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ISOLATION OF THE L-FORM OF *VIBRIO CHOLERAE* FROM CLINICAL SPECIMENS IN THE PHILIPPINES[1]

KEN-ICHI KOTERA[2], JESUS C. AZURIN and HIROSHI OGONUKI

Philippine-Japan-WHO Joint Cholera Laboratory, Bureau of Quarantine, Port Area, Manila, The Philippines

CEZAR V. UYLANCO

San Lazaro Hospital, Manila, The Philippines

KAZUMINE KOBARI

WHO Regional Office for the Western Pacific, Manila, The Philippines
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SUMMARY During a survey in the Philippines from February to May, 1970, the L-form of *V. cholerae* was isolated from two cases: a long-term carrier (bile) and a patient with gastroenteritis (rectal swab). Both isolated specimens reverted to vibriones on transfer to Kligler or Thiosulfate-Citrate-Bile salts-Sucrose (TCBS)-agar.

The L-form was again isolated from bile of the same carrier in a survey from October to December of the same year, but no L-form was found in patients with, or convalescing from cholera.

INTRODUCTION

The L-form of bacteria was first described by Klieneberger in 1935. Since then there have been many reports on this form in the fields of microbiology, immunology, biochemistry and genetics (Klieneberger-Nobel, 1951). The importance of recovering the L-forms of bacteria from clinical materials has attracted increasing attention. Nelson and Pickett (1951) recovered the L-form from blood of a case of brucellosis, which seemed to them to play a rôle in the recurrence of the disease and in establishment of chronic infection. Wittler et al. (1960) isolated the L-form of *Corynebacterium* sp. from blood specimens of a case of subacute endocarditis who had been treated with large amounts of penicillin. During a two year observation period, they found that *Corynebacterium* sp. was isolated when the patient was exhibiting symptoms, whereas only the L-form was recovered in the asymptomatic stage.

Based on these and other observations, it was

1 This work was supported by the Colombo Plan, Overseas Technical Cooperation Agency of Japan

2 Present Address: Culture Collection Room, Research Institute for Microbial Diseases, Osaka University, and Department of Clinical Pathology, Research Foundation for Microbial Diseases of Osaka University, Yamada-Kami, Suita, Osaka, Japan.

thought that the L-form of *V. cholerae*, if this form exists, might be carried by convalescent patients, and cause renewed outbreak of cholera. To examine this possibility we carried out bacteriological surveys of patients with cholera and carriers in the Philippines from February to May, 1970 and from October to December of the same year, under the support of the Columbo Plan. We were able to isolate and identify the L-form of *V. cholerae* from specimens from two cases: a patient with cholera and a carrier. The procedure used and results are reported in this paper.

MATERIALS AND METHODS

1. *Clinical specimens*

Bile was taken by duodenal tube from a carrier in Bacolod who has been excreting *V. cholerae* into the bile for seven years. Stools or rectal swabs were obtained from patients with cholera in San Lazaro Hospital, Manila who were at various stages of the disease. In the second survey, care was taken to obtain materials successively at regular intervals from patients under treatment.

2. *Media*

For isolating the L-form, PPLO broth and PPLO agar (Difco Laboratories or Kitasato Phamaceutical Industry. Co.) were used. In the first severy, horse serum (20%), penicillin (100–500 IU/ml) and sucrose (10%) were added to the above media, while in the second survey heat-treated (56 C for 30 min) horse serum (20%), lactose (10%) and $CaCl_2 \cdot 2H_2O$ (0.001 M) were added instead.

For enrichment culture of the L-form in the second survey, the broth described above was used at double strength.

For isolation of *V. cholerae*, Thiosulfate-Citrate-Bile salts-Sucrose (TCBS) agar and Nutrient agar, pH 8.2 (Eiken Chemical Co., Ltd., Tokyo) were used in the first survey, in the second survey the latter was replaced by Heart Infusion agar (Eiken Chemical Co.)

3. *Isolation of the L-form*

1) First survey

The procedures used to isolate the L-form from bile of a long-term excretor are shown in Fig. 1. Bile was diluted 10-fold with PPLO broth and filtered through a millipore filter (0.45 μ). Then 0.2 ml of the filtrate was streaked on PPLO agar, and incubated at 37 C for 7 days. The remainder of the filtrate was incubated at 37 C for 7 days and then inoculated onto PPLO agar, to see if typical colonies formed. When some growth was observed, its reversibility to the vibrionic form was tested and its biological and serological characters were examined.

The procedure for isolation of the L-form from stools or rectal swabs of cholera patients, given in Fig. 2, was essentially the same as that described above. However, the PPLO broth used for diluting the specimen before filtration did not contain penicillin, and penicillin (500 IU/ml) was added to the filtrate.

2) Second survey

Faecal materials were diluted twofold with enrichment medium and filtered through a Millipore membrane filter (0.3 μ). Then 0.2 ml of the filtrate was streaked on each agar plate to isolate the L-form, and plates were incubated at 37 C for 2–5 days. Each plate was examined microscopically for the appearance of L-form colonies.

The remainder of the filtrate in a sterile tube was incubated similarly, and an aliquot was streaked on the plate and cultivated at 37 C for a further 2–5 days.

Bile from the carrier was treated like faecal materials.

When minute colonies resembling those of the L-form were observed, they were transferred to a plate of Heart Infusion agar. The biological and serological characters of the revertant cultures obtained on the plate were tested.

4. *Isolation of V. cholerae*

Specimens after enrichment culture in alkaline peptone water or without enrichment culture, were inoculated onto TCBS agar or Nutrient agar, pH 8.2 and Heart Infusion agar, and cultivated at 37 C for 18–24 hr.

5. *Bacteriological characterization of strains*

To characterize the isolated and revertant strains, the " points of differentiation between *V. cholerae* and *V. eltor* " of the IAMS Subcommittee on Taxonomy of Vibrios were applied. The methods cited in their Minutes (Feeley 1966) were followed

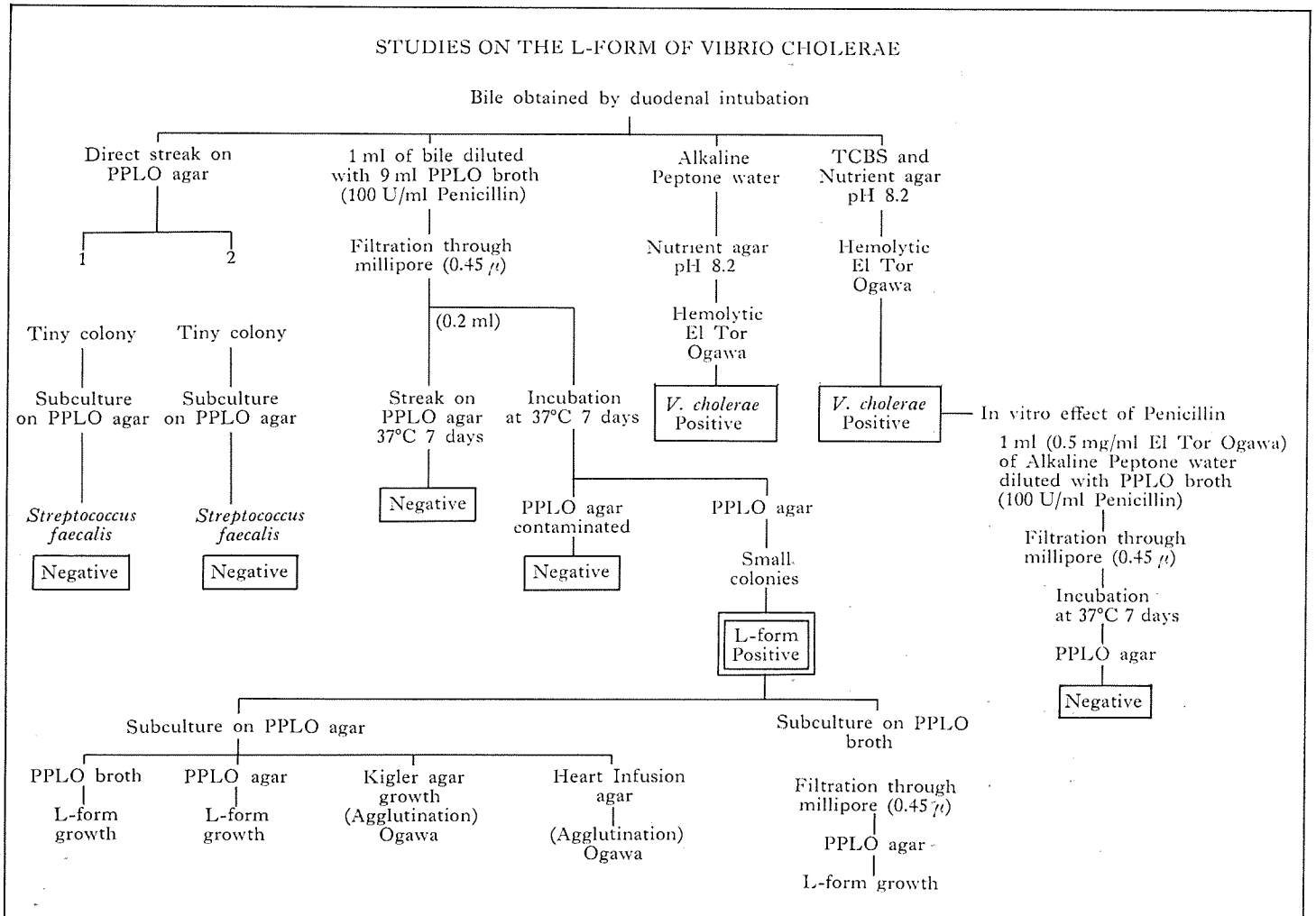

FIGURE 1. *Procedures for isolation of Vibrio from a long term excreter (DM) in the first survey*

in the present study.

RESULTS

1. *First survey*

A 0.2 ml sample of filtered bile material from a long-term excreter (D.M.), formed small colonies on PPLO agar. Two of these were subinoculated onto PPLO agar. However, these colonies were deduced to be *Streptococcus faecalis* from Gram-stained smears and from their biological properties.

On the other hand, a liquid culture of filtered bile material from the same carrier became slightly turbid 6 days after inoculation. Two PPLO Agar plates were inoculated with samples of this culture. On one of the two plates, small nipple-like colonies developed, which looked like those of mycoplasmata. (The other plate became contaminated and was discarded). On transfer to Kligler agar and Heart Infusion agar, colonies of vibrios developed after 48 hr incubation at 37 C. These were specifically agglutinated by Ogawa-antiserum and were essentially identical in biological properties and phage susceptibility to the *V. cholerae* strain isolated from the same specimen, as shown in Table 1. They were identified as biotype eltor (serotype 1, Ogawa). These small mycoplasma-like colonies on PPLO Agar were cultured in PPLO broth for 48 hr, the culture was filtered through a Millipore filter (0.45 μ) and the filtrate again gave mycoplasma-like colonies on subinoculation onto PPLO agar. Thus, this agent was filtrable and was identified as the L-form of *V. cholerae*.

To see if the L-form appeared in the filtrate *in vitro* as a result of the action of penicillin present in the PPLO broth used for diluting the specimen, an isolated culture of *V. cholerae* from the same specimen was suspended in PPLO broth containing 100 IU/ml penicillin.

TABLE 1. *Biological characters of isolated strains of V. cholerae and of the revertant from the L-form (first survey).*

Character \ Strain	D・M		O・B	
	Isolated on TCBS-agar	Revertant from L-form	Isolated on TCBS-agar	Revertant from L-form
Serotype	1・Oawa	1・Ogawa	1・Ogawa	1・Ogawa
Chicken red cell agglutination	+	+	+	+
Sensitivity to Polymyxin B	−	−	−	−
Lysogeny of Group IV phage	−	−	−	−
Lysogeny of Kappa phage	+	+	+	+
Acid production from				
Lactose	−	−	−	−
Saccharose	+	+	+	+
Mannose	+	+	+	+
Arabinose	−	−	−	−
Glucose	+	+	+	+
Voges-Proskauer	+	+	+	+
Methyl red reaction	−	−	−	−
Indophenol oxidase	+	+	+	+
Indole produced	+	+	+	+
Motility (SIM)	−	−	+	+
H_2S produced	−	−	−	−
Haemolysis (sheep RBC)	+	+	+	+

This suspension, containing about 0.5 mg of organisms per ml, was stood at 34 C for ten minutes, to expose it to penicillin, and then filtered through a Millipore filter (0.45 μ). No growth was obtained within 7 days on incubation at 37 C. Therefore, it seems unlikely that recovery of the L-form was due to an action of penicillin *in vitro* under the present conditions.

V. cholerae biotype eltor (serotype 1, Ogawa) was also isolated from a rectal swab from a patient with gastroenteritis when the swab was enriched in alkaline-peptone water (pH 8.2), and inoculated on to TCBS-agar (Fig. 2). PPLO broth inoculated with the same material and filtered through a Millipore filter (0.45 μ), became slightly turbid after 48 hr, and was subinoculated onto PPLO agar. The mycoplasma-like colonies grown on the PPLO agar were found to be unstable on transfer to Heart Infusion agar. They were identified as the L-form of *V. cholerae* by their ability to pass through a Millipore filter (0.45 μ) and by the biological and serological properties of the revertant cultures.

2. *Second survey*

No L-form was recovered from faecal materials of patients with, or convalescing from cholera.

However, bile of the carrier D. M. who has been excreting *V. cholerae* in the bile for 7 years, formed minute colonies when cultivated on plates. On microscopic examination these were seen to be nipple-like, resembling L-form colonies, as shown in Fig. 3.

One of these colonies was transferred to a plate of Heart Infusion agar. Large colonies appeared, which were identified by their biological characters and agglutination with antisera to *V. cholerae* serotype 1, and by phage-typing, as *V. cholerae* serotype 1, Ogawa.

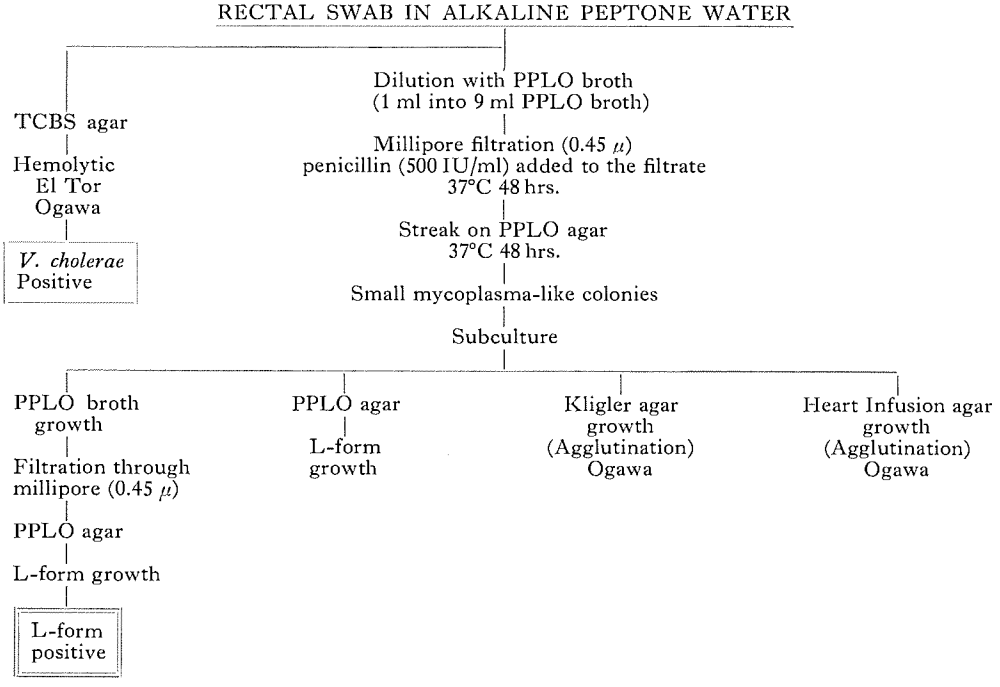

FIGURE 2. *Procedures for isolation of Vibrio from rectal swab of a case of gastro-enteritis patient: O.B. 3 years, Female*

FIGURE 3. *Colonies developed on PPLO agar plate after 5 days' incubation at 37C. Magnification 400×*

Their characters completely coincided with those of *V. cholerae* isolated on TCBS agar from the same material.

The bile contained 2×10^4 cells of *V. cholerae*/ml. In all nine L-form colonies were obtained on 5 plates, each inoculated with 0.2 ml of filtrate of two-fold diluted material. Thus, the bile must contain not more than 5 L-form cells/ml.

Repeated attempts to isolate the L-form from stools of the same person, made over a period of five days, were unsuccessful.

DISCUSSION

There have been no previous reports of isolation of the L-form of *V. cholerae* from patients or carriers. In this study, we concluded that our isolated culture was the L-form of *V. cholerae* for the following reasons:

1) Colonies were grown from material which had passed through a Millipore filter (0.3 μ or 0.45 μ).

2) *V. cholerae* isolated from the same specimen formed flat colonies of 3 mm in diameter. However, only small colonies (0.5 mm in diameter) developed from the filtered material and these were only just visible after 48 hr, appearing like the nipple-like colonies of mycoplasma.

3) The biological and serological properties of the revertants obtained from these small colonies were essentially identical to those of *V. cholerae* strains isolated from the same specimen.

The case from which we could isolate the L-form in both the first and second surveys was a long-term carrier of *V. cholerae*. She presumably harboured the organism in her gallbladder. She had a high titer antibody (Inaba: 1: 1280, Ogawa: 1: 640) in her serum, which might have inhibited cell wall synthesis of *V. cholerae* and thus resulted in formation of L-forms, since Ianetta and Wedgwood (1967) found that L-forms of *V. cholerae* formed on treatment of vibriones with complement plus lysozyme. Alternatively, the presence of bile itself might have induced the emergence of L-forms.

The presence of horse serum in the isolation medium, may have resulted in conversion of vibriones to the L-form. However, the effect of its complement activity, at least, was excluded in the second survey by use of heat-inactivated serum.

Moreover, it was found that under the conditions used in the present study the vibrionic form was not converted to the L-form by exposure to penicillin *in vitro*. However, the use of pencillin was discontinued in the second survey to exclude the possibility that the L-forms might be formed by conversion *in vitro*. Thus, care was taken in the second survey to exclude factors which might influence the conversion of vibriones to the L-form during the isolation procedure.

In the second survey no L-form was obtained from faecal materials of patients with, or convalescing from cholera. These negative results might be due to the use of chloramphenicol and furaluzine in treatment of these patients, since these drugs might have reduced the whole population of *V. cholerae* to so low a level that no chance of the L-form formation could be left thereafter.

In any way, the number of L-form in the specimen, if present, might have been below the level that could be detected by our method.

Further studies seem necessary to see at

what stage of the disease the L-form appears and to what extent it can be isolated from cholera patients.

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REFERENCES

Feeley, J. C. 1966. Minutes of IAMS Subcommittee on Taxonomy of vibrios. Int. J. Syst. Bacteriol. 16: 135–142.

Ianetta, A., and R. J. Wedgwood. 1967. Culture of serum-induced spheroplasts from *Vibrio cholerae*. J. Bacteriol. 93: 1688–1692.

Klieneberger-Nobel, E. 1951. Filterable forms of bacteria. Bacteriol. Rev. 15: 77–103.

Nelson, E. L., and M. J. Pickett. 1951. The recovery of L forms of brucella and their relation to brucella phage. J. Infect. Dis. 89: 226–232.

Wittler, B. R. C., W. F. Malizia, P. E. Kramer, J. D. Tuckett, H. N. Pritchard, and H. J. Baker. 1960. Isolation of a Corynebacterium and its transitional forms from a case of subacute bacterial endocarditis treated with antibiotics. J. Gen. Microbiol. 23: 315–333.