ISOLATION AND INCIDENCE OF PLEUROPNEUMONIA-LIKE ORGANISMS FROM THE HUMAN ORAL CAVITY

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ABSTRACT

SHKLAIR, I. L. (U. S. Naval Training Center, Great Lakes, Ill.), M. A. MAZZARELLA, R. R. GUTEKUNST, AND E. M. KIGGINS. Isolation and incidence of pleuropneumonia-like organisms from the human oral cavity. J. Bacteriol. 83:785-788. 1962.—Pleuropneumonia-like organisms (PPLO) were isolated from the oral cavity of 87.2% (184 of 211) of young male naval recruits, by use of an enriched medium under anaerobic conditions. Aerobically, the organisms were isolated from 75% (78 of 104) of the subjects. However, aerobic cultivation was not the method of choice for the isolation of these organisms, as only a few colonies of PPLO were found. These colonies were atypical, or incomplete, and could not be further subcultured either aerobically or anaerobically. With anaerobic procedures, typical PPLO colonies developed and they could be subcultured anaerobically.

The PPLO were isolated by adding saliva or gingival scrapings to 8 ml of PPLO broth enriched with 10% horse serum and 1% yeast autolysate. In addition, thallium acetate (1:2,000) and penicillin (3,000 units/ml) were added to prevent bacterial overgrowth. The broth was incubated at 37 C for 48 hr; 0.1 ml was then spread on PPLO agar enriched with the above nutrients and thallium acetate. The plates were incubated at 37 C for 48 hr. The broth and agar plates were adjusted to a pH of 7.8 to 8.0. Both PPLO and L forms were isolated by the above procedure.

Pleuropneumonia-like organisms (PPLO) have been isolated from the mouth and pharynx (Nicol and Edward, 1953; Morton et al., 1951; Smith and Morton, 1951; Dienes and Madoff, 1953; Burnett and Gilmore, 1959), but as yet no significance can be attached to their presence. There are two divergent thoughts on the method of isolation of these organisms from the oral cavity. Morton et al., Smith and Morton, and Dienes and Madoff reported the isolation of these organisms under aerobic conditions. However, Nicol and Edward could isolate the oral strains only under anaerobic conditions with the addition of thymic nucleic acid. Burnett and Gilmore and Gilmore and Burnett (1959) isolated PPLO and L forms from saliva and gingival debris grown in a veal-thioglycolate broth incubated in a normal atmosphere. Growth from the mid-portion of the anaerobic zone of the broth was transferred to a PPLO agar plate and incubated in a candle jar. To date, the discrepancy between these conflicting procedures has not been explained. This study was undertaken to determine the better method of isolating these organisms from saliva and gingival debris.

MATERIALS AND METHODS

Specimens of saliva and gingival debris were collected from 211 male naval recruits, 17 to 23 years old, with an average age of 18 years. Initially, the procedure of Morton et al. (1951) was followed; however, this procedure was abandoned because no PPLO were observed and excessive bacterial overgrowth was encountered, even though the medium contained potassium tellurite and crystal violet as bacterial inhibitors.

From various modifications of the above isolation methods, the following media and procedures evolved. The inoculum, either 0.2 ml of saliva or the equivalent of 500 to 600 µg (dry wt) of gingival debris, was placed in 8 ml of Difco PPLO broth enriched with 10% horse serum and 1% yeast autolysate (Basamin-Busch powder; Anheuser-Busch, Inc., St. Louis, Mo.). In addition, thallium acetate (1:2000) and penicillin...
(3000 units/ml) were added to prevent bacterial overgrowth (Edward, 1947; Dienes, 1947; Morton and Lecee, 1953). The broth was incubated aerobically for 48 hr at 37 C; 0.1 ml was then transferred to Difco blood agar base or PPLO agar, enriched with the above nutrients and adjusted to pH 7.8. Thallium acetate, but no penicillin, was added to prevent bacterial contamination and to minimize growth of L forms. The plates were incubated aerobically for 48 hr at 37 C and examined microscopically (150 X).

The same procedures, using the same media, were then carried out under anaerobic conditions of incubation. The anaerobic method used was a modification of both Morton’s (1943) and Rogosa’s (1956) techniques. The plates and tubes were incubated at 37 C for 48 hr in Brewer jars which were fitted with covers modified by the addition of two-way stopcocks and removal of the electric platinum catalyst. The jars were evacuated to a pressure of 250 mm of mercury, and a gas composed of 95% nitrogen and 5% carbon dioxide (Ohio Chemical and Surgical Equipment Co., Cleveland, Ohio) was added; this oxygen-free gas mixture was then flushed through the jars for 3 min at a flow rate of 3 liters/min. The cycle of evacuation, replacement, and flush was performed three times. A positive pressure of 20 mm of mercury was maintained during the incubation period.

A number of the original aerobic broth tubes were stored at 30 C for preservation, and were studied at convenient intervals.

RESULTS

Only atypical colonies grew on the aerobically inoculated medium (Fig. 1). These colonies had the appearance of the dark central portion of a PPLO colony, but lacked the periphery which gives the PPLO colony its typical “fried egg” appearance (Fig. 2). These atypical colonies could not be further subcultured either aerobically or anaerobically. Under anaerobic conditions, typical PPLO colonies were isolated which could be subcultured using the anaerobic techniques described. The typical PPLO isolates, when subcultured under aerobic conditions, produced the atypical colonies which could not be further subcultured using either the aerobic or anaerobic procedures.

The “aerobic tubes,” which were initially found to contain PPLO and were stored at -30 C, yielded typical PPLO colonies with anaerobic methods. Some other specimens that did not produce any colonial growth aerobically did so under complete anaerobiosis.

With anaerobic cultivation methods, 184 of the 211 (87.2%) specimens yielded PPLO colonies. In addition, 104 of the 211 samples were examined by aerobic techniques for the presence of PPLO; PPLO were found in 78 (75%) of the samples. Even though aerobic cultivation produced a high rate of PPLO isolation, it was not considered the method of choice because growth was usually
very poor, there being only a few colonies per plate and the organisms could not be subcultured. Anaerobiosis usually increased growth by more than 100-fold. Growth was greatly stimulated by the addition of the yeast autolysate. No growth was observed aerobically unless the yeast autolysate was present. Yeast extract (Difco) could not act as replacement for the autolysate. Horse serum or 30% ascitic fluid (Difco) produced better growth than did PPLO serum fraction (Difco).

During the anaerobic phase of the study, some unusual large bodies, which proved to be L forms, were isolated (Fig. 3, 4, and 5). These produced bacillar forms when subcultured aerobically or anaerobically in PPLO broth void of penicillin and thallium acetate. They were not further identified. The “typical” PPLO colonies, when grown as above, usually did not produce bacterial growth. The “typical” PPLO colonies which did not revert to bacterial forms were considered PPLO. This arbitrary classification must be used until the uncertainties between the genetic and morphological differences of the L forms and PPLO are resolved. It is known that Proteus (Dienes, 1949), Salmonella (Dienes, 1948; Weinberger, Madoff, and Dienes, 1950), and possibly other microorganisms produce the 3A L-form type of colony which has been very difficult to revert to its bacterial form. To what extent the bacteria found in the oral cavity produce the 3A or other stable L forms is unknown, although Gilmore and Burnett have produced and isolated a variety of L forms from the oral cavity.

**DISCUSSION**

On the basis of the above results, one may speculate that the so-called aerobic procedures of Morton and his collaborators were, in reality, anaerobic. That is, the oxidation-reduction potential of the medium may have been reduced as a result of synergistic bacterial action, deepness of the tubed medium, or a combination of these factors. The PPLO were in a rapid-growth phase when grown in broth; when they were transferred to the genuine aerobic conditions on the surface of agar, they continued to grow for a short time and then died. Hence, only the dark center without the periphery was seen in the atypical colonies. This lack of PPLO development was further demonstrated by the absence of bacterial and PPLO growth upon attempts to subculture the original aerobic broth culture into fresh uninoculated broth. This was probably due to the lack of viable bacteria acting synergistically, which, if present, would reduce the oxidation-reduction potential of the medium and allow the PPLO to grow.

This may explain some of the discrepancies between the work of Morton et al. and Dienes and Madoff, who grew PPLO from saliva and gingival scrapings aerobically, and Nicol and Edward, who could grow them only anaerobically. It may also explain why Sabin and Johnson (1940) isolated PPLO colonies aerobically from excised tonsils. These colonies, which were referred to as “X” colonies, could not be subcultured aerobically.

Recently, Barile and Sheingorm (1960) reported the isolation of PPLO from an infected pulp in a human tooth. The isolation of this organism was also accomplished under anaerobic conditions. However, they could not isolate PPLO from saliva.

**LITERATURE CITED**


