation the latter ought to be incubated for a period of not less than 18 and not more than 24 hours. To use the growths after a shorter incubation period is not advisable in view of the claim made by Friedberger & Luerssen (1905) [2] that then a " pseudo-agglutination " of the organisms under test might prove misleading.

The question whether live or killed antigens should be used for the agglutination tests has not been uniformly answered.

The early workers (see, for instance, Koch, Kirchner & Kolle, 1902) invariably used live organisms not only for their orientative tests, made with the aid of hanging-drop preparations, but also for tube agglutination. The practice of using live vibrios was also generally recommended by some modern workers—for instance, by Seal (1935), Linton & Seal (1935), and Sugio & Shimomura (1936), and for testing the sera of cholera-vaccinated individuals by Brounst & Maroun (1949) and by Gallut & Brounst (1949) [3]—and is, of course, universally resorted to for the preliminary identification of cholera-suspect colonies with the aid of rapid slide tests.

Gardner & Venkatraman (1935), having to rely for the purposes of their studies solely upon tube agglutination tests, established the important fact that, in contrast to what was the case in the Salmonella group, formol

[1] See *Bull. Wld Hlth Org.* 1955, **12**, 994.
[2] See *Bull. Wld Hlth Org.* 1955, **12**, 983.
[3] See *Bull. Wld Hlth Org.* 1955, **12**, 1003.

did not inhibit the O-agglutinability of *V. cholerae*, and therefore worked with formolized suspensions.

In the experience of Burrows et al. (1946) it was fully satisfactory to use suspensions of classical cholera vibrios killed by the addition of 0.2% formol for tube agglutination. However, these observers noted that, in the case of the *V. El Tor*, agglutination tests with live suspensions gave more consistent results than those with formol-killed organisms.

Gallut (1949), working both with live vibrios and with suspensions to which 5 per mille of formol had been added, obtained equally good results with both. He stated, however, that the formolized suspensions were more practical because they could be kept for one week at least without undergoing appreciable autolysis. It is noteworthy, however, that, when dealing again with the technique of tube-agglutination tests in his 1954 instructions for cholera laboratory work, Gallut advocated the use of " preferably not formolized " suspensions. These were apparently also recommended by Ahuja et al. (1950, 1951), while Kauffmann (1950) stated that he had resorted to formolized suspensions for his tube-agglutination tests.

As has been discussed before, Gardner & Venkatraman were not in favour of using O antigens obtained through boiling of the suspensions for 2 hours for tube agglutination tests, because this prolonged exposure to heat depressed the O-agglutinability of the vibrios. They added that, as a few experiments had convinced them, exposure of H+O suspensions to 95°-100° C for a few minutes sufficed to remove their H-agglutinability and that formolized broth cultures treated in this manner were " excellent reagents for detecting and measuring O agglutinins ". Nevertheless, Gardner & Venkatraman advocated the use of H+O suspensions for cholera-diagnostic work and this procedure has been adopted by all modern workers except Tang, Chu & Wong (1944), who employed *V. cholerae* suspensions which had been boiled for two hours for their type-identification tests. Still, though this technique apparently proved satisfactory for the purposes of Tang and co-workers, there can be no doubt that for routine cholera diagnosis H+O suspensions ought to be used in combination with O-agglutinating sera. As has been noted above, the latest advice seems to be that live vibrios ought to be employed in preference to formolized suspensions.

The simple technique universally adopted for the performance of rapid slide-agglutination tests consists of placing drops of normal saline and of diluted O-agglutinating serum on slides and distributing material taken with the aid of a loop from cholera-suspect colonies first in the saline and then in the immune serum. The appearance of agglutination in the latter, which takes place almost immediately, should be observed with the aid of a hand lens or preferably under the low power of the microscope. A conglomeration of the organisms under test taking place also in the saline control drops, which usually indicates a transition of the vibrios into the

rough state, renders the specimens in question unfit for a rapid presumptive diagnosis.

Since, as has been discussed in the fourth study,[1] organisms other than vibrios may become paragglutinated by cholera-immune sera, it is indispensable to confirm positive results of slide-agglutination tests through bacterioscopic examinations. The specimens necessary for these can easily be prepared by (*a*) spreading out the drops in which the organims under test have been distributed, specially the saline drops, in thin layers; (*b*) fixing the films thus made, after they have become air-dry, by heat; and (*c*) staining them with dilute carbol-fuchsin.

It is obvious that bacterioscopic examinations are also essential in cases where slide-agglutination tests made with material from macroscopically suspect colonies give a negative result, because otherwise the presence of cholera-like vibrios in the stool or water samples examined would be overlooked.

Though occasionally lower serum dilutions have been used for rapid slide tests, most modern cholera workers recommend dilutions ranging from 1 : 50 to 1 : 100. It is important to note in this connexion that in the experience of Burrows et al. (1946):

" Preliminary investigation indicated that the occurrence of low titer agglutinin for the cholera vibrio was common in normal rabbit serums. Titers varied from 1 : 20 to 1 : 80 in individual serums and pools almost invariably showed a titer of not less than 1 : 50. No normal rabbit serums were found to agglutinate the vibrios to a titer of 1 : 100, however, and this relatively high level was taken as the lower limit of significance."

In view of these observations it would be well to adopt a serum dilution of 1 : 100 as the standard for rapid slide agglutination tests, even though many workers, including the present writer, obtained fully clear-cut results when using a dilution of 1 : 50 for a preliminary examination of cholera-suspect growths.

The technique of tube-agglutination tests may be exemplified by describing the methods adopted for this purpose by the following workers:

1. Koch and co-workers (1902): The test sera are diluted with 0.8% saline (passed two times in succession through hardened paper filters for the purpose of complete clarification) in proportions of 1 : 50, 1 : 100, 1 : 500, 1 : 1000, and 1 : 2000. Loopfuls of the agar cultures to be tested are evenly distributed into 1-ml amounts of these serum dilutions put into agglutination tubes. These are examined after an incubation at 37°C for one hour, preferably by holding them in a slanting position and inspecting them with the aid of a weakly magnifying lens from below in the daylight reflected from the ceiling of the room.

Controls must be made with (*a*) the suspect culture and the normal serum of the animal species which served for serum manufacture; (*b*) with saline alone; and (*c*) with a known cholera culture incubated as long as that under test.

2. As summarized in the 1932 volume of the *Tropical Diseases Bulletin*, Kiribayashi (1931) recommended the following method of tube agglutination, which he claimed to

[1] See *Bull. Wld Hlth Org.* 1955, 12, 1005.

be not only expedient but more sensitive than the ordinarily used procedures:
" A series of 10 tubes is set up, each containing 2 cc. of 1 per cent. peptone water. A dilution of cholera agglutinating serum is made at 1 in 25 with the same 1 per cent. peptone water, and 2 cc. of the serum-dilution is added to No. 1 of the series of tubes. Successive transferences of 2 cc. of mixtures made in each of the tubes, except the last, which remains as a control, give dilutions of cholera serum in peptone water ranging from 1/50 to 1/12,800. A suspicious colony from a plate culture spread with test faeces is suspended in 1 cc. peptone water and one drop added to each of the 10 serial tubes. The tubes are then incubated for 3 hours and readings taken of the resultant agglutination."

3. Gardner & Venkatraman (1935) stated that they had used the agglutination method of Dreyer (1906, 1909) as described by Gardner (1931). The tubes were incubated in a water-bath at 51°-53°C and readings were taken after 4-5 hours and finally after 18-24 hours. As the two workers added, " the end-point recorded was the last definite trace of agglutination visible with a weak lens by artificial light against a dark background ".

4. To ascertain the presence of cholera agglutinins in the population of certain rural areas of India, Read & Pandit (1941) tested the sera, which had been collected in the field and sent to the laboratory in ampoules, in dilutions of 1/25, 1/50, and 1/125 against formolized and boiled suspensions of Inaba and Ogawa strains of *V. cholerae*. Readings were taken after the tubes had been kept for 2 hours in the incubator and for a further 22 hours at room temperature.

5. Tang, Chu & Wong (1944) used for their agglutination tests 24-hour agar cultures of the strains to be examined. After saline suspensions made from these growths had been boiled for 2 hours, they were adjusted in a standard comparator with barium sulphate emulsions to a density of about 2000 millions of vibrios per ml. Dilutions ranging from 1 : 20 to 1 : 2560 were made from Inaba and Ogawa monospecific sera, and 0.5 ml of each dilution was mixed in an agglutination tube with an equal amount of the vibrio suspensions. Readings were taken after the tubes had been kept at 56°C for 2 hours and at 37°C for a further 18-24 hours.

6. Referring to the agglutination method they used for the purpose of their investigations, Burrows et al. (1946) stated the following:

" The usual method of setting up the agglutination test based on tube-to-tube dilution to give serum dilutions as reciprocals of 2^n is slow and cumbersome and is impractical when large numbers of titrations are carried out. We have, therefore, employed a method... which makes possible more rapid and accurate serum dilutions. The tubes are set up in groups of 3 on large boards and saline, serum and antigen added in the amounts shown in [the following] table...

Serum dilution	*Tube number*	*Saline (cc)*	*Serum (cc)*	*Antigen (cc)*	*Final dilution*
1 : 50	1	0	0.50	0.5	1 : 100
	2	0.25	0.25	0.5	1 : 200
	3	0.40	0.10	0.5	1 : 500
1 : 500	4	0	0.50	0.5	1 : 1,000
	5	0.25	0.25	0.5	1 : 2,000
	6	0.40	0.10	0.5	1 : 5,000
1 : 5,000	7	0	0.50	0.5	1 : 10,000
	8	0.25	0.25	0.5	1 : 20,000
	9	0.40	0.10	0.5	1 : 50,000."

Burrows et al. pointed out that only three pipettes were required for the preparation of these serum dilutions and that dispensing could be facilitated by using burettes.

A further important observation made by these workers was that

" there appeared to be no difference between agglutinations incubated at 37°C and 53°C. The former was preferable since it allowed the routine use of the usual incubator. At either temperature agglutination was incomplete in 6 hours but complete at 15-18 hours. The tests were then incubated at 37°C overnight, and were read against a dark background without hand lens ".

The technique recommended by Ahuja et al. (1950, 1951) for cholera-diagnostic work was as follows:

" Agglutination tests are carried out in a waterbath at 52°C with Inaba and Ogawa anti-O agglutinating sera. Young cultures of freshly isolated strains show a tendency to rapid lysis and, if a number of tests are being done at a time, it helps to place each rack in the waterbath as soon as the addition of the culture suspension has been made without waiting for the whole series of additions to be completed. The ' O ' agglutination of cholera usually appears very early and a preliminary reading may be taken at the end of 2 hours, but the final reading is taken next day, after the racks have been stood at room temperature overnight. The suspensions should agglutinate at over 50%-75% of the titre of either Inaba or Ogawa anti-O serum."

Similarly, Gallut (1954) prescribed the following agglutination technique:

A suspension of an 18-24-hours-old agar culture in 8.5 per mille saline and containing about 2000 millions of cholera vibrios per ml is utilized for the tests. The serum dilutions should vary from 1 : 100 up to the maximum titre of the sera used (Inaba and Ogawa anti-O sera and absorbed monovalent anti-O Inaba and Ogawa sera). Agglutination generally appears quite rapidly so that preliminary readings can be made after the tubes have been kept in the water-bath at 52°C for 2 hours. A second reading is taken after the tubes have been kept at room temperature overnight.

It is certain that tube-agglutination tests are altogether indispensable for establishing the diagnosis of cholera in early and sporadic cases. It would also be most desirable to use this elaborate method as a matter of routine during outbreaks. However, as exemplified by the experiences of the present writer in the cholera epidemics raging in China during the Second World War, which often, with lightning speed, involved wide regions or even whole provinces, it may be impossible to make tube-agglutination tests with the growths isolated from each patient. In the face of such emergency situations it was often unavoidable to base the final diagnosis of cholera upon the outcome of rapid slide tests combined with the cumulative evidence procured through simple confirmatory tests. Provided that its use was restricted to clinically typical cases, this simplified procedure appeared to give sufficiently reliable results.

(c) Identification of dissociated cholera vibrios. Commenting upon the results obtained in cholera-diagnostic work by various observers in India with O-agglutinating sera (see Gardner & White, 1937), White (1937) stressed that

(*a*) " Certain variants [of *V. cholerae*], selectively produced through the action of cholera-phages B to M or combinations of the latter may show a reduced agglutinability with anti-cholera O sera "; and

(*b*) " The rough variant of the cholera vibrio, which is selectively produced through the widely distributed A type of choleraphage and which no longer synthesizes the specific polysaccharide responsible for the O-serological reactions of the smooth form, is not agglutinable by O-agglutinating serum raised against the smooth type of *V. cholerae* ".

White admitted that any doubts which might arise on account of a reduced agglutinability of the cholera vibrios under the action of cholera-phages B-M could and should be overcome with the aid of agglutinin absorption tests. However, in view of the antigenic difference existing between the S and R forms of *V. cholerae* it was indispensable for the identification of the latter to resort to special agglutinating sera manufactured with rough cholera vibrios. Moreover, in view of the instability of the latter in 0.85% normal saline, it was necessary for all serological work with rough cholera vibrios to use solutions with a NaCl content of only 0.4%-0.5%.

Ahuja (1951; see also Singh & Ahuja, 1951) also emphasized the necessity of using rough as well as smooth O-agglutinating sera for cholera-diagnostic work. Ahuja pointed out in this connexion that the SR variation

" may display different degrees of roughness. Some strains which, according to all accepted criteria, morphological, physical, serological, etc., appear to be smooth cholera vibrios, display the presence in varying degrees of rough element when tested against cholera rough ' O ' serum. They even lack the characteristic property, that of salt instability in 0.85 per cent sodium chloride, which is considered one of the usual characters of a rough strain. The colonial appearance may be that of a typical smooth vibrio and yet the strain may have varying degrees of rough antigen in its make-up ".

As has been described in the fourth study,[1] Ahuja (1951) and Singh & Ahuja (1951) recommended a test based on the vibriocidal action of guinea-pig serum on rough or partially rough cholera vibrios for the detection of the R state. However, though the experiences they made with this method were fully satisfactory, Gallut (1953) did not confirm its reliability. Be this as it may, it is certain that for the routine purposes of cholera diagnosis agglutination tests with rough O sera are rather more expedient than the quite elaborate procedure of Singh & Ahuja.

Haemolysis tests

The problem of haemolysis and the general principles underlying the carrying out of haemolytic tests, which are indispensable for a differentiation of the classical *V. cholerae* from the El Tor vibrios in the strict sense, have already received full attention earlier in these studies.[2] It seems sufficient, therefore, to quote here the methods recommended by Ahuja et al. (1950, 1951) for the performance of haemolysis tests with vibrios isolated for the purpose of cholera diagnosis:

[1] See *Bull. Wld Hlth Org.* 1955, 12, 985.
[2] See *Bull. Wld Hlth Org.* 1955, 12, 824, 990.

" The haemolysis test is done by adding 1 ml of 24-hour culture in isotonic Douglas broth to 1 ml of a 5% suspension of washed sheep or goat erythrocytes, incubating the mixture at 37°C for 2 hours followed by overnight storage in the cold room. It is essential to make sure that the erythrocytes used in the test are not fragile in 0.65% saline. Alternatively, a saline suspension of a 24-hour growth on nutrient agar, standardized to contain 8,000 million organisms per ml, may be used. To 1 ml of this vibrio suspension is added 1 ml of a 3% suspension of washed erythrocytes and the mixture [is] treated as above. Krishnan, who recently made a detailed study of the various factors involved in the tests, has shown that more consistent results may be obtained by using broth cultures in preference to saline suspensions of agar cultures and that a 24-hour growth in broth is preferable to a 48- or 72-hour growth."

It would be most desirable if the method ascribed in this statement to Krishnan alone, but actually recommended by Krishnan & Gupta (1949),[1] would be adopted as standard. It is important to note that in the experience of these two workers the use of the more easily available sheep erythrocytes was preferable to that of goat erythrocytes.

Bacteriophage tests

In the course of a lecture on the problems of cholera bacteriology Finkelstein (1931) reported that according to unpublished observations by Clark cholera and cholera-like vibrios did not behave uniformly when subjected to the action of a choleraphage which had been supplied by Morison. Though 30 *V. cholerae* strains were lysed by this phage, 8 were not, whereas out of 23 cholera-like strains 11 were lyso-sensitive and 12 resistant.

Combiesco-Popesco & Wisner (1933), on the other hand, found all the 15 cholera-like strains they were able to examine resistant to the action of a choleraphage received from Egypt, while not less than 94% of their 67 cholera strains were lysed by this.

Commenting upon these observations, which had been made in his laboratory, Cantacuzène (1933) pointed out that

" Lysosensitivity by phage appears to be a phenomenon most often, *but not invariably*, linked with the authenticity of the cholera strains. One ought to consider it as a phenomenon of great probability but not of certitude ". [Trans.]

The limited diagnostic value of bacteriophage tests was also admitted by Seal (1935), who made in this connexion the following statement:

" Study regarding the bacteriophage is yet far from complete. New phages are still being discovered and added to an already long list... Different vibrios have been found to behave differently with these phages. They may be lysable by one or more of these phages or may be completely resistant to all. 'A'-phage lysability has been held as a criterion for complete smoothness, but smooth vibrios have later on been found by Pasricha and his collaborators [1932b], which are not A-phage lysable. The difficulty

[1] See *Bull. Wld Hlth Org.* 1955, 12, 826.

specially arises with those vibrios which are phage resistant. Non-agglutinable vibrios obtained from cholera cases, convalescents, carriers, water, etc., have often been found phage resistant or lysable only by few or mixed phages. On the whole, phage lysability may be utilized as an additional confirmatory test and this is always done nowadays in all experimental work."

A consideration of these statements and also of the great difficulty of maintaining suitable phage strains in readiness makes it clear that tests with choleraphages are of no practical value in cholera laboratory work. It is not surprising, therefore, that no reference to their use has been made by any modern authority.

Confirmatory Tests

Tests for roughness

Before dealing with the tests customarily used for confirming the laboratory diagnosis of cholera, it is indicated to refer to two simple methods which have been recommended for the detection of the rough state of microbial organisms including the *V. cholerae*—namely, tests with Millon's reagent and with trypaflavine.

Millon's reaction. According to the standard works on organic chemistry, the reagent of Millon (1849), which is widely used in chemical work for the detection of various substances containing the hydroxy-phenyl group, including tyrosine, phenol, and thymol, is prepared by (*a*) dissolving 1 part of mercury in 2 parts of strong nitric acid, (*b*) adding 2 volumes of water, and (*c*) decanting the clear fluid after standing. The reagent seems to have been used first for bacteriological work by White (1929) in the course of a study on the smooth and rough races of intestinal bacteria. White stated in this connexion that:

" When a large loopful of rough growth is emulsified in approximately 3 c.c. of water and about 1 c.c. of Millon's solution is added thereto, the bacilli are at once clumped and, on boiling the mixture, collected in dense masses and quickly assume the deep red-pink coloration of the positive test. Under the same conditions typically smooth bacilli of the *Salmonella*, colon and dysentery groups are relatively little affected; the bacilli remain dispersed and the colour of their suspension does not deepen during a few minutes of boiling beyond a yellowish or ochre tint. The deposit which settles on standing commonly varies in colour from yellow to ochre; occasionally it is pale pink—a variation probably related to the degree of smoothness—; in all cases it is fine and readily dispersible.

" Smooth bacilli from which the soluble specific non-protein factor has been extracted (*e.g.* by prolonged boiling with dilute acid) or the proteins thrown down by acid from a solution of smooth bacilli in NaOH solution react precisely like fresh rough bacilli and it would seem to be an inevitable conclusion that in the smooth organism the soluble specific carbohydrate intervenes—probably mechanically—between the tyrosin-containing complex and the reagent."

Taking advantage of tests with Millon's reagent in the course of their studies on cholera bacteriophage, Asheshov et al. (1933b) found that these compared favourably in reliability as well as in ease of application with

other procedures used for detecting the roughness of *V. cholerae* cultures, including observation of the appearance of the colonies, the character of growth in fluid media, the influence of NaCl concentration, and the type of agglutination. Asheshov et al. used for the purposes of their bacteriophage work the following modified method of preparing Millon's reagent:

> Solution of 1 part of metallic mercury in 2 parts by weight of nitric acid of 36° according to Baumé's scale was done in a fume chamber or in the open air, because during this process dense fumes of nitrous oxide are given off. After dissolution had become complete, the green liquid obtained was diluted with 2 parts of water and then poured into a large photographic dish, where it was left to aerate for about 24 hours.

To make tests with this reagent, Asheshov et al. proceeded as follows:

> " A loopful of 24 hours' growth of microorganisms on agar is emulsified in 2 c.c. of tap-water, 0.2 c.c. of Millon's reagent is added, the tube is left for about one minute, and then heated nearly to boiling point. The heat is maintained for about one minute more, but without boiling."

Commenting upon their method, Asheshov et al. stressed that their well-aerated reagent did not produce a red colouration when acting upon rough vibrios. This was desirable, because in their experience the addition of non-aerated Millon's reagent even to smooth cholera cultures led to the appearance of a red colour owing to the presence of extraneous organic matters as well as of a few rough elements. When the aerated reagent was used, reliance had to be placed upon the presence or absence of two other phenomena thus described by Asheshov and his colleagues:

> " 1. *Flocculation.* With pure rough culture this appears even without heating, the flocculi slowly falling to the bottom. On heating, the flocculi ' coagulate ', partly floating on the froth, partly falling to the bottom, clearing the liquid more or less completely. The more complete the coagulation and the clearer the liquid the more rough is the culture.
>
> " 2. ' *Creeping* '. After heating, it will be observed that a film, more or less granular, is ' creeping ' up along the wall of the test-tube above the surface of the liquid. It consists of coagulated vibrios which float on the surface of a thin layer of the liquid adhering to the wall. The more marked the phenomenon the more rough is the culture."

Trypaflavine reaction. Trypaflavine, first considered by Alessandrini & Sabatucci (1931) as a means of distinguishing between supposedly different *Brucella* species, was recognized by Pampana (1931, 1933) as a reagent suitable for a differentiation between the smooth and rough forms of one and the same bacterial species.

Describing his technique, Pampana (1933) stated:

> " The reagent consists of a 1 : 500 solution of *trypaflavine*, in normal saline. A drop of the solution is put on a slide. Close to the drop, but not in the drop, we depose a minute fraction of a loopful of the bacterial colony to be examined. We then flame the loop, and, when it is cool again, we moisten it gently with the trypflavine and gradually emulsify the material on the slide. Finally we mix it with the whole droplet of trypaflavine solution. If the colony contained the "*R* " variant, agglutination takes place immediately or within a few seconds. The reaction is very easily read, the more so if the surface of the slide is illuminated by oblique light against a dark background."

Pampana added that his test could also be performed by mixing equal amounts of 1 : 500 trypaflavine solution and of the bacterial suspension to be examined in a test-tube, but found this procedure less sensitive than the above described " drop-agglutination " method.

Working with 100 vibrio cultures, including El Tor and cholera-like vibrios besides classical *V. cholerae* strains, Popiesco-Combiesco & Soru (1934) confirmed the value of the latter test, with the aid of which they were able to distinguish between smooth, SR, and rough organisms. The former two types produced a uniformly turbid growth in broth, the latter a granular growth. Results obtained with the acid-agglutination method of Damboviceanu (1933) [1] paralleled those of Pampana's test, also permitting a classification of the vibrios into three groups. However, in contrast to the trypaflavine reaction, Damboviceanu's method was too elaborate to be of practical value.

Bhaskaran (1953) who had the opportunity of examining variant strains of *V. cholerae* obtained through cultivation on Aronson-type media (see page 742 above) with rough O-agglutinating sera as well as with Millon's and Pampana's reagents, commented thus on the results of these combined tests:

" From all the strains of *V. cholerae*, rough variants were obtained during cultivation in B.C. agar [i.e., basic-fuchsin/sodium-carbonate agar] which gave positive results with Millon's and trypaflavin tests and agglutinated wholeheartedly with rough ' O ' serum. Further, every grade of intermediate condition between smoothness and roughness was seen to exist. At one end of the scale were the typically rough strains which reacted only with rough ' O ' sera, while not being clumped by smooth ' O ' sera at as low a dilution as 50. The majority of the variants, however, represented intermediate stages between smoothness and roughness. These variants, while satisfying Millon's and trypaflavin tests, agglutinated with rough as well as the homologous ' O ' sera. At the other end of the scale were a few smooth strains which were recovered unaffected after cultivation in B.C. agar."

As far as one may judge from these findings made with artificially dissociated variants of *V. cholerae*, tests with Millon's and Pampana's reagents were as reliable for detecting the presence of rough elements in these growths as those with rough O-agglutinating sera. However, attention has been drawn above (page 770) to the observations of Ahuja (1951), who found, among the cholera strains at his disposal, some reacting with rough O-serum even though they lacked all other characteristics indicating roughness. In view of this and more still because, as confirmed by Bhaskaran, cholera strains do occur which react solely with rough O-sera, there can be no doubt that tests with these have to be resorted to in cholera-diagnostic work regardless of whether tests with Millon's and Pampana's reagents are utilized as well.

[1] See *Bull. Wld Hlth Org.* 1955, 12, 1018.

Cholera-red reaction

It is of historical interest to note that the technique now generally adopted of performing the cholera-red reactions with organisms cultivated in peptone water was not used by the pioneer workers. Poehl (1886), who first recorded that addition of hydrochloric acid to *V. cholerae* growths led to the appearance of a red " pigment ", worked with Koch's nutrient gelatin. This was also used by Brieger (1887), while Bujwid (1887), who independently described the reaction, resorted to cultivation in broth. However, Dunham (1887), systematically searching for an optimal technique to produce the cholera-red reaction, recommended the use of 1% peptone water, with which he said he obtained positive results after cultivation for four hours or once even after an incubation of only 3 hours. Dunham noted, on the other hand, that gelatin stab-cultures of *V. cholerae* gave a cholera-red reaction only after they had become entirely liquefied, whereas the presence of even inconsiderable remnants of undissolved gelatin led to the appearance of a brown colouration.

Another important proposal made by Dunham, which has been adopted by modern observers (see, for instance, Ahuja et al., 1950, 1951; and Gallut, 1954), was to use concentrated sulfuric acid in place of hydrochloric or other acids for the performance of the cholera-red test.

As already alluded to above (see page 758), the early observers inclined to the belief that a typical cholera-red reaction was given solely by *V. cholerae* and that consequently a positive outcome of such tests sufficed for the identification of this organism. This view was expressed in particular by Bujwid (1888), who reached the now quite amazing conclusion that by (*a*) cultivating material from cholera-suspect stools in 10 ml of 2% peptone water, (*b*) taking after 24 hours a loop from the surface of the culture for subcultivation in the same medium, (*c*) successively making in this manner two further peptone water subcultures, and (*d*) using the third subculture for the cholera-red test, one was able to establish the laboratory diagnosis of cholera without the aid of a microscope.

However, the belief in the specificity of the cholera-red reaction was soon discredited by observations showing that it was also given by cholera-like vibrios, such as the *V. metchnikovi*, isolated by Gamaléia (1888) from fowls, and water vibrios such as the *V. berolinensis* (Neisser, 1893), the *V. danubicus* (Heider, 1893), and, according to Prausnitz (1903), the majority of the strains isolated from water samples in Hamburg. Hence, as the last mentioned observer maintained with much reason, the cholera-red test was mainly valuable as an easy means of ruling out the cholera nature of vibrio strains which reacted negatively, whereas a positive result obtained in the case of strains morphologically and culturally identical with *V. cholerae* was not conclusive.

It was sometimes claimed that the nitroso-indole reaction, if done with

one and the same strain upon successive occasions, was apt to give variable results (see, for instance, Pottevin, 1913, and summary by Pollitzer, 1934), but, as far as the present writer can judge, such differences were of a quantitative rather than a qualitative nature. It is, however, of the utmost importance to realize that for various extrinsic reasons cholera-red tests done with authentic strains of *V. cholerae* may give falsely negative results.

In the first place, as was recognized by Bujwid (1887), it is necessary to use pure cultures of the organisms under examination for the performance of the tests, because the presence of extraneous organisms is apt to interfere with the reaction. Tobey (1908) and Logie (1913) showed that, in particular, the presence of nitrite-destroying organisms like *E. coli* exerted such an untoward influence. The direct use of cholera stools for cholera-red tests, even though it may lead to positive results, is therefore inadvisable.

Another indispensable prerequisite for the proper performance of cholera-red tests is the use of suitable media. Experience has shown that not all brands of peptone are adequate in this respect, because they may be lacking in tryptophane, which is indispensable for the formation of indole (Mackie, 1929c). A proper content of the media in nitrates is also of crucial importance. Bleisch (1893), who made a profound study of this question, maintained that besides avoiding too low a nitrate content, it was also essential to guard agaist an excess of nitrates or preformed nitrites. He considered broth media, apt to show an inconstant composition, to be altogether unsuitable substrates for cholera-red tests and insisted upon the use of specially prepared 2% peptone water media to which exactly determined amounts of diluted potassium nitrate solution had been added to bring the nitrate content to a proper level. Ample practical experiences have shown, however, that—provided that they had been manufactured with suitable brands of peptone—the peptone water media ordinarily used for cholera laboratory work prove reliable for the performance of cholera-red tests as well. Their suitability may be simply ascertained by growing a known cholera-red-positive strain of *V. cholerae* in them for 24 hours and then adding one drop of strong sulfuric acid per ml of the medium (see, for instance, Taylor, Pandit & Read, 1937). However, even if the lot of peptone used has been found suitable through such preliminary tests, it is advisable always to inoculate a control tube with a known nitroso-indole-positive culture of *V. cholerae* when making cholera-red tests with unknown vibrio strains.

Tests for indole

As has been stated in the third of these studies,[1] tests for indole are of no differential-diagnostic value in cholera laboratory work because

[1] See *Bull. Wld Hlth Org.* 1955, **12**, 818.

(*a*) in addition to intestinal bacteria belonging to other genera, many cholera-like strains as well as *V. cholerae* show evidence of indole production; and (*b*) naturally, all cholera-red-positive vibrio strains are indole producers. However, as is to be expected as well, cholera-like strains which prove negative in nitroso-indole tests do not react uniformly when being examined for indole formation, yielding either negative or positive results (Taylor, Pandit & Read, 1937).

As far as could be ascertained, former cholera workers followed the method introduced by Böhme (1901) to demonstrate the presence of indole in their cultures (see, for instance, Mackie, 1929c).

This method consisted of the use of two reagents composed as follows:

(1) *p*-dimethylamidobenzaldehyde	4 parts
96% ethanol	380 parts
Concentrated hydrochloric acid	80 parts;
(2) Potassium persulfate in saturated aqueous solution (used as oxidant).	

Böhme's procedure was to add to about 10 ml of a broth culture of the organisms to be tested, first, 5 ml of solution 1, then the same amount of reagent 2, and to shake well. The presence of indole was indicated by the appearance of an intense red colour becoming visible at once or within a few minutes.

Taylor, Pandit & Read resorted to a modified procedure: (*a*) using 24-hours-old peptone water cultures of their vibrio strains instead of broth cultures; (*b*) superimposing Böhme's reagent 1 on the culture fluids instead of admixing it; and (*c*) omitting the addition of potassium persulfate solution. In their experience slight heating was useful to hasten the appearance of a positive reaction.

Animal experiments

The value of animal experiments for the differentiation of cholera from cholera-like vibrios was emphasized by Koch (1893). He insisted in this connexion that, in order to obtain reliable results, it was necessary to work with material from agar cultures and not from fluid cultures. A dose of one loop (about 1.5 mg) of the former growths, suspended in 1 ml of broth, was adequate for the intraperitoneal infection of guinea-pigs weighing 300-350 g, but increased doses had to be used for heavier animals. Maintaining that this mode of experimentation invariably led to the appearance of a typical collapse and eventually to the death of the animals, Koch stated that:

" Since one or a few well-developed [cholera] colonies are able to furnish sufficient material for an animal experiment, the great value of making agar cultures early becomes evident. One must rate the method of animal experimentation highly because, in analogy with the cholera-red test, it makes manifest a property possessed exclusively by the cholera bacteria. Among all curved, i.e., spirilla-like, bacteria which come into question

in cholera laboratory work, so far none has been found which produces in the above-mentioned dose symptoms even remotely similar to those of the cholera bacteria." [Trans.]

A determined stand against these postulations of Koch was taken by Gruber (1894), who denied the specificity of the method of animal experimentation devised by Koch and, moreover, stressed that the virulence of cholera cultures as well as the susceptibility of the test animals was variable.

Prausnitz (1903), besides suggesting that under certain circumstances, e.g., through immersion in water for some time, the cholera vibrios might lose their virulence, laid greater stress upon the fact that many of the cholera-like strains isolated from water samples at Hamburg had shown a high pathogenicity for guinea-pigs.

Subsequent observations have supported the views of Gruber and Prausnitz rather than those of Koch. It is true that there is no great likelihood of meeting avirulent strains among the cholera vibrios isolated from the faeces of patients in the acute stage of the disease and that consequently negative results of animal experiments obtained under these conditions speak strongly against the presence of *V. cholerae*. However, a positive result obtained through inoculation of test animals with vibrios isolated from the patients' stools cannot be considered conclusive. It is obvious, moreover, that it would be altogether impossible to make routine use of animal experimentation during a major outbreak of cholera. To resort to this method when dealing with early or sporadic cases would be within the realm of practical policy but, as far as the present writer can judge, it would be better then to use the available guinea-pigs for Pfeiffer's test rather than for simple intraperitoneal inoculation.

Carbohydrate tests

From the exhaustive description of the saccharolytic effects produced by cholera and cholera-like vibrios in the third of these studies [1] it will be gathered that for the practical purposes of cholera diagnosis it suffices to make tests with only three sugars, namely, saccharose, mannose, and arabinose. It has been shown that with very few exceptions strains which failed to acidify the former two substances or acidified arabinose were not cholera vibrios. At the same time, however, it has to be stressed that these characteristic reactions were given not only by *V. cholerae*, but also by a considerable number of cholera-like strains.

As has been suggested in the third study, it would be well to adopt the method of Heiberg (1934) as standard when making tests with these three sugars. The procedure of this worker was (*a*) to grow the strains to be tested in peptone water into which the various carbohydrates had been

[1] See *Bull. Wld Hlth Org.* 1955, 12, 819.

incorporated at a concentration of 0.5% (equivalent—according to Gallut, 1954—to 3 drops of a 30% solution of the sugars per 10 ml of the medium); (*b*) to add a few drops of a 0.2 per mille solution of bromothymol blue in ethanol as indicator; and (*c*) to take initial readings after an incubation of not more than 20 hours at 37° C so as to be able to distinguish between rapid and late acidifications—an essential distinction for diagnosis. As has been noted, Heiberg worked with peptone water media of comparatively low alkalinity (pH 8.0-8.4). It would be preferable for the sake of uniformity and expediency to use peptone water of a pH of 9.2 for these tests as well as for cholera laboratory work in general (see page 717 above).

Voges-Proskauer reaction

The important results which can be obtained through an examination of classical cholera vibrios, El Tor vibrios, and cholera-like vibrios with the Voges-Proskauer reaction (originally described in 1898) as well as the modern technique preferable for carrying out this method have been fully dealt with in the third study.[1] While it had to be admitted that it was impossible to distinguish with the Voges-Proskauer reaction alone between cholera and cholera-like vibrios, attention could be drawn to the cardinally important conclusions reached by Taylor, Pandit & Read (1937) when considering the results of such tests in combination with those elicited with the aid of the cholera-red reaction and fermentation tests with saccharose, mannose, and arabinose. As these workers found, it was possible

" on biochemical evidence alone, to obtain presumptive diagnosis of the serology of the typical *V. cholerae*; if it gives fermentation reactions of Heiberg type I [i.e., acidification of saccharose and mannose, but not of arabinose], is cholera-red positive and negative to the modified V-P [Voges-Proskauer] test, it is very probably an agglutinable vibrio ".

From the foregoing, it transpires that a typical result of such combined tests goes a long way to support a presumptive diagnosis of cholera arrived at through rapid slide agglutination.

[1] See *Bull. Wld Hlth Org.* 1955, **12**, 822.

Annex

It seems well to append to the present study a valuable statement on the laboratory diagnosis of cholera which was compiled by a group of outstanding cholera experts: Lt-Col. M. L. Ahuja, Director, Central Research Institute, Kasauli, India; Dr K. V. Krishnan, Professor of Microbiology, All-India Institute of Hygiene and Public Health, Calcutta, India; Dr S. R. Pandit, Director, Institut Pasteur, Shillong, India; and Dr K. V. Venkatraman, Director, King Institute, Guindy, Madras, India. Having been submitted to the Joint OIHP/WHO Study-Group on Cholera at its third session, it was first published in the report on this meeting and was afterwards republished in a slightly modified but technically unaltered form in the Indian Journal of Medical Research (Ahuja et al., 1951). The present text is that of the original version. It would have been redundant to reprint the appendix to this document, in which the various new media recommended were described. Instead of this, attention is drawn to the pages of the present study on which these media have been discussed.

LABORATORY DIAGNOSIS OF CHOLERA

Bacteriological Procedures

The bacteriological diagnosis of cholera includes the isolation of *V. cholerae* from the stool or vomit of a case under investigation, and the identification of the vibrio by morphological, cultural, biochemical, and serological procedures. The isolation of *V. cholerae* may be very easy or remarkably difficult depending upon whether one is dealing with an established case of cholera in its acute stage during an epidemic or with a convalescent or a contact some time after the subsidence of the epidemic. Likewise, the identification of the organism can more readily be assumed from a few simple tests, including a test of agglutinability with a specific 'O' agglutinating serum, during an epidemic of cholera, but should be based upon a complete examination in the case of the occurrence of a case of cholera-like disease during non-epidemic times or at the beginning of an epidemic and more especially in areas usually free from cholera. Various procedures have been followed in the isolation and identification of *V. cholerae* with more or less satisfactory results; those preferred by the authors after more thorough investigation are presented here.

Collection of specimen: The isolation of *V. cholerae* from a suspected case is most readily made from a specimen of freshly passed stool or one obtained by the insertion of a rectal swab the handle of which is protected by a piece of rubber beyond the anal sphincter. If facilities for adequate bacteriological examination are available locally the material is plated directly on a suitable solid medium. Selective media mentioned later can

bear a comparatively heavy inoculum. If, however, a delay of some hours is anticipated, or if the specimen has to be sent to a distant laboratory by post, a small portion of the stool is placed in a bottle containing 10 ml of a preserving medium of potassium chloride solution, boric acid, caustic soda, and sea salt.[1] In the case of a specimen obtained by rectal swabbing, the swab is immersed in the preserving medium and squeezed out against the side of the bottle. In the case of examination of convalescents or contacts it is more satisfactory to add a specimen of stool (about 1-3 g) to the preserving medium. Specimens of vomit, or shreds of clothing soiled with stool or vomit of a case of cholera, may similarly be added to the preserving medium.

Microscopic examination of a stained film (stained with diluted carbol-fuchsin) and a wet film from washed flakes of mucus present in the rice-water stool of a case of cholera, revealing the presence of numerous actively motile vibrios, may form the basis of a provisional diagnosis of cholera. Too much reliance should not, however, be placed on morphological examination alone; the most characteristic appearances are found only in those cases where clinically a diagnosis of cholera is almost certain, and non-choleraic vibrios indistinguishable morphologically from true *V. cholerae* abound in almost all the water in the plains of India and are to be found mixed with stool specimens.

In the cultural examination of a specimen for the isolation of *V. cholerae* from an acute case, direct plating of fresh material or material held in the preserving medium is preferred to plating after enrichment. Either plates of alkaline nutrient agar, or a differential medium such as Aronson's medium, or a modified Wilson & Reilly solid medium may be used.[2] The superiority of the results obtained with direct plating over those obtained with preliminary enrichment followed by plating has been pointed out by Pandit, who examined 430 specimens of stool samples simultaneously by both the methods, using in each case alkaline agar, Aronson medium, and the modified Wilson & Reilly solid medium, for plating; he found that 96% of the positives were picked out by direct plating, whereas only 89% were picked out by the procedure utilizing enrichment followed by plating. Our experience has also been similar. Of the plating media mentioned above, both the Aronson medium and the modified Wilson & Reilly medium have an inhibitory effect on many organisms other than vibrios, the latter medium being superior to the former in that it inhibits also the El Tor vibrio to some extent and many inagglutinable vibrios, though not all. While generally reliable, certain batches of Aronson's medium turn out to be poorly supportive of the growth of *V. cholerae*.

In the case, however, of the examination of stools of convalescents and contacts, a preliminary enrichment is desirable. Read's modification of

[1] See page 710 above.

[2] See page 748 above.

Wilson & Blair's fluid enrichment medium has been most useful in our experience.[1] This is particularly valuable when vibrios are very scanty in the stool. The fluid enrichment medium is plated with 10 ml of the preserving medium containing the entire stool specimen, and a few loopfuls of the enriched culture are subcultured on Wilson & Reilly's solid medium after 18-20 hours' incubation. The necessity for the readjustment of the pH of culture during enrichment after about 4-6 hours of incubation pointed out by Read does not arise when the buffer solution is used for collecting the stool sample.

When very large numbers of stool specimens of contacts have to be examined expeditiously, it is convenient to examine them in groups of ten. The contents of 10 specimen bottles are added to a flask containing the required amount of the fluid enrichment medium and a few loopfuls of the enriched culture plated after 18-20 hours' incubation on modified Wilson & Reilly's medium. Adequate care must be taken in pouring the contents to avoid contamination of the original bottles, which are set aside until the results of the group are known. In the event of the isolation of *V. cholerae* from any group, the small remnant of the specimen contained in each of the bottles constituting the group is individually treated by enrichment and plating to determine which was contributory to the positive isolation of the group.

The colonies of *V. cholerae* have a characteristic appearance on both Aronson's and Wilson & Reilly's media. Portions of such colonies are picked out with a platinum needle and tested by slide agglutination against a suitable dilution (1: 50 to 1: 100) of a cholera agglutinating serum containing both Inaba and Ogawa anti-O agglutinins or preferably against two agglutinating sera, one for Inaba and the other for Ogawa. During an epidemic, it is generally adequate to base a provisional diagnosis of cholera on a positive agglutination obtained by this procedure pending confirmation by subsequent testing of cultures raised from colonies picked out by this screening.

For further steps in the identification of the isolated vibrio, a few colonies which give a positive slide agglutination test are subcultured on agar slants and in tubes of isotonic Douglas broth (tryptic digest). A suspension of an 18- to 24-hour growth of agar in 0.85% salt solution containing 0.2% formalin, adjusted to contain approximately 2000 million organisms per ml (Brown's opacity tube No. 2), is used for serological identification and a 24-hour growth in Douglas broth for the haemolysis test.

Agglutination tests are carried out in a water-bath at 52° C with Inaba and Ogawa anti-O agglutinating sera. Young cultures of freshly isolated strains show a tendency to rapid lysis and, if a number of tests are being done at a time, it helps to place each rack in the water-bath as soon as the

[1] See page 721 above.

addition of the culture suspension has been made without waiting for the whole series of additions to be completed. The 'O' agglutination of cholera usually appears very early and a preliminary reading may be taken at the end of 2 hours, but the final reading is taken the next day, after the racks have been stood at room temperature overnight. The suspensions should agglutinate at over 50%-75% of the titre of either Inaba or Ogawa anti-O serum.

The haemolysis test is done by adding 1 ml of 24-hour culture in isotonic Douglas broth to 1 ml of a 5% suspension of washed sheep or goat erythrocytes, incubating the mixture at 37° C for 2 hours followed by overnight storage in the cold room. It is essential to make sure that the erythrocytes used in the test are not fragile in 0.65% saline. Alternatively, a saline suspension of a 24-hour growth on nutrient agar, standardized to contain 8000 million organisms per ml, may be used. To 1 ml of this vibrio suspension is added 1 ml of a 3% suspension of washed erythrocytes and the mixture treated as above. Krishnan, who recently made a detailed study of the various factors involved in the tests, has shown that more consistent results may be obtained by using broth cultures in preference to saline suspensions of agar cultures and that a 24-hour growth in broth is preferable to a 48- or 72-hour growth.

The culture in Douglas broth is also utilized for the inoculation of fermentation tubes for the determination of the range of action of the vibrio under test. *V. cholerae* produces acid without gas in glucose, mannose, saccharose, and maltose, but not in lactose and arabinose. Cholera-red reaction can be demonstrated by the addition of a few drops of pure sulfuric acid to a 24-hour peptone water culture of the organism, but the test is not distinctive of *V. cholerae*. Many other vibrios also give the reaction. The Voges-Proskauer reaction is negative.

Although speed in diagnosis of cholera is very important, it is equally essential that judgement should be reserved and only a provisional report returned until the essential tests for the identification of the *V. cholerae* are completed, more especially in areas where cholera is not usually prevalent. Under favourable conditions it is possible to offer a provisional diagnosis, by the procedures outlined, in 16-20 hours. A definitive diagnosis takes 48 hours more.

RÉSUMÉ

Cette étude est consacrée au diagnostic du choléra en laboratoire. L'auteur décrit les méthodes de prélèvement et de conservation des fèces, d'enrichissement du matériel et de culture sur milieux solides. Il mentionne aussi les procédés applicables à la recherche du vibrion dans les vomissures et dans l'eau. Les tests permettant d'établir le diagnostic (agglutination, hémolyse, bactériophage) sont ensuite décrits, ainsi que les épreuves destinées à le confirmer (réaction de Millon, de la trypaflavine, de l'indol, etc.). En annexe sont discutées les techniques bactériologiques de diagnostic à appliquer suivant les circonstances.

REFERENCES

Abel, R. & Claussen, R. (1895) Untersuchungen über die Lebensdauer der Cholera-vibrionen in Fäkalien. *Zbl. Bakt. 1. Abt.* **17**, 77, 118

Ahuja, M. L. (1951) *A note on the serological analysis of* V. cholerae *with particular reference to a new test for the identification of roughness in cholera strains* (Unpublished working document WHO/Cholera/11)

Ahuja, M. L. et al. (1950) Laboratory diagnosis of cholera. Bacteriological procedures. In: *Wld Hlth Org. techn. Rep. Ser.* **18**, p. 10

Ahuja, M. L. et al. (1951) Laboratory diagnosis of cholera. A note on bacteriological procedures. *Indian J. med. Res.* **39**, 135

Alessandrini, A. & Sabatucci, M. (1931) La tripaflavina quale mezzo di differenziazione dei microbi del genere Brucella. *Ann. Igiene*, **41**, 29

American Public Health Association (1950) *Diagnostic procedures and reagents ; technics for the laboratory diagnosis and control of the communicable diseases*, 3rd ed., New York, p. 25

Andrade, E. (1906) Influence of glycerin in differentiating certain bacteria. *J. med. Res.* **14**, 551

Arens (1893) Über den Nachweis weniger Cholerakeime in grösseren Mengen Trink-wassers. *Münch. med. Wschr.* **40**, 190

Aronson, H. (1915) Eine neue Methode der bakteriologischen Choleradiagnose. *Dtsch. med. Wschr.* **41**, 1027

Asheshov, I. N. et al. (1933a) Studies on cholera bacteriophage. Part I. General tech-nique. *Indian J. med. Res.* **20**, 1101

Asheshov, I. N. et al. (1933b) Studies on cholera bacteriophage. Part III. Virulence and development of bacteriophage. *Indian J. med. Res.* **20**, 1159

Babes, V. (1914) Studien über Cholerabekämpfung. *Z. Hyg. InfektKr.* **77**, 501

Baerthlein, K. (1912) Über die Differentialdiagnose der choleraähnlichen Vibrionen. *Berl. klin. Wschr.* **49**, 156

Baerthlein, K. & Gildemeister, E. (1915) Über Choleraelektivnährböden. *Zbl. Bakt. 1. Abt. Orig.* **76**, 550

Bandi, I. (1910) Le epidemie coleriche delle Puglie e di Napoli. *Riv. crit. Clin. med.* **11**, 770, 785, 802

Baumgarten, A. & Langer-Zuckerkandl, H. (1917) Über elektive Choleranährböden. *Z. Hyg. InfektKr.* **83**, 389

Bengston, I. A. (1924) The adaptibility of various American peptones for use in cholera media. *Bull. U.S. Hyg. Lab.* No. 139, p. 37 (Summarized in *Trop. Dis. Bull.* 1926, **23**, 188)

Bhaskaran, K. (1953) Studies on vibrio dissociation. Part I. Smooth rough dissociation of *V. cholerae* in rosaniline agar. *Indian J. med. Res.* **41**, 143

Bleisch, M. (1893) Über einige Fehlerquellen bei Anstellung der Cholerarothreaktion und ihre Vermeidung. *Z. Hyg. InfektKr.* **14**, 103

Boccolari, A. & Olivi, G. (1916) Il terreno di Aronson per la diagnosi del colera. *Ann. Med. nav. colon.* **2**, 13 (Quoted in *Trop. Dis. Bull.* 1917, **10**, 84)

Böhme, A. (1901) Die Anwendung der Ehrlichschen Indolreaktion für bakteriologische Zwecke. *Zbl. Bakt. 1. Abt. Orig.* **40**, 129

Bötticher, E. (1915) Die bakteriologische Choleradiagnose unter besonderer Berück-sichtigung der von Aronson und Lange neuerdings angegebenen Choleranährböden. *Dtsch. med. Wschr.* **41**, 1303

Bose, S. (1939) Note on the preparation of an unpurified mannose solution for bacterio-logical use. *Indian J. med. Res.* **27**, 73

Brieger, L. (1887) Zur Kenntniss der Aetiologie des Wundstarrkrampfes nebst Bemer-kungen über das Choleraroth. *Dtsch. med. Wschr.* **13**, 303

Brounst, G. & Maroun, T. (1949) Recherche d'anticorps chez des sujets vaccinés contre le choléra. *Ann. Inst. Pasteur*, **76**, 554

Bürgers, T. J. (1910) Bakteriologische Ergebnisse der Choleraepidemie 1909 in Ostpreussen. *Hyg. Rund. (Berl.)*, **20**, 169

Bujwid, O. (1887) Eine chemische Reaktion für die Cholerabakterien. *Z. Hyg.* **2**, 52

Bujwid, O. (1888) Neue Methode zum Diagnosticiren und Isoliren der Cholerabakterien. *Zbl. Bakt.* **4**, 494

Burrows, W. et al. (1946) Studies on immunity to Asiatic cholera. II. The O and H antigenic structure of the cholera and related vibrios. *J. infect. Dis.* **79**, 168

Cantacuzène, J. (1933) Diagnostic microbiologique du vibrion cholérique et choix d'un antigène pour la préparation d'un sérum agglutinant. *Bull. Off. int. Hyg. publ.* **25**, 984

Ch'i, C. T. & Zia, S. H. (1949) Further studies of a differential medium for the isolation of *V. cholerae*. *Chin. med. J.* **67**, 496

Combiesco-Popesco, C. & Wisner, B. (1933) Recherches sur l'agglutinabilité et la sensibilité au bactériophage des vibrions cholériques et paracholériques. *C. R. Soc. Biol. (Paris)*, **113**, 484

Craster, C. V. (1913) Ship-borne cholera. The sea as factor in the transmission of cholera. *J. Amer. med. Ass.* **61**, 2210

Creel, R. H. (1911) Method employed at New York Quarantine for the detection of cholera carriers. *J. Amer. publ. Hlth Ass.* **1**, 899

Crendiropoulo, M. (1912) *Rapport sur l'examen des selles des voyageurs provenant des pays infectés de choléra.* (Conseil sanitaire, maritime, et quarentenaire d'Egypte, Alexandrie) (Quoted in *Zbl. Bakt. 1. Abt. Ref.* **55**, 361)

Crendiropoulo, M. & Panayotatou, A. (1910) Sur un nouveau milieu pour le diagnostic du choléra. *Zbl. Bakt. 1. Abt. Orig.* **55**, 248

Dahmen, M. (1892) Die Nährgelatine als Ursache des negativen Befundes bei Untersuchung der Faeces auf Cholerabacillen. *Zbl. Bakt.* **12**, 620

Damboviceanu, A. (1933) Agglutination par les acides de vibrions cholériques et paracholériques. *C. R. Soc. Biol. (Paris)*, **113**, 485

Deeleman, M. (1897) Der Einfluss der Reaktion des Nährbodens auf das Bakterienwachstum. *Arb. GesundhAmt (Berl.)*, **13**, 374

Deycke, H. (1893) Über einen neuen elektiven Nährboden für Cholerabazillen. *Dtsch. med. Wschr.* **19**, 888

Dieudonné, A. (1909) Blutalkaliagar, ein Elektivnährboden für Choleravibrionen. *Zbl. Bakt. 1. Abt. Orig.* **50**, 107

Dieudonné, A. & Baerthlein, K. (1912) Über Choleraelektivnährböden. *Münch. med. Wschr.* **59**, 1752

Dishon, T. (1951) A selective medium for the isolation of " Vibrio cholerae ". *Bull. Res. Coun. Israel*, **1**, No. 1-2, 158

Douglas, S. R. (1914) On a method of making cultivation media without prepared peptone and on a peptone-free medium for growing tubercle baccilli. *Lancet*, **2**, 891

Dreyer, G. (1906) Om anvendelse af draebt kultur til Widal-Reaktion. *Hospitalstidende*, **14**, 532

Dreyer, G. (1909) Widal's reaction with sterilized cultures. *J. Path. Bact.* **13**, 331

Drigalski, K. W. von & Conradi, H. (1902) Über ein Verfahren zum Nachweis der Typhusbazillen. *Z. Hyg. InfektKr.* **39**, 283

Dunbar (1896) Bericht über die Arbeiten des im Herbst 1892 anlässlich der Cholera-Epidemie in Hamburg errichteten provisorischen hygienischen Instituts. *Arb. GesundhAmt (Berl.)*, **10**, Appendix 9, 142

Dunham, E. K. (1887) Zur chemischen Reaktion der Cholerabakterien. *Z. Hyg.* **2**, 337

Eijkman, C. (1901) Über Enzyme bei Bakterien. *Zbl. Bakt. 1. Abt.* **29**, 841

Endo, S. (1904) Über ein Verfahren zum Nachweis der Typhusbacillen. *Zbl. Bakt. 1. Abt. Orig.* **35**, 109

Esch, P. (1910) Zum bakteriologischen Choleranachweis mittels Blutalkali-Nährböden. *Dtsch. med. Wschr.* **36**, 559

Esch, P. (1912) Zur Frage der Choleraelektivnährböden. *Dtsch. med. Wschr.* **38**, 1682

Esch, P. (1915) Fleischnatronagar als Choleraelektivnährböden. *Münch. med. Wschr.* **62**, 790

Escherich, T. (1884) Klinisch-therapeutische Beobachtungen aus der Cholera-Epidemie in Neapel. *Ärztl. Intell.-Bl. (Münch.)*, **31**, 561 (Quoted by Kolle, 1904)

Felsenfeld, O. et al. (1951) Studies on recently isolated cholera vibrios. Re-evaluation of culture methods. *J. Bact.* **62**, 175

Finkelstein, M. H. (1931) Problems in the bacteriology of cholera and cholera-like infections. *Trans. roy. Soc. trop. Med. Hyg.* **25**, 29

Flügge, C. (1893) Die Verbreitungsweise und Verhütung der Cholera auf Grund der neueren epidemiologischen Erfahrungen und experimentellen Forschungen. *Z. Hyg. InfektKr.* **14**, 122

Fraenkel, C. (1892) Nachweis der Cholerabakterien im Flusswasser. *Dtsch. med. Wschr.* **18**, 925

Fraenkel, E. (1892) Über die Diagnose der Cholera asiatica. *Dtsch. med. Wschr.* **18**, 880

Friedberger, E. & Luerssen, A. (1905) Zur bakteriologischen Choleradiagnose. *Dtsch. med. Wschr.* **31**, 1597

Fügner, I. (1914) Über den modifizierten Dieudonnéschen Choleranährboden von Hofer & Hovorka. *Zbl. Bakt. 1. Abt. Orig.* **74**, 354

Fürst, T. (1916) Lentzsches Blutalkalitrockenpulver zur Bereitung von Choleranährböden in Feldlaboratorien. *Dtsch. med. Wschr.* **42**, 226

Gallut, J. (1949) Contribution à l'étude de l'antigène thermostable du vibrion cholérique. Applications pratiques de l'analyse antigénique O. *Ann. Inst. Pasteur*, **76**, 122

Gallut, J. (1953) Sur le pouvoir vibriocide du sérum de cobaye considéré comme révélateur du caractère " R " du " Vibrion cholerae ". *Ann. Inst. Pasteur*, **84**, 363

Gallut, J. (1954) Les éléments du diagnostic bactériologique du choléra. *Rev. colon. Méd. Chir.* **26**, 158

Gallut, J. & Brounst, G. (1949) Sur la mise en évidence des agglutinins cholériques. *Ann. Inst. Pasteur*, **76**, 557

Gallut, J. & Grabar, P. (1943) Recherches immunochimiques sur le vibrion cholérique. I. Etude quantitative de la réaction de précipitation de l'antigène glucidolipidique par l'immunsérum de lapin. *Ann. Inst. Pasteur* **69**, 250

Gamaléia, M. N. (1888) Vibrio Metchnikovi (n.sp.) et ses rapports avec le microbe du choléra asiatique. *Ann. Inst. Pasteur*, **2**, 482

Gardner, A. D. (1931) *Technique of serological reactions.* In: Great Britain, Medical Research Council, *A system of bacteriology in relation to medicine*, vol. 9, p. 184

Gardner, A. D. & Venkatraman, K. V. (1935) The antigens of the cholera group of vibrios. *J. Hyg. (Lond.)*, **35**, 262

Gardner, A. D. & White, P. B. (1937) Résumé des résultats obtenus dans l'Inde par l'emploi des sérums " O " agglutinant de vibrion cholérique préparés avec des antigènes " O " standard provisoires. *Bull. Off. int. Hyg. publ.* **29**, 1855

Ghedini, G. (1916) A proposito del terreno di Dieudonné. *Pathologica*, **8**, 191 (Quoted in *Trop. Dis. Bull.* **8**, 167)

Gibson, H. G. (1916) A new solid medium for the isolation of the cholera vibrio. *Brit. med. J.* **2**, 454

Glaser, E. & Hachla, J. (1911) Ist der Dieudonnésche Nährboden nur für die Choleravibrionen elektiv? *Zbl. Bakt. 1. Abt. Orig.* **57**, 371

Gohar, M. A. (1941) The bacteriostatic, bactericidal and possible chemotherapeutic properties of potassium tellurite with special reference to a method for the isolation of the cholera vibrio. *J. trop. Med. Hyg.* **44**, 96

Gohar, M. A. (1947) A rapid method for the bacteriological diagnosis of cholera. *J. roy. Egypt. med. Ass.* **30**, 553

Gohar, M. A. (1948) Isolation of the cholera vibrio. *J. trop. Med. Hyg.* **51**, 59

Gohar, M. A. (1951) *Laboratory diagnosis of cholera. Enrichment with potassium tellurite.* (Unpublished working document WHO/Cholera/23)

Gohar, M. A. & Makkawi, M. (1947) Potassium tellurite in the isolation of the cholera vibrio. *J. roy. Egypt. med. Ass.* **30**, 556

Gohar, M. A. & Makkawi, M. (1948a) Cholera in Egypt. Laboratory diagnosis and protective inoculation *J. trop. Med. Hyg.* **51**, 95

Gohar, M. A. & Makkawi, M. (1948b) Isolation of the cholera vibrio. *J. roy. Egypt. med. Ass.* **31**, 462

Goldberger, J. (1913) Some new cholera selective media. *Bull. U.S. Hyg. Lab.* No. 91, p. 19

Gordon, M. H. (1906) Note on the ability of *V. cholerae asiaticae* to decompose starch. *Zbl. Bakt. 1. Abt. Orig.* **42**, 5

Gradwohl, R. B. H. (1948) *Clinical laboratory methods and diagnosis. A textbook on laboratory procedures with their interpretation*, 4th ed., vol. 2, p. 1365

Great Britain, Ministry of Health, Public Health Laboratory Service (1947) Bacteriological examination of stools for *Vibrio cholerae. Monthly Bull. Minist. Hlth (Lond.)*, **6**, 225 (Quoted in *Trop. Dis. Bull.* 1948, **45**, 336)

Greig, E. D. W. (1913) An investigation of cholera convalescents and contacts in India. *Indian J. med. Res.* **1**, 65

Greig, E. D. W. (1917) The results of the bacteriological examination of the stools of 659 cases of cholera at Calcutta. *Indian J. med. Res.* **4**, 651

Gruber, M. (1887) Bakteriologische Untersuchung von choleraverdächtigen Fällen unter erschwerenden Umständen. *Wien. med. Wschr.* **37**, 185, 221

Gruber, M. (1894) Cholerastudien II. Über die bakteriologische Diagnostik der Cholera und des Choleravibrio. *Arch. Hyg. (Berl.)*, **20**, 123

Gruber, M. & Durham, H. E. (1896) Eine neue Methode zur raschen Erkennung des Choleravibrio und des Typhusbacillus. *Münch. med. Wschr.* **43**, 285

Hach, J. W. (1924) Versuche über die Anwendung der Ottolenghischen Gallennährflüssigkeit als Elektivnährboden in der praktischen Choleradiagnostik. *Z. Hyg. InfektKr.* **103**, 518

Hachla, J. & Holobut (1909) Beitrag zur Frage elektiver Nährböden für Choleravibrionen. *Zbl. Bakt. 1. Abt. Orig.* **52**, 299

Haendel & Baerthlein (1912) Vergleichende Untersuchungen über verschiedene Choleraelektivnährböden. *Arb. GesundhAmt (Berl.)*, **40**, 357

Hall, H. C. (1916) Ist es möglich sofort einen brauchbaren Dieudonné-Agar herzustellen ohne die Zusammensetzung des Substrates zu verändern? *Berl. klin. Wschr.* **53**, 217

Harris, N. M. (1925) The preparation of Endo's medium. *Milit. Surg.* **57**, 280 (Quoted in *Trop. Dis. Bull.* 1926, **23**, 188)

Heiberg, B. (1934) Des réactions de fermentation chez les vibrions. *C. R. Soc. Biol. (Paris)*, **115**, 984

Heider, A. (1893) Vibrio danubicus. *Zbl. Bakt.* **14**, 341

Heim, L. (1892) Zur Technik des Nachweises der Choleravibrionen. *Zbl. Bakt.* **12**, 353

Heim, L. (1901) Zum Nachweise der Choleravibrionen. *Zbl. Bakt. 1. Abt.* **30**, 570

Hesse, E. (1920) Vergleichende Untersuchungen über Choleraelektivnährböden. *Arb. ReichsgesundhAmt*, **52**, 596

Hetsch, H. (1903) Beitrag zur Frage der Leistungsfähigkeit des Peptonwasser-Anreicherungsverfahrens in der praktischen Choleradiagnostik. *Z. Hyg. InfektKr.* **45**, 348

Hirschbruch, A. & Schwer (1903) Die Choleradiagnose mit Hilfe eines Spezialagars. *Zbl. Bakt. 1. Abt. Orig.* **34**, 585

Hirschbruch, A. & Schwer (1904) Bemerkungen über feste Nährböden zum Zwecke der Choleradiagnose. *Zbl. Bakt. 1. Abt. Orig.* **36**, 144

Hofer, G. & Hovorka, J. (1913) Versuche zur elektiven Ausgestaltung des Dieudonnéschen Choleranährbodens. *Zbl. Bakt. 1. Abt. Orig.* **71**, 103

Huntemüller (1909) Der Dieudonnésche Blut-Alkali-Agar. *Zbl. Bakt. 1. Abt. Orig.* **50**, 109

Indian Research Fund Association, Scientific Advisory Board (1942) *Cholera treatment enquiry under the Director, School of Tropical Medicine, Calcutta.* In: *Report... for the year... 1942*, New Delhi, p. 1

Indian Research Fund Association, Scientific Advisory Board (1949) *Inquiry on cholera under the Director, School of Tropical Medicine, Calcutta.* In: *Report... for the year 1949*, New Delhi, p. 7

Ito, T. (1914) [On cholera media.] *Nippon Eiseigaku-Zasshi*, **10**, No. 3 (Quoted by Takano, Ohtsubo & Inouye, 1926)

Kabeshima, T. (1913) Über einen Hämoglobinextract-Soda-Agar als Elektivnährboden für Choleravibrionen. *Zbl. Bakt. 1. Abt. Orig.* **70**, 202

Kabeshima, T. (1922) [On peptone water of the proliferation of *V. cholerae*.] *Jap. Z. Mikrobiol. Path.* **16**, No. 5 (Summarized in *Jap. med. Wld*, **2**, 322 and in *Trop. Dis. Bull.* 1923, **20**, 369)

Karliński, J. (1890) Zur Kenntniss der Tenacität der Choleravibrionen. *Zbl. Bakt.* **8**, 40

Kauffmann, F. (1950) On the serology of the *Vibrio cholerae*. *Act. path. microbiol. scand.* **27**, 283

Kiribayashi, S. (1931) [Notes about the early diagnosis of cholera. Part I. Especially on the agglutination test when peptone-water is used as the medium.] *J. med. Ass. Formosa*, **30**, 80 (Summarized in *Trop. Dis. Bull.* 1932, **29**, 378)

Kiribayashi, S. (1933) [Supplementary report on the biological pecularities of *Vibrio cholera*. II. On the development of *Vibrio cholera* on starch-agar-media.] *J. med. Ass. Formosa*, **32**, 66 (Quoted in *Trop. Dis. Bull.* 1934, **31**, 47)

Klein, E. (1905) Über einen neuen tierpathogenen Vibrio—Vibrio cardii. *Zbl. Bakt. 1. Abt. Orig.* **38**, 173

Koch, R. (1884) In: Die Conferenz zur Erörterung der Cholerafrage. *Dtsch med. Wschr.* **10**, 499, 519; (Also in: Conferenz zur Erörterung der Cholerafrage. *Berl. klin. Wschr.* 1884, **21**, 477, 493, 509)

Koch, R. (1893) Über den augenblicklichen Stand der bakteriologischen Choleradiagnose. *Z. Hyg. InfektKr.* **14**, 319

Koch, R., Kirchner, M. & Kolle, W. (1902) Erlass des Ministers der geistlichen, Interrichts- und Medizinal-Angelegenheiten betreffend Anleitung für die bakteriologische Feststellung der Cholerafälle, vom 6. November 1902. *MinistBl. preuss. MedAngeleg.* No. 12 (reprinted by Kolle, 1904)

Koch, W. & Kaplan, D. (1952) A cholera medium with more than tenfold yield. *Bull. Wld Hlth Org.* **7**, 353

Koch, W. S. & Kaplan, D. (1953) Improved media for Vibrio cholerae and salmonella. *Amer. J. trop. Med. Hyg.* **2**, 279

Kodama, T. (1921) [On a new specific medium for *Vibrio cholerae*.] *Jap. J. Hyg. infect. Dis.* **17**, No. 1 (Quoted by Takano, Ohtsubo & Inouye, 1926, p. 12 and in *Trop. Dis. Bull.* 1922, **19**, 381)

Kodama, T. (1922a) [Contribution to the knowledge of the new specific medium for *V. cholerae*, together with a critical review of Aronson's medium.] *Jap. J. Hyg. infect. Dis.* **17**, No. 3 (Quoted in *Jap. med. Wld*, **2**, 176 and in *Trop. Dis. Bull.* 1922, **19**, 738)

Kodama, H. (1922b) Ein neuer elektiver Nährboden für Choleravibrionen. *Zbl. Bakt. 1. Abt. Orig.* **88**, 433

Kolle, W. (1903) Über den jetzigen Stand der Choleradiagnose. *Klin. Jb.* **11**, 357

Kolle, W. (1904) *Cholera asiatica.* In: Kolle, W. & Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, vol. 3, p. 1

Kolle, W. & Gotschlich, E. (in collaboration with Hetsch, H., Lentz, O. & Otto, R.) (1903) Untersuchungen über die bakteriologische Choleradiagnostik und Spezifität des Koch'schen Choleravibrio. *Z. Hyg. InfektKr.* **44**, 1

Kolle, W. & Prigge, R. (1928) *Cholera asiatica.* In: Kolle, W., Kraus, R. & Uhlenhuth, P., *Handbuch der pathogenen Mikroorganismen*, 3rd ed., Jena, vol. 4, part 1, p. 1

Kolle, W. & Schürmann, W. (1912) *Cholera asiatica.* In: Kolle, W. & Wassermann, A. von, *Handbuch der pathogenen Mikroorganismen*, 2nd ed., Jena, vol. 4, p. 1

Ko-Ran (1922a) [A new cholera medium]. *Tokyo med. News*, No. 2291 (Quoted by Takano, Ohtsubo & Inouye, 1926, p. 13)

Ko-Ran (1922b) [Special culture medium for cholera asiatica prepared after a new method.] *J. med. Ass. Formosa*, No. 223, pp. 4 & 35 (Quoted in *Trop. Dis. Bull.* 1923, **20**, 369)

Kraus, R., Zia, Z. & Zubrzciky, J., von (1911) Über einen flüssigen Nährboden zur Anreicherung von Choleravibrionen (Blutalkalibouillon). *Wien. klin. Wschr.* **24**, 1084

Krishnan, K. V. & Gupta, M. S. (1949) *A standard haemolytic test for diagnosis of* V. cholerae. (Unpublished document)

Krombholz, E. & Kulka, W. (1912) Zur Anreicherung der Choleravibrionen, insbesondere über Ottolenghi's Galleverfahren. *Zbl. Bakt. 1. Abt. Orig.* **62**, 521

Krumwiede, C. Jr., Pratt, J. S. & Grund, M. (1912) Cholera—simple methods of bacteriological diagnosis. *J. infect. Dis.* **10**, 134

Lange, C. (1915) Ein neuer Nährboden für die Choleradiagnose. *Dtsch. med. Wschr.* **41**, 1119

Lange, C. (1916) Ein neuer Nährboden für die Choleradiagnose. *Z. Hyg. InfektKr.* **81**, 138

Lefebvre, M. A. & Gallut, J. (1937) Sur l'emploi d'un milieu électif pour l'isolement du vibrion cholérique. *Bull. Soc. méd.-chir. Indochine*, **15**, 1069 (Summarized in *Trop. Dis. Bull.* 1938, **35**, 740)

Lentz, O. (1915) Bereitung des Dieudonné-Agars mit Hilfe eines Blutalkali-Trockenpulvers. *Dtsch. med. Wschr.* **41**, 425

Lieou, Y. (1938) Sur un vibrion cholérique isolé par inoculation au cobaye du contenu gastrique. *Bull. Soc. Path. exot.* **31**, 212

Linton, R. W. & Seal, S. C. (1935) The effect of the use of living or dead suspensions of vibrios on the agglutination titre. *Indian med. Gaz.* **70**, 68

Logie, W. J. (1913) On the inhibition of the cholera-red reaction by certain nitrite-destroying organisms and on the mutual inhibition of *B. dysenteriae* (Flexner) and *V. cholerae* when grown together. *J. Hyg. (Lond.)*, **13**, 162

Lubarsch, O. (1892) Zur Epidemiologie der asiatischen Cholera. *Dtsch. med. Wschr.* **18**, 978

MacConkey, A. (1905) Lactose-fermenting bacteria in faeces. *J. Hyg. (Lond.)*, **5**, 333

Mackie, T. J. (1929a) *The cholera vibrio and related organisms. Morphology and staining reactions of* V. cholerae. In: Great Britain, Medical Research Council, *A system of bacteriology in relation to medicine*, London, vol. 4, p. 346

Mackie, T. J. (1929b) *The cholera vibrio and related organisms. Cultivation of* Vibrio cholerae *and its cultural characters.* In: Great Britain, Medical Research Council, *A system of bacteriology in relation to medicine*, London, vol. 4, p. 350

Mackie, T. J. (1929c) *The cholera vibrio and related organisms. Biochemical properties.* In: Great Britain, Medical Research Council, *A system of bacteriology in relation to medicine*, London, vol. 4, p. 362

McLaughlin, A. J. (1916) The control of Asiatic cholera on international trade routes. *Amer. J. trop. Dis.* **3**, 392

Maitra, G. C. & Basu, J. B. (1924) A short note on the method of successfully cultivating cholera vibrio from cases of clinical cholera. *Calcutta med. J.* **19**, 1

Millon, E. (1849) Sur un réactif propre aux composées protéiques. *C. R. Acad. Sci. (Paris)*, **28**, 40

Mitsutake, S. (1912) [Über einen neuen Differentialnährboden für Choleravibrionen.] *Z. Militärärz.* No. 29, p. 48 (quoted by Kolle & Prigge, 1928)

Moldovan (1912) Praktische Ergebnisse der bakteriologischen Cholerauntersuchungen in Dalmatien in 1911. *Öst. Sanitätsw.* No. 8 (Quoted by Haendel & Baerthlein, 1912)

Müller, P. T. (1915) Über Choleramassenuntersuchungen. *Münch. med. Wschr.* **62**, 1659
Narayanan, E. K. (1941) Some observations on the preparation of mannose. *Indian J. med. Res.* **29**, 1
Neisser, M. (1893) Über einen neuen Wasser-Vibrio, der die Nitrosoindol-Reaktion liefert. *Arch. Hyg. (Berl.)*, **19**, 194
Neufeld, F. & Woithe (1910) Über elektive Choleranährböden, insbesondere den Dieudonnéschen Agar. *Arb. GesundhAmt (Berl.)*, **33**, 605
Neumann, R. O. (1915) Über die Cholerabekämpfung in Rumänien. *Arch. Hyg. (Berl.)*, **84**, 1
Nicholls, L. (1917) The chemical affinities of *V. cholerae*. *Lancet*, **2**, 563
Ottolenghi, D. (1911) Über eine neue Methode zur Isolierung der Choleravibrionen aus den Faeces. *Zbl. Bakt. 1. Abt. Orig.* **58**, 369
Pampana, E. J. (1931) La dissociazione microbica e la tripaflavine come suo reattivo. *Ann. Igiene*, **41**, 537
Pampana, E. J. (1933) Microbic dissociation: Detection of the "*R*" variant by means of a specific drop-agglutination. *J. Hyg. (Lond.)*, **33**, 402
Pandit, S. R. (1934) A device for filtering bacteriophage. *Indian J. med. Res.* **22**, 17
Pandit, S. R. (1941) *Cholera field enquiry in Bengal under Dr. S. R. Pandit at the All-India Institute of Hygiene and Public Health, Calcutta.* In: Indian Research Fund Association, Scientific Advisory Board, *Report... for the year...* 1941, New Delhi, p. 1
Panganiban, C. S. & Schoebl, O. (1918) Preservation of cholera stool specimens for delayed bacteriological examination. *Philipp. J. Sci., Sec. B*, **13**, 275
Panja, G. (1942) A new method of isolation of vibrios from cholera stool. *Indian J. med. Res.* **30**, 391
Panja, G. & Ghosh, S. K. (1943) A modified medium for isolation of dysentery, enteritis and cholera organisms. *Indian med. Gaz.* **78**, 55
Panja, G. & Ghosh, S. K. (1947) Isolation of cholera vibrios from Hooghly river water at Calcutta. *Indian J. med. Res.* **35**, 1
Panja, G., Malik, K. S. & Paul, B. M. (1942) Examination of cholera vomit. *Indian med. Gaz.* **77**, 347
Pasricha, C. L., De Monte, A. J. & Gupta, S. K. (1932a) Mutation of cholera vibrios. (The characters of the population of a freshly isolated cholera colony, with a note on some colony variants of cholera and cholera-like vibrios). *Indian med. Gaz.* **67**, 64
Pasricha, C. L., De Monte, A. J. & Gupta, S. K. (1932b) Cholera and cholera-like vibrophages. *Indian med. Gaz.* **67**, 487 (quoted by Seal, 1935)
Petri, R. J. (1887) Eine kleine Modification des Koch'schen Plattenverfahrens. *Zbl. Bakt.* **1**, 279
Petri, R. J. (1890) Über die Verwerthung der roten Salpetrigsäure-Indolreaction zur Erkennung der Cholerabakterien. *Arb. GesundhAmt (Berl.)*, **6**, 1
Petruschky, J. (1896) Bacillus faecalis alcaligenes (n. sp.). *Zbl. Bakt. 1. Abt.* **19**, 187
Pfeiffer, R. (1895) Die Differentialdiagnose der Vibrionen der Cholera asiatica mit Hilfe der Immunisierung. *Z. Hyg. InfektKr.* **19**, 75
Pilon, P. (1911) Blut-Soda-Agar als Elektivenährboden für Choleravibrionen. *Zbl. Bakt. 1. Abt. Orig.* **60**, 330
Piras, L. (1913) Bakteriologische Beobachtungen, die während der Choleraepidemie zu Genua im Jahre 1911 gemacht worden sind. *Hyg. Rund. (Berl.)*, **23**, 641
Poehl, A. (1886) In: Chemico-biology of micro-organisms and ptomaines. (Editorial) *Lancet*, **2**, 830
Pollitzer, R. (1926) Laboratory reports. In: Wu Lien-teh, Chun, J. W. H. & Pollitzer, R. Preliminary report on the 1926 cholera epidemic. *Nat. med. J. China*, **12**, 413 (439)
Pollitzer, R. (1934) *Laboratory aspects.* In: Wu Lien-teh, Chun, J. W. H., Pollitzer, R. & Wu, C. Y. *Cholera—a manual for the medical profession in China*, Shanghai
Popesco-Combiesco, C. & Soru, E. (1934) Recherches sur l'agglutinabilité des vibrions cholériques et paracholériques par la trypaflavine. *C.R. Soc. Biol. (Paris)*, **115**, 1317

Pottevin, H. (1913) Contribution à l'étiologie du choléra. *Bull. Off. int. Hyg. publ.* **5**, 1158
Pottevin, H. (1915) Instructions pour le prélèvement, l'envoi et l'examen des fèces en vue de la recherche du vibrion cholérique. *Bull. Soc. Path. exot.* **8**, 98
Prausnitz, C. (1903) Zum gegenwärtigen Stand der Choleradiagnose unter besonderer Berücksichtigung derjenigen Vibrionen, deren Unterscheidung von Choleravibrionen Schwierigkeiten bereitet. *Z. Hyg. InfektKr.* **43**, 239
Read, W. D. B. (1939) Differential isolation of *V. cholerae*. *Indian J. med. Res.* **26**, 851
Read, W. D. B. & Pandit, S. R. (1941) Distribution of *V. cholerae* and El Tor type strains in certain rural areas in India. *Indian J. med. Res.* **29**, 403
Read, W. D. B. et al. (1939) Growth and survival of *V. cholerae* with special reference to growth and survival in water. *Indian J. med. Res.* **27**, 1
Rivas, D. & Smith, A. J. (1912) The detection of cholera bacillus from faeces and water in twenty-four to forty-eight hours. *New Orleans med. surg. J.* **65**, 273
Schoebl, O. (1915) Practical experiences with some enriching media recommended for the bacteriological diagnosis of Asiatic cholera. *Philipp. J. Sci. Sec. B*, **10**, 127
Schottelius, M. (1885) Zum mikroskopischen Nachweis der Cholerabacillen in Dejectionen. *Dtsch. med. Wschr.* **11**, 213
Schürmann, W. & Abelin-Rosenblat, S. (1913) Die bakteriologische Choleradiagnose auf Grund von Prüfungen neuer Anreicherungs- und Differenzierungsmethoden. *Med. Klinik*, **9**, 138
Schürmann, W. & Fellmer, T. (1915) Zur bakteriologischen Choleradiagnose. *Dtsch. med. Wschr.* **41**, 1183
Seal, S. C. (1935) Difficulties in the bacteriological diagnosis of cholera vibrios. *Indian med. Gaz.* **70**, 614
Seal, S. C. (1939) A preliminary note on the relative efficiency of bismuth-sulphite medium and peptone-water enrichment in the isolation of *V. cholerae* from human and other sources. *Indian J. med. Res.* **27**, 297
Seneca, H. & Henderson, E. (1949) Laboratory diagnosis of cholera. *Amer. J. trop. Med.* **29**, 921
Sgalitzer, M. & Loewy, O. (1913) Über die Verwendbarkeit der Blutalkalibouillon als Anreicherungsmittel für Choleravibrionen. *Zbl. Bakt. 1. Abt. Orig.* **69**, 556
Shahin, M. (1933) L'examen bactériologique des selles des pèlerins égyptiens pour l'année 1932, à leur départ et à leur retour du pèlerinage. *Bull. Off. int. Hyg. publ.* **25**, 85
Singh, G. & Ahuja, M. L. (1950) A note on the antigenic relationship to *V. cholerae* of the so-called "A" type of vibrio (Burrows) and "B" type of vibrio (Gallut). *Indian J. med. Res.* **38**, 317
Singh, G. & Ahuja, M. L. (1951) A new test for the identification of roughness in *V. cholerae*. *Indian J. med. Res.* **39**, 417
Soda, Y. et al. (1936) Sur le délai dans lequel les selles doivent être examinées pour la recherche du vibrion cholérique. *Bull. Off. int. Hyg. publ.* **28**, 64
Sokhey, S. S., Habbu, M. K. & Bharucha, K. H. (1950) Hydrolysate of casein for the preparation of plague and cholera vaccines. *Bull. Wld Hlth Org.* **3**, 25
Stern, W. (1915) Vergleichende Untersuchungen mit festen Cholera-Elektivnährböden. *Wien. klin. Wschr.* **28**, 1383
Stokes, W. R. & Hachtel, F. W. (1913) The use of a modified Hesse's medium for isolating the typhoid bacillus and the cholera spirillum from stools. *Zbl. Bakt. 1. Abt. Orig.* **69**, 346
Straus & Roux (1884) Exposé des recherches sur le choléra à Toulon. *Bull. Acad. Méd. Paris*, 2nd series, **15**, 1047
Sugio, K. & Shimomura, H. (1936) [The studies on the agglutination and agglutinin absorption test of B. cholera.] *J. med. Ass. Formosa*, **35**, 534 (Quoted in *Trop. Dis. Bull.* **33**, 863)
Takano, R., Ohtsubo, I. & Inouye, Z. (1926) *Studies of cholera in Japan.* Geneva (League of Nations publication C.H. 515)

Tanda, G. (1911) Bakteriologische Beobachtungen bei der Choleraepidemie in Molfetta (Apulien) von September bis November 1910. *Hyg. Rund. (Berl.)*, **21**, 829

Tang, F. F., Chu, C. M. & Wong, Y. W. (1944) A study of *V. cholerae* isolated from the 1942 Kunming epidemic with special reference to serological types. *Indian J. med. Res.* **32**, 1

Taylor, J. (1937) Recherches récentes sur le choléra dans l'Inde. *Bull. Off. int. Hyg. publ.* **29**, 1843 (Quoted by Wilson & Reilly, 1951)

Taylor, J. & Ahuja, M. (1938) Incidence and characters of vibrios in waters in Northern India. *Indian J. med. Res.* **26**, 1

Taylor, J., Pandit, S. R. & Read, D. B. (1937) A study of the vibrio group and its relation to cholera. *Indian J. med. Res.* **24**, 931

Teague, O. & Travis, W. C. (1916) A new differential culture medium for the cholera vibrio. *J. infect. Dis.* **18**, 601

Tobey, E. N. (1908) Cholera-red reaction as affected by mixed cultures. *J. med. Res.* **19**, 505

Tokunaga, M. (1911) [Value of strong alkaline media for diagnosis of cholera]. *Osaka Igakkai Zasshi*, **10**, Nos. 1 and 5 (Quoted by Takano, Ohtsubo & Inouye, 1926)

Toyoshima, T. (1914) [On selective media for cholera.] *Jap. Z. Mikrobiol. Path.* **2**, Nos. 1-4 (Quoted by Takano, Ohtsubo & Inouye, 1926)

Vardon, A. C. & Datta Roy, B. K. (1938) A papain-casein culture medium for the preparation of bacteriophage, and for general laboratory use. *Indian J. med. Res.* **26**, 379

Vedder, A. & van Dam, W. (1932a) Studien über Elektivnährböden für Choleravibrionen. I. Mitteilung: Die Ursachen der Elektivität und Reifung des Dieudonné-Nährbodens, *Zbl. Bakt. 1. Abt. Orig.* **126**, 145

Vedder, A. & van Dam, W. (1932b) Studien über Elektivnährböden. II. Mitteilung: Neue Elektivnährböden für die Choleradiagnostik. *Zbl. Bakt. 1. Abt. Orig.* **126**, 450

Venkatraman, K. V. (1949) *Enquiry on cholera under Dr. K. V. Venkatraman, Director, King Institute, Guindy, Madras.* In: Indian Research Fund Association, Scientific Advisory Board, *Report ... for the year 1949*, New Delhi, p. 5

Venkatraman, K. V., Krishnaswami, A. K. & Ramakrishnan, C. S. (1941) Occurrence of Vibrio El Tor in natural sources of water in the absence of cholera. *Indian J. med. Res.* **29**, 419

Venkatraman, K. V. & Pandit, C. G. (1938) An epidemic of cholera in a rural area in South India caused by the " Ogawa " type of *V. cholerae*. *Indian J. med. Res.* **25**, 585

Venkatraman, K. V. & Ramakrishnan, C. S. (1941) A preserving medium for the transmission of specimens for the isolation of *V. cholerae*. *Indian J. med. Res.* **29**, 681

Verzár, F. & Weszecky, O. (1916) Zur Stuhluntersuchung auf Typhus- und Cholerabazillen. *Dtsch. med. Wschr.* **42**, 476

Violle, H. (1915) Sur un nouveau milieu de culture de séparation pour le vibrion cholérique (milieu sodo-glycériné). *Bull. Soc. Path. exot.* **8**, 52

Voges, O. & Proskauer, B. (1898) Beitrag zur Ernährungsphysiologie und zur Differentialdiagnose der Bakterien der haemorrhagischen Septicaemie. *Z. Hyg. InfektKr.* **28**, 20

Volpino, G. (1916) L'uso del terreno di Aronson nella diagnosi rapida del vibrione colerigeno. *Policlinico, Sez. prat.* **23**, 549

Wahbi, S. (1938) Rapport bactério-parasitologique sur l'examen des pèlerins au laboratoire de la quarantaine à Najaf (février-mars 1938). *Bull. Off. int. Hyg. publ.* **30**, 2531

Weisskopf, A. (1911) Zur Methodik der bakteriologischen Choleradiagnose. *Wien. klin. Wschr.* **24**, 1185

White, P. B. (1929) Notes on intestinal bacilli with special reference to smooth and rough races. *J. Path. Bact.* **32**, 85

White, P. B. (1937) Commentaire sur les résultats négatifs ou douteux des recherches bactériologiques dans le choléra clinique. *Bull. Off. int. Hyg. publ.* **29**, 1861

White, P. B. (1948) Bacteriological and immunological aspects of cholera (Summary). *Proc. roy. Soc. Med.* **41**, 176

Wilson, W. J. & Blair, E. M. M. (1931) Further experience of the bismuth-sulphite media in the isolation of *Bacillus typhosus* and *B. paratyphosus* B from faeces, sewage and water. *J. Hyg. (Lond.)*, **31**, 138

Wilson, W. J. & Reilly, L. V. (1940) Bismuth sulphite media for the isolation of *V. cholerae*. *J. Hyg. (Lond.)*, **40**, 532

World Health Organization, Joint OIHP/WHO Study-Group on Cholera (1950). *Wld Hlth Org. techn. Rep. Ser.* **18**

Yen, A. C. H. (1933) Phenolphthalein starch medium for rapid isolation of *V. cholerae*. *Proc. Soc. exp. Biol. (N.Y.)*, **30**, 884

Yen, A. C. H. (1947) A differential medium for the isolation of *V. cholerae*. *Chin. med. J.* **65**, 133

Yoshida, K. (1911) [On cholera media]. *Gunidan Zasshi*, No. 24 (Quoted by Takano, Ohtsubo & Inouye, 1926)

Ziehl, F. (1882) Zur Färbung des Tuberkelbacillus. *Dtsch. med. Wschr.* **8**, 451

Zirolia, G. (1911) Beobachtungen über die Dauer des Vorkommens von Choleravibrionen in den Entleerungen von Cholerarekonvalescenten und über ihr Wiederauftreten infolge der Verabreichung von Abführmitteln. *Hyg. Rund. (Berl.)*, **21**, 769