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Recovery of PPLO of Atypical Pneumonia on Artificial Agar Medium.* (27575)

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Epidemiologic and volunteer studies have strongly suggested that the Eaton agent is etiologically associated with the syndrome of cold agglutinin positive atypical pneumonia of man(1,2,3). After its recognition in 1944 this filterable agent was classified as a virus, although it is of large size (200-250 mμ) and sensitive to streptomycin and tetracycline(4, 5,6). Recently the organism was cultivated on an artificial agar medium and shown to be a PPLO, *i.e.*, a member of the genus *Mycoplasma*(7). Growth on agar was achieved with a strain of Eaton agent (FH) which had been passaged many times in eggs and subsequently adapted to monkey kidney tissue culture(8,9). The present study was initiated to determine if naturally occurring strains of Eaton PPLO resembled the laboratory adapted strain as regards growth on an agar medium. In addition it was important to learn if PPLO agar was a sensitive medium for the recovery of strains from infected individuals.

Materials and methods. Agar medium. The complete medium consisted of 7 parts Difco PPLO agar, 2 parts uninactivated horse serum and 1 part 25% yeast extract. The extract was prepared from active baker's yeast by boiling with distilled water, followed by clarification through filter paper. This material was then autoclaved and stored at −20°C until used. The agar was prepared in 70 ml quantities and sterilized by autoclaving. After cooling to approximately 45°C the agar was supplemented with the yeast extract and horse serum. The final medium contained 500 units of penicillin and 5 μg of amphotericin per ml and thallium acetate at a final concentration of 1:2000. Approximately 6 to 7 ml of the agar medium was added to a plastic plate which measured 5 cm in diameter.

Cultivation. One-tenth ml of throat swab fluid was inoculated onto the agar and spread by streaking with a bacteriologic loop. The inoculated plates were incubated at 35°. One series of plates was incubated in a desiccator jar; a lighted candle was placed in the jar before the lid was secured in order to produce an increased CO_2 tension. Other inoculated plates were incubated aerobically without further manipulation.

* The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

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Identification of isolates. Isolates were passaged on agar plates until sufficient colonies were available for identification by the indirect fluorescent antibody technic. Colonies from the agar plate were then transferred to glass slides by the technic described by Clark *et al.*, and fixed with acetone for 10 minutes at room temperature(10). Identification was accomplished with 2 pairs of acute and convalescent sera from patients who developed a rise in Eaton fluorescent stainable antibody and from whom the agent was recovered. The acute serums were used at a 1:10 dilution and the convalescent serums were used at a 1:20 dilution. Acute phase sera failed to stain Eaton PPLO colonies while intense fluorescence was observed with the convalescent sera. In addition the isolates were tested with a 1:40 dilution of an Eaton hyperimmune rabbit serum. Identification was confirmed when fluorescence was observed with this serum and when a normal rabbit serum failed to stain the colonies. The rabbit hyperimmune serum was prepared by 8 biweekly intramuscular inoculations of 4 ml of a 10% Eaton (FH strain) chick embryo lung suspension.

Fluorescent antibody technic. The indirect method was used in all tests. Horse anti-human globulin and sheep anti-rabbit globulin fluorescein conjugates were obtained from Progressive Laboratories. Titration of fluorescent stainable Eaton antibody with infected chick embryo lung frozen sections was performed as described previously(2).

Specimens. Throat swabs were collected from Marine recruits with pneumonia and from volunteers experimentally infected with a 2nd monkey kidney tissue culture passage isolate. The history of these individuals has been described(2,3,11). Throat swab specimens were collected in trypticase soy broth with 0.5% bovine albumin and 500 units of penicillin per ml. The specimens were stored at –60°C for 12 to 18 months before being tested for PPLO.

Results. Recovery of PPLO from volunteers. Throat swab specimens were obtained from 10 volunteers on the 9th day following administration of 640 egg doses of a strain of Eaton agent in its 2nd monkey kidney tissue culture passage(3). The agent was isolated in tissue culture from each of these specimens at time of collection(3). Each of the volunteers developed a rise in fluorescent stainable antibody. After storage at –60°C for one year the throat swab specimens were thawed and examined for the presence of PPLO using the artificial agar medium. Each specimen was inoculated onto 2 agar plates which were incubated at 35°C. One plate was held in a candle jar while the other was incubated aerobically.

Eaton PPLO were re-isolated on agar plates from each of the volunteers (Table I). Isolates were generally detected earlier under aerobic conditions than when incubation was carried out with increased CO_2 tension. In addition, the organism was recovered from one volunteer using the former but not the latter type of incubation. In view of these findings aerobic incubation was employed routinely during subsequent isolation attempts.

Recovery of PPLO from naturally infected individuals. Three groups of Marine recruits with pneumonia were selected for study. The

TABLE I. Use of Agar Plates for Recovery of Eaton PPLO from Experimentally Infected Volunteers.

Volunteer	Days of incubation before PPLO detected		Approx. No. of PPLO colonies per .1 ml of throat swab fluid	
	Aerobic incub.	Increased CO_2 incub.*	Aerobic incub.	Increased CO_2 incub.*
1	10	11	220	31
2	10	11	54	30
3	10	13	20	10
4	11	10	150	60
5	11	13	7	5
6	11	13	100	20
7	11	15	30	6
8	15	15	2	2
9	15	18	2	6
10	19	—†	1	—

* Candle jar.

† Eaton PPLO not recovered. Approximately 200 colonies of a non-Eaton PPLO were observed when this specimen incubated under increased CO_2 tension.

Note: Throat swab specimens collected from 10 volunteers 9 days following inoculation of 2nd monkey kidney tissue culture passage Eaton PPLO. Specimens were stored at –60°C for 1 yr prior to testing.

TABLE II. Use of Agar Plates for Recovery of Eaton PPLO from Naturally Infected Patients with Pneumonia.

Diagnosis	Immunofluorescence with chick embryo sections: Reciprocal of titer Acute	Conval.	No. in group	No. from whom Eaton PPLO recovered	Days of incubation before PPLO detected*	Approx. No. of PPLO colonies per .1 ml of throat swab fluid
Eaton infection	<10	80 or >	13	12	6, 6, 7, 7, 7, 7, 7, 7, 8, 12, 12	550, 520, 200, 173, 140, 80, 47, 46, 18, 6, 21, 22
Indeterminate	40–80	40–80	4	2	7, 11	20, 36
Not Eaton infection	10–20	20	4	0	—	—
	<10–10	<20	10	0	—	—

* Aerobic incubation.

Note: Throat swab specimens collected from Marine recruits with pneumonia before 5th day of illness and stored at −60°C for 18 mo. prior to testing.

first consisted of individuals with serologic evidence of Eaton infection. The status of the second group was indeterminate since moderate to high levels of fluorescent stainable antibody were present in both acute and convalescent phase serums. Recruits in the 3rd group were serologically negative. Throat swab specimens were stored at −60°C for 18 months prior to testing for presence of PPLO. Each specimen was inoculated onto an agar plate which was incubated aerobically at 35°C.

Eaton PPLO were recovered from almost all of the pneumonia patients with definite serologic evidence of infection (Table II). The agent was not recovered from the 14 patients who were without serologic evidence of Eaton infection. Eaton PPLO were recovered from 2 of the patients in the indeterminate group, suggesting that the organism may be present in the pharynx late in infection at a time when maximal levels of antibody have been attained.

Properties of recovered PPLO. PPLO recovered from volunteers produced the same colonial morphology as did naturally occurring strains of the organism. The colonies were homogeneously granular and the center was often embedded into the agar (Fig. 1). The morphologic appearance of the strains from volunteers and naturally infected individuals was identical with that previously described for the laboratory adapted FH strain (7). Colonies were rarely encountered which had the "fried egg" or "nippled" appearance typical of most human oral and genital PPLO. Human oral PPLO with "fried egg" or "nippled" morphology are shown in Fig. 2 and 3.

When first detectable, Eaton PPLO colonies measured approximately 10 μ in diameter. At maturation the colonies varied in size from 20 to 100 μ. The smaller colonies were generally seen in the densely infected areas of the plate while the larger colonies tended to be isolated.

During isolation naturally occurring strains of Eaton PPLO usually required 6 to 7 days of incubation before colonies were detectable; in 3 instances the interval was 8 to 12 days. The strain recovered from volunteers grew somewhat more slowly; the usual interval before colonies were recognized was 10 to 11 days and in 3 instances, 15 to 19 days. Growth of the 24 isolates shown in Tables I and II was more rapid during the 2nd and 3rd passages on agar, and by the 4th passage 100 or more colonies were recognizable by the 4th to 5th day of incubation. In all instances sufficient growth occurred by the 3rd or 4th passage to permit identification of the organism by the fluorescent antibody technic.

Discussion. Identification of the Eaton agent as a PPLO (genus *Mycoplasma*) was established in a recent study in which an egg and tissue culture adapted laboratory strain was propagated on an agar-yeast extract-horse serum medium(7). Serum was found to be necessary for growth and the colonies which grew on and into the agar stained with the Dienes stain(7).

In the present investigation naturally occurring strains of Eaton PPLO were also shown to propagate on the agar medium. This medium appeared to be a sensitive system for

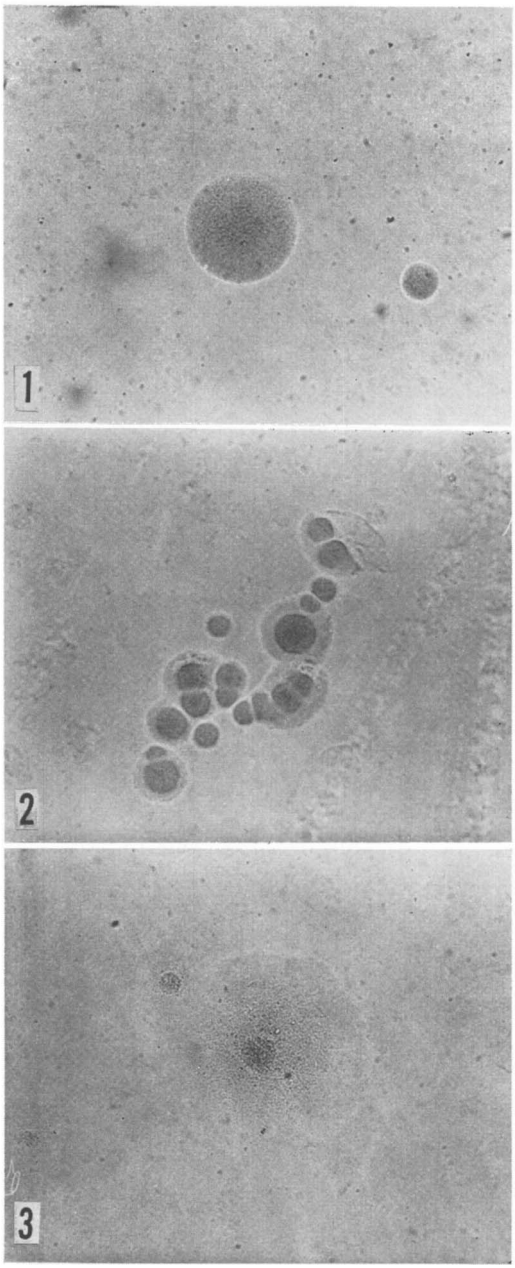

FIG. 1. Eaton PPLO. 90 ×.
FIG. 2. Non-Eaton PPLO from oropharynx—"fried egg" morphology. 90 ×.
FIG. 3. Non-Eaton PPLO from oropharynx—"nippled" morphology. 90 ×.

recovery of Eaton PPLO from infected individuals. Isolates were obtained from 22 of 23 individuals with serologic evidence of infection. In unpublished studies, additional strains have been recovered from patients with pneumonia in North Carolina and the District of Columbia(12,13).

Previously embryonated eggs or tissue culture were employed for isolation of Eaton agent(1,9). Both methods are laborious since they require the preparation of frozen sections of chick embryo lung. For this reason isolation attempts have been limited and what is known of the natural history of infection has been acquired primarily from serologic investigations. Undoubtedly our understanding of the behavior of Eaton agent in man will be hastened now that an easily prepared agar medium is available for recovery of the organism. It is now feasible to investigate the communicability of infected individuals, the period of persistence of the agent in the pharynx, and finally the possible relationship of Eaton agent to chronic lung disease. Chronic involvement of the lung would not be surprising since this is characteristic of PPLO infection in birds and certain mammalian species(14,15).

The morphologic appearance of colonies produced by strains from naturally infected individuals was the same as that previously observed with the well adapted laboratory strain. All Eaton strains recovered from infected individuals produced small to moderate sized homogenously granular colonies. The non-Eaton PPLO recovered from the pharynx on our standard agar medium have produced colonies with a characteristic "fried egg" or "nippled" appearance. The distinctive colonial appearance of Eaton PPLO may prove useful in presumptive identification of isolates during diagnostic or epidemiologic studies. It is clear, however, that many additional Eaton and non-Eaton PPLO must be compared before colonial morphology can be adopted for purposes of identification.

Summary. Naturally occurring Eaton PPLO was shown to propagate on an agar-yeast extract-horse serum medium. The agent was recovered directly on agar from 12 of 13 serologically positive patients with pneumonia. Eaton PPLO was not recovered from 14 serologically negative pneumonia patients. All Eaton PPLO isolates produced small homogenously granular colonies which could be easily differentiated from the usual

"fried egg" or "nippled" colonies produced by non-Eaton oral PPLO. The distinctive colonial morphology of Eaton PPLO may ultimately prove useful for its presumptive identification.

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C^{14}-Labeled Streptococcal Hyaluronic Acid in the Guinea Pig.* (27576)

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Synovitis has been produced in guinea pigs (1) by polysaccharides and polysaccharide complexes of gram-negative bacteria. Some of the exudative material in the joint space apparently consisted of masses of hyaluronic acid and protein(2). Little is known concerning the local production and resorption of hyaluronic acid in synovial fluid. A reasonable assumption would be that the half-life of hyaluronic acid in synovium would be similar to that of rabbit skin–namely, 2 to 3 days (3,4). As an approach to the study of synovial hyaluronic acid, the fate of parenterally injected C^{14}-labeled streptococcal hyaluronic acid in the guinea pig has been investigated. As might be expected with a linear polymer of N-acetyl glucosamine-glucuronic disaccharide, streptococcal hyaluronic acid appeared to be identical with that of animals (5), although a lower content of uronic acid has been reported(6).

Methods and materials. C^{14}-labeled hyaluronic acid was obtained from Streptococcus, Group A 111 grown in 2.5% Difco heart infusion broth containing 1% glucose, 0.1% sodium acetate and 6.3 millicuries of sodium acetate-1-C^{14} per liter. During the 30-hour period of growth at 37°C, the culture was neutralized at intervals with sodium hydroxide. The bacterial cells were removed by centrifugation and the crude hyaluronic acid was precipitated from the culture media by addition of 4 volumes of 95% ethanol saturated with potassium acetate according to the method of Pike(7). The precipitated potassium hyaluronate was washed 4 times with 95% ethanol and twice with absolute ethanol. The hyaluronic acid was then purified by the procedure of Roseman *et al.*(6) with removal of protein by phosphomolybdic acid, precipitation of hyaluronic acid from aqueous solution with 3 volumes of acetone and treatment with Amberlite IR-120. The final product was again precipitated as potassium hyaluronate, then washed 4 times with 95% ethanol and twice with ether. The uronic acid content was determined by procedure of Dische(8) and glucosamine content by the

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