ORIGINAL ARTICLES

The Coconut Water Egg Malachite Green Medium (CEM) for the Isolation of Mycobacterium Tuberculosis*

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TUBERCULOSIS in all forms is the second leading cause of deaths in this country, fourth in causing disease and the number one disease killer of our children. On its early diagnosis depends its cure and control and an assured healthy people. Toward this end, government and private agencies have joined efforts in the detection and treatment of infectious cases as well as the institution of preventive measures. The most utilized tools of detection are sputum microscopy and x-ray. While sputum microscopy has been the least expensive and most efficient tool in mass case detection, there is **still need for other laboratory tools in the more scientific study of detected cases.** The isolation of **Mycobacterium tuberculosis is one of the more reliable aids in the diagnosis of symptomatic cases with negative sputum for acid fast bacilli.** For those cases under therapy with apparently no clinical or bacteriological improvement, a bacteriological work-up is necessary to determine sensitivity or resistance to the drug being used, or where a possible change of drug is contemplated. For those where actual identification of the infecting organisms is necessary, a bacteriological work-up is the only tool.

Today less than 10 clinical laboratories all over the country can afford to continuously and regularly do the routine bacteriological isolation of **Mycobacte-**

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rium tuberculosis. Currently these laboratories are utilizing a medium principally composed of eggs or other protein sources, mineral salts and a dye. With the exception of eggs and the distilled water, the reagents and chemicals that make up the formulas have to be procured from abroad entailing difficulties like irregular supply, increased cost, and because too few laboratories are using the special medium, there is very little interest to make the purchase from abroad.

The formula of the medium currently in use for the isolation of **M. tuberculosis** may be one of the following:

1. Lowenstein-Jensen medium, modified

KH_2PO_4	2.4 gm.
$MgSO_4.H_2O$	0.24
Magnesium citrate	0.60
Aspargine	3.6
Glycerol	12 ml.
Malachite green 2% aq. solution	20 ml.
Potato flour	30 gm.
Distilled water	600 ml.
Eggs (fresh, whole)	1000 ml.

2. Lowenstein-Jensen medium, modified

Mineral salt solution

KH_2PO_4	4.0 gm.
$MgSO_4$	0.4
Magnesium citrate	1.0
Aspargine	6.0
Glycerol	20.0 ml.
Distilled water	1000.0 ml.

Complete formula

Mineral salt	300.0 ml.
2% malachite green	10.0 ml.
10 beaten eggs, approx.	500.0 gm.

3. American Trudeau Society (ATS medium, modified)

Potato peeled and diced	140 gm.
Glycerol, reagent grade 2%	335 gm.
Egg yolk (fresh) with) 3 white	400 ml.
2% malachite green	10 ml.

4. Petragnani medium

a. Pasteurized, homogenized whole milk	275.0 ml.
Potato starch (Fisher)	20.0 gm.
Aspargine (Difco)	1.9 gm.
b. Fresh whole eggs	10
Fresh egg yolk	3
Glycerine	30 ml.
c. 2% aq. sol. malachite green	

5. Middle-brook-Cohn 7H-10 agar

Solution [1]

Monopotassium phosphate, ACS	15 gm.
Disodium phosphate, ACS	15 gm.
Distilled water	250 ml.

Solution 2

Ammonium sulfite, ACS	5.0 gm.
Monosodium gllutamate	5.0 gm.
Sodium citrate (2 H_2O) USP	4.0 gm.
Ferric ammonium citrate	0.4 gm.
Magnesium sulfate (7 H_2O)	5.0 gm.
Biotin (in 2 ml. 10% ammonium hydroxide)	5.0 gm
Distilled water	250.0 ml.

Solution 3

Calcium chloride (2 H_2O), ACS	50.0 mg.
Zinc sulfate (7 H_2O), ACS	100.0 mg.
Copper sulfate (5 H_2O), ACS	100.0 mg.
Pyrodoxine HCL	100.0 mg.
Calcium panthothenate	100.0 mg.
Distilled water	100.0 ml.

Solution 4

Reagent grade glycerine

Solution 5

Malachite green, 0.01% aq. sol.

Solution 6

Albumin-oleate dextrose solution
50 gm. bovine albumin, fraction V
in 900 ml. sterile saline
Sodium oleate solution
50% aq. solution of dextrose

A glance through the above formulas will reveal their varying complexity, the cost it will entail in their preparation, the time involved in the weighing of each constituent especially those where an analytical balance is required, the physical facilities for the preparation and the need for personnel of higher technical training. The preparation of formula nos. 4 and 5 require more skill. Formula no. 3 or ATS medium is a more simple one, but still it makes use of potatoes which have to be peeled and diced besides eggs.

METHODS

In answer to the need for maximizing the utilization of isolation and culture of **M. tuberculosis** in all laboratories, a new medium, the coconut water egg malachite green medium (CEM) has been devised. It has the following formula:

Coconut water ..... 300 ml.
Whole eggs 10
(approx. 500 gm.)
2% malachite green

Water from young green coconut is sterilized by boiling or autoclaved for 10 min. at 10 lbs. pressure. The eggs are cleansed thoroughly with soap and water; rinsed well and soaked in 70% alcohol, and dried with sterile towel. The eggs are aseptically broken into a sterile conical flask or an Erlenmeyer flask with a pipette or glass rod. The flask is shaken to break up the eggs and the coconut water added with malachite green solution enough to produce a light green color of the mixture. The contents are shaken well to mix, then filtered through sterile gauze. The mixture is

aseptically distributed into screw-capped test tubes and inspissated at 80° to 85°C one hour in a slanting position.

The sputum swab culture method devised by Nassau was followed in the evaluation of this medium for the isolation of **M. tuberculosis** from sputum. The modified Lowenstein-Jensen medium, formula 2, was used as control. One or two sterile swabs are moistened with sterile distilled water and both held in one hand, dipped into the sputum sample and vigorously rotated, mixing well the sample. The swabs are then placed in tubes, two-thirds full with 5% sterile oxalic acid and allowed to stand at room temperature for 35 minutes. Then the swabs are transferred to another tube also two-thirds full with sterile 5% sodium citrate and allowed to stand in the solution for 10 minutes. Two slopes of CEM and of Lowenstein-Jensen media are inoculated with each sample. The swabs are firmly rubbed on each slope while the swab is being rotated.

RESULTS

Laboratory standard strains of **Mycobacterium** like H37Rv, **M. xenopei, M. avium, M. kansasii,** a Scotochromogenic strain, 607 strain, BCG strain and **M. leprae murium** have been cultured and maintained in CEM medium and **M. tuberculosis** have been successfully isolated from sputum samples using CEM. Illustrations of these cultures may be seen in Plates 1—4. It has been noted that the growth of the **Mycobacterium** strains in CEM has been very good.

The standard laboratory human Saranac virulent strain H37Rv has maintained the rough characteristic of its colonies light buff color, somewhat dry in CEM as in the Lowenstein-Jensen medium. A Group II Scotochromogenic strain of the Runyon group of atypical mycobacteria has shown its moist, smooth, confluent colonies with yellow to orange pigments in both CEM and Lowenstein-Jensen media. **Mycobacterium avium,** a virulent avian tubercle bacilli, produced smooth glistening colonies with cream or buff non-photochromogenic confluent colonies in both CEM and Lowenstein-Jensen media. Another atypical mycobacterium **Mycobacterium xenopei** in both CEM and Lowenstein—Jensen media produced smooth, pale yellow non-photochromogenic, moist growth. Another laboratory strain used by the NIST is strain no. 607 of Runyon Group IV which maintains its confluent, finely irregular, light buff colored colonies in CEM as in Lowenstein-Jensen medium. **Mycobacterium kansasii** of Runyon Group I showed yellow pigmented somewhat rough colonies when exposed to light in both media. A-20, a strain of the Scotochromogenic group isolated locally from sputum did not lose its yellow orange pigmented colonies when grown in CEM.

CEM was found as efficient as Lowenstein-Jensen medium in the isolation of Mycobacterium from sputum samples; buff colored rough colonies were produced. At no instance were negative results obtained from CEM when Lowenstein-Jensen medium was positive for isolation.

Using the sputum swab culture technique of Nassau, contamination from other bacteria presented no problem in utilizing CEM or Lowenstein-Jensen medium.

CEM was provided by the authors to another independent worker and he was successful in growing both **M. leprae murium** and BCG strain.

Plate I — Shows the characteristic growth of **M. xenopei, M. avium, M. kansasii, H37Rv, H607** and a Scotochromogen strain in CEM and LJ media.

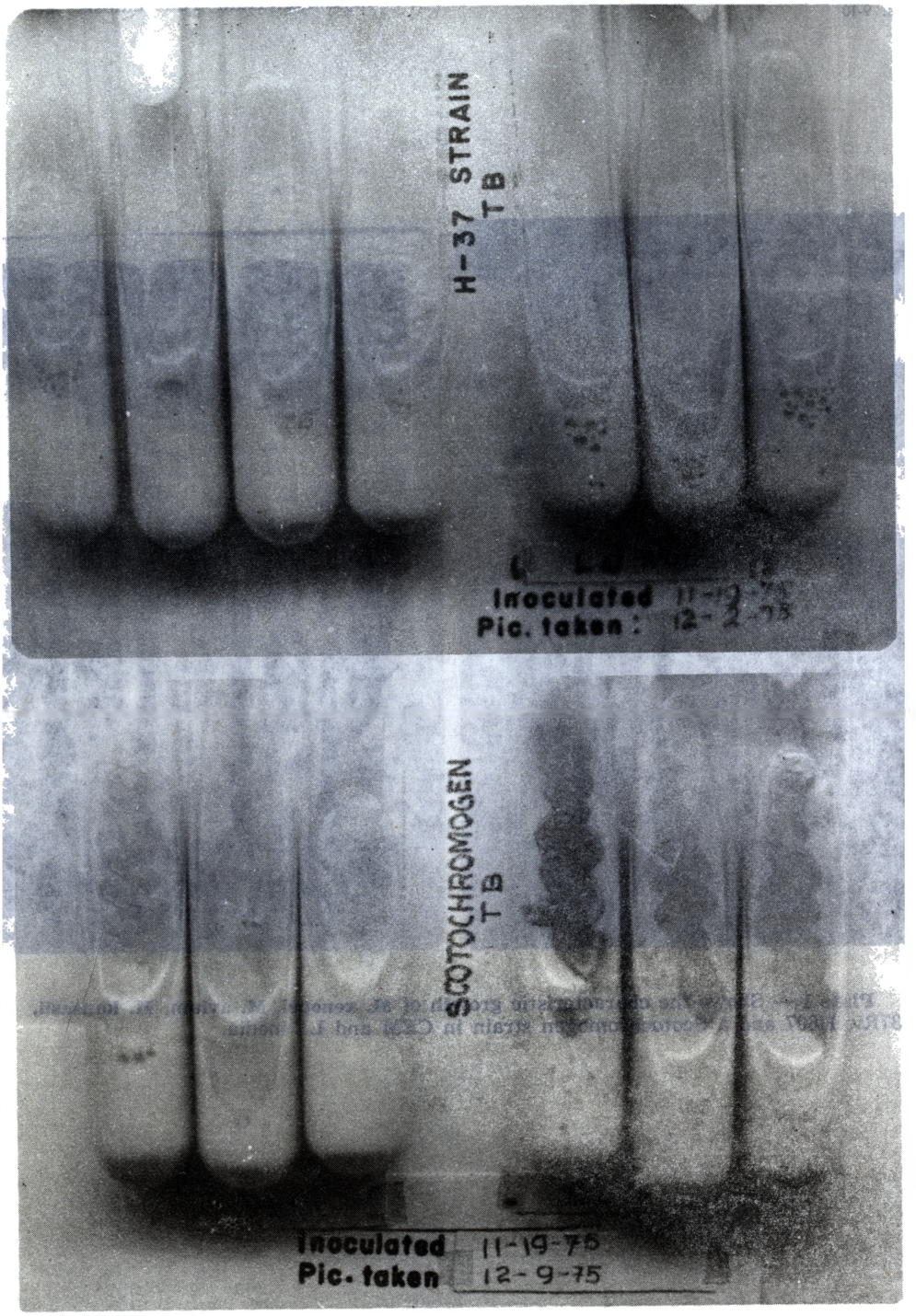

Plate II — Shows a close-up of H37Rv and a Scotochromogen strain in both CEM and LJ media.

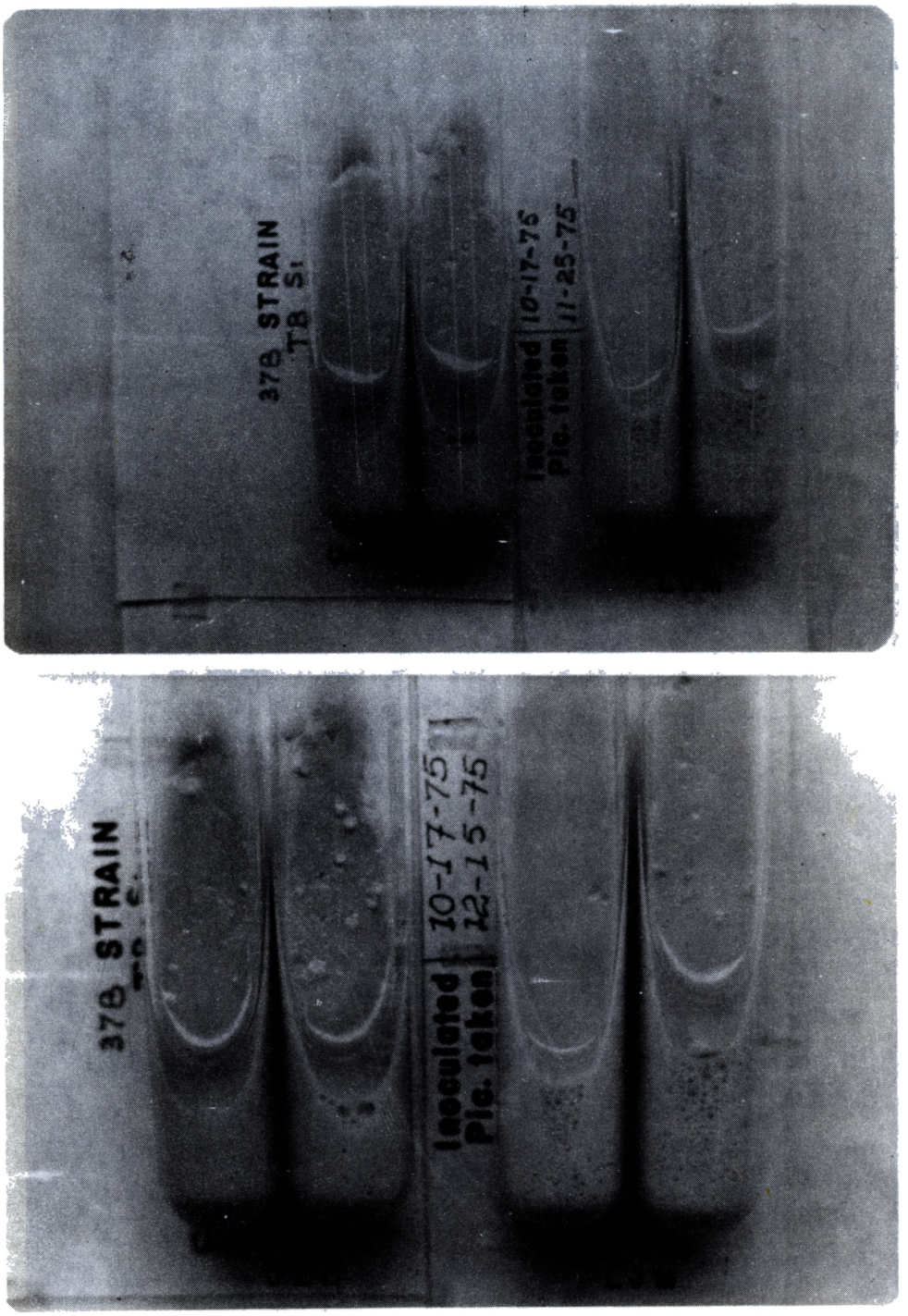

Plate III — Shows the isolates from a sputum sample (378) on different days after inoculation of CEM and LJ media.

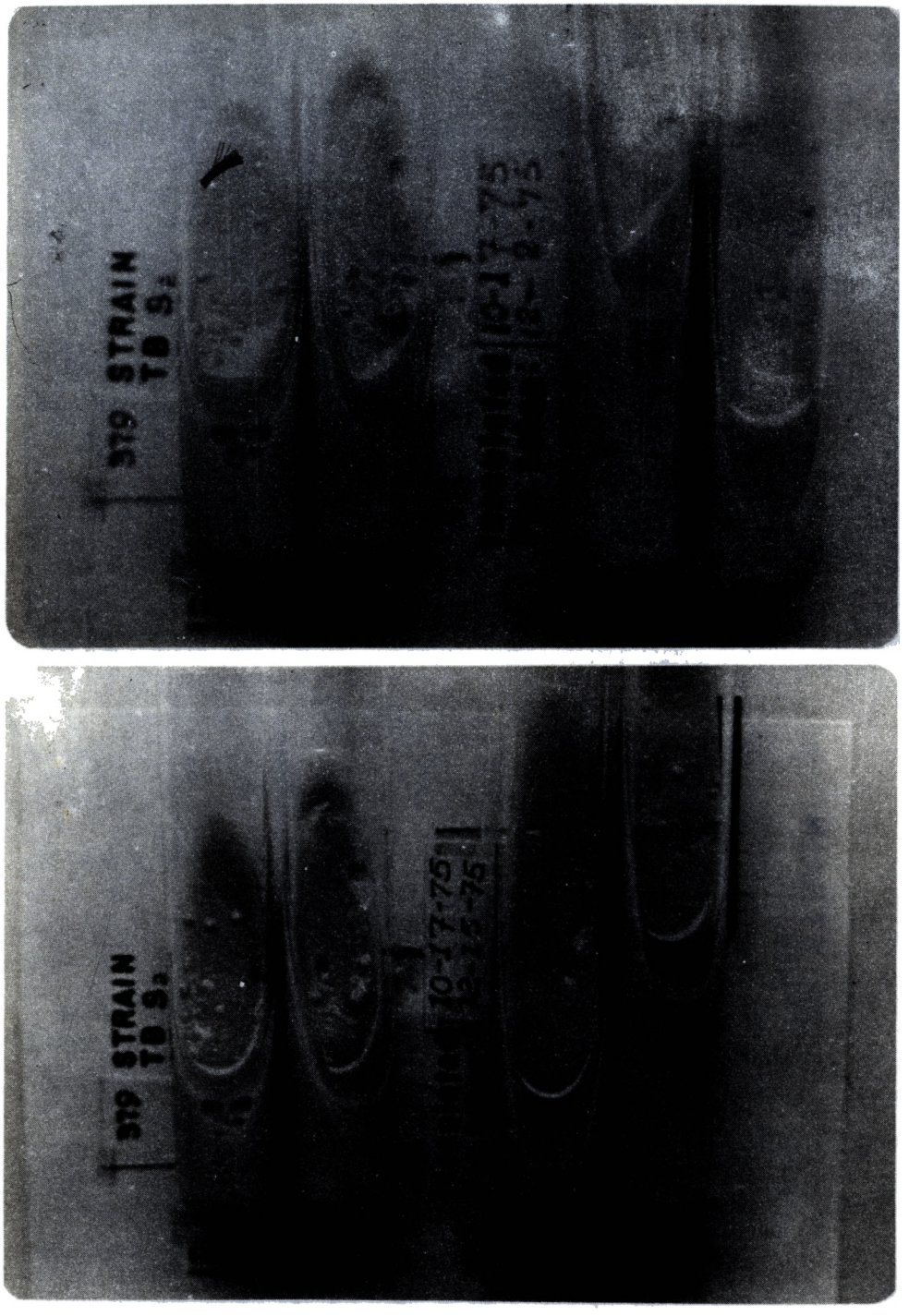

Plate IV — Shows the isolates from a sputum sample (379) on different days after inoculation of CEM and LJ media.

DISCUSSION

CEM utilizes coconut water as its main and only source of mineral salts in addition to its nutrients like protein and carbohydrates, to mix with whole eggs as a culture medium for a fastidious group of organism like the **mycobacteria.** It was definitely shown in this work that used with eggs it can support the growth of **mycobacteria.**

Blauvett[1] reported that non-cooked, non-sterilized coconut water from "ripe fresh fruit" added to ordinary nutrient agar and broth nearly doubled the culture qualities of the latter in growing **Staphylococcus aureaus, B. faecalis alcalis alcaligenes,** and **B. welchii.** Paguio et al[2] in 1970 utilized coconut water medium for the isolation of **Vibrio cholerae.** Agasan[3] in 1975 has proven the sensitivity of coconut water medium in detecting coliforms in waters and foods and the growth of **Salmonella, Shigella** and **Klebsiella** in the same medium.

Several workers have analyzed the composition of coconut water from young green coconuts. Peters[4] gave the range of concentration of the important components of coconut water per 100 ml. as follows:

Protein	0.23 — 0.43 gm.%
Carbohydrates	3.68 — 5.0 gm.%
Fats	0.64 — 0.8 gm.%
Calcium	0.03 gm.%
Phosphorous	0.01 — 0.22 gm.%

Pradera and his co-workers[5] reported the mineral content of 100 ml. of coconut water as follows:

Calcium	29.0 — 46.0 mg.
Chlorine	105.0 — 160.0 mg.
Phosphorous	5.5 — 9.0 mg.
Potassium	134.0 — 220.0 mg.

Child and Nathaniel[6] reported the constituents to be:

Water	95.50%
Nitrogen	0.50
Phosphoric acid	0.60
Calcium oxide	0.69
Magnesium oxide	0.59
Iron	0.50 mg. in 100 gm.
Total solids	4.71 gm./100 ml.
Red sugar as invert sugar	0.80 gm./100 ml.
Add. sugar as sucrose	1.28 gm./100 ml.
total sugar	2.08 gm./100 ml.
ash	0.62 gm./100 ml.
Unidentified organic solids	2.01 gm./100 ml.
Ascorbic acid (Vit. C)	2.20 — 3.70 mgm./100 ml.
Nicotinic acid	0.640 microgram/100 ml.
Panthotenic acid	0.520 microgram/100 ml.
Folic acid	0.003 microgram/100 ml.

Vanderbelt[7] found the following amount of vitamin B complex as:

Nicotinic acid	0.64 microgram/ml.
Panthotenic acid	0.52 microgram/ml.
Biotin	0.02 microgram/ml.
Riboflavin	0.01 microgram/ml.
Folic acid	0.003 microgram/ml.

Pradera and his co-workers (5) made a thorough study of the amino acid content of the water and found them present as peptones based on dry protein content of the water to be as follows:

Glutamic acid	9.76 — 14.50 gm.%
Arginine-1	12.75 gm.%
Leucine	1.95 — 4.18 gm.%
Lysine	1.95 — 4.57 gm.%
Proline	1.21 — 4.12 gm.%
Aspartic acid	3.60
Tyrosine	2.83 — 3.00
Alanine	2.41
Histidine	1.95 — 2.05
Phenylalanine	1.23
Serine	0.59 — 0.91
Cysteine	0.97 — 1.17

The present work has shown for the first time the full utilization of coconut water to support the growth of a fastidious group of organisms not just the use of its "growth factor". Quite a number of workers have shown this growth factor as capable of stimulating growth of certain bacteria. Ramakrishnan, et al[8] in 1958 has shown that coconut water even at a dilution of 1 in 10,000 when used as a supplement to the ordinary medium for the cultivation of **Mycobacterium tuberculosis** showed maximum growth in 12 days only instead of the usual 20 days. We failed to elicit any change in the growth period of the **Mycobacterium** in CEM compared to Lowenstein-Jensen although in some cases growth has been more luxuriant in CEM especially in isolations from sputum samples. In our particular work the growth factor in coconut water is only an incidental advantage when the water is utilized as a whole.

Green coconuts can be procured anywhere especially in the provinces. The simplicity of the formula and preparation does not require much technical skill nor physical laboratory facilities which suit local conditions.

A laboratory that is going to start a TB bacteriology service would need to invest in the following if it will utilize Lowenstein-Jensen medium:

Materials	Cost
KH_2PO_4	₱ 42.00/lb.
$MgSO_4$	56.70/lb.
Magnesium Citrate	45.00/lb.
Aspargine	150.00/100 gm.
Glycerol	95.00/480 cc.
Distilled water	2.50/liter
Malachite green	50.00/25 gm
10 eggs	4.50

However, if it will utilize CEM, the cost estimate would only be:

Green coconut	₱ 0.30 — 0.70/nut depending on where you procure it
10 eggs	4.50 — if procured in the city
malachite green	50.00/25 gm.

So that it is very apparent that one has to have about ₱445.00 to start with the Lowenstein-Jensen medium and only about ₱56.00 or only 1/8 of the former, for CEM. With CEM, even if malachite green is not available, the medium can still be used. CEM is a medium that can be produced from materials locally available.

Since it is a cheap medium, the preparation of CEM may be done at a central laboratory and tubes of the medium dispensed to peripheral areas. Actual production of the media can even be done in a small laboratory. The tubes can be inoculated in the peripheral area using the sputum swab culture technique of Nassau. The inoculated media are then transported to more developed laboratories where it can be incubated, observed and studied. Instead of screw-capped tubes, ordinary tubes with rubber stopper may be used, thus further lowering the cost of each slope. CEM keeps very well at room temperature for at least 3 months and sterile coconut water for about 6 months. The addition of malachite green improves the gross visibility of the colonies. The green color of the media brings out very well the colonial characteristics.

CEM is an ideal medium for the isolation of **mycobacteria** since it supports early and eugonic growth for small inoculum; enables easy recognition of organisms; is easily prepared and inexpensive; and keeps the growth of contaminating organisms to a minimum. CEM medium is most useful wherever tuberculosis is a problem; wherever there is need for a more scientific work-up of detected cases especially for antimicrobial studies; wherever there is a lack or shortage of technical skills and physical laboratory facilities; wherever there is difficulty in the procurement of chemicals and reagents produced or manufactured in developed countries; wherever there is need to lower the cost of medical care delivery and wherever coconut is grown. CEM answers all these needs, which will not only be in the Philippines but in many developing countries where tuberculosis is a health problem.

SUMMARY

For the first time a coconut water egg malachite green medium (CEM), has been devised for the isolation of **Mycobacterium tuberculosis.**

CEM utilizes all the coconut water as its main and only source of mineral salts in addition to its nutrients like protein and carbohydrates to mix with whole eggs as a culture medium for a fastidious group of organisms like **mycobacteria.** Laboratory standard strains of **mycobacteria** like H37Rv, **M. xenopei, M. avium, M. kansasii,** a Scotochromogenic strain, No. 607 strain, BCG strain and **M. leprae murium** have been cultured and maintained in CEM. **Mycobacterium tuberculosis** has been successfully isolated from sputum samples using CEM.

CEM is simple and inexpensive nor does it require much technical skill and physical laboratory facilities to prepare. It is an ideal medium for the isolation of **Mycobacterium** since it supports early

and encourage growth for small inoculum; enables easy recognition of organism and keeps the growth of contaminating organisms to a minimum. It utilizes materials locally available. CEM will be most useful not only in the Philippines but in other developing countries where tuberculosis is a health problem.

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