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ISOLATION OF *BORRELIA REFRINGENS* IN PURE CULTURE FROM PATIENTS WITH CONDYLOMATA ACUMINATA

ALBERT W. HANSON AND GEORGE R. CANNEFAX

Research and Development Laboratories, Venereal Disease Research Laboratory, Venereal Disease Branch, Communicable Disease Center, U.S. Public Health Service, Atlanta, Georgia

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Abstract

Hanson, Albert W. (Communicable Disease Center, Atlanta, Ga.), and George R. Cannefax. Isolation of *Borrelia refringens* in pure culture from patients with condylomata acuminata. J. Bacteriol. **88**:111-113. 1964.—*Borrelia refringens* was isolated from five of ten clinical specimens by use of a modification of the Noguchi technique. An enriched anaerobic medium is described for the cultivation of *B. refringens* with retention of morphological characteristics.

The bacterial flora of the infected genitalia is of interest to the clinician from the diagnostic view and to the scientist because of the paucity of information concerning the cultural requirements of this flora and its role in disease production. Of the bacteria found on the infected genitalia, the Treponemataceae have been studied the least, owing to the problems associated with primary isolation and maintenance in artificial media. To study the relationship of *Borrelia refringens* to the host, it is necessary that methods be developed to obtain this organism in pure culture with ease and to maintain its morphological characteristics.

The methods for the isolation of the Treponemataceae are modifications of the principle advocated by Noguchi (1912*a*, *b*), which depended upon the ability of the spiral organisms to migrate away from the contamination in soft agar or gel. More recently, Rosebury et al. (1951) proposed a modification of the Noguchi method; they used small petri plates as containers for primary cultures with the wall of a central well in an agar plate as the site of inoculation. This method eliminated the effects of disruption of the agar by the gas-producing bacteria and greatly improved the isolation procedures.

B. refringens has been described frequently in the literature. As early as 1905, it was described as being found with the causative agent of syphilis, *Treponema pallidum*. Noguchi (1912*c*) described the isolation and cultivation of *Spirochaeta refringens*. In *Bergey's Manual of Determinative Bacteriology* (Breed, Murray, and Smith, 1957) the organism is described as having cells 0.5 to 0.75 by 6.0 to 20.0 μ, with coarse and shallow spirals. The spirals are generally smoothly rounded and regular, tapering towards the end into fine projection, and motile with an active serpentine rotating motion with marked flexion. The organism stains easily with the common dyes, and, according to *Bergey's Manual*, the cultivation at any time in the past is uncertain.

In this study, a technique for the isolation and cultivation of *B. refringens* with maintenance of the general clinical morphology is described.

Materials and Methods

Lesion material from patients with condylomata acuminata of the genitalia was examined by dark-field smears to confirm the presence of *B. refringens*. When these organisms were found, cultures of the condylomata acuminata lesions were taken with sterile cotton swabs and placed in the holding medium. The clinical material was held at room temperature during the time of collection and transportation to the laboratory (2 to 5 hr).

Owing to the length of time between collection of the clinical material and its arrival at the laboratory, it was found necessary to use a holding medium to maintain viability of the desired organisms. The holding medium consisted of 45% spirolate broth (BBL), 45% Brain Heart Infusion (BBL) broth with 0.05% sodium thioglycolate added, 10% normal rabbit serum inactivated for 30 min at 56 C, and 20 units of polymyxin B sulfate (Chas. Pfizer & Co., Inc., New York, N. Y.) per ml, in a total volume of 3 ml in screw-cap tubes (13 by 100 mm). Experiments demonstrated that the antibiotic in the concentration used did not inhibit the motility of *B. refringens*

or *T. pallidum*, but did inhibit the motility and growth of some of the bacillary flora found in the clinical material.

Two primary isolation media were used: (i) the holding medium with the same antibiotic concentration and 0.2% agar to give a soft gel; (ii) a coagulated serum medium consisting of 75% sterile hog serum and 25% of a 0.85% sterile saline solution containing 0.002% calcium chloride, 0.1% dextrose, 0.9% asparagine (Difco), and 0.9% Tryptone (BBL). The second mixture was inspissated in a water bath (80 C) until a solid coagulation occurred (approximately 10 min). The primary isolation media were prepared daily before use, and were tubed in 5-ml amounts in screw-cap tubes (13 by 100 mm).

A 1-ml amount of the inoculated holding medium was used in each of the two primary isolation media. After incubation of 48 to 96 hr at 35 C, dark-field examination of the growth was made to verify an increase in numbers of *B. refringens* over the original inoculum. When adequate numbers of *B. refringens* were produced in the primary cultures, about 2×10^5 per ml, well plates were prepared in petri plates (15 by 100 mm) by use of the holding medium with the addition of 0.025% asparagine, 0.025% Tryptone, 0.8% agar, and without the antibiotic. The depth of the medium was 12 to 14 mm, and the diameter of the central well was approximately 10 mm. The wells were filled with the primary isolation media, placed in Brewer jars, and degassed and gassed three times with a 95% hydrogen and 5% carbon dioxide gas mixture. The hydrogen was reacted with any free oxygen remaining in the jars by activating the heating element in the Brewer jar lid. This method is a modification of two previously described techniques, those of Rosebury et al. (1957) and Hampp (1943). After 5 to 7 days of incubation at 35 C, growth was observed as a delicate white haze 1 to 3 cm in distance about the well. Smears of the outer edge of this haze were prepared, and a pure culture of *B. refringens* was confirmed by dark-field examination. Agar plugs containing the pure culture were punched from the well plates just beyond the edge of the visible haze by use of a sterile capillary pipette 1 to 2 mm in diameter. The agar plugs were transferred to tubes of medium of the same composition as well plates, with the agar concentration reduced to 0.1%. The point of inoculation of the agar plugs was midway in the column of soft medium.

RESULTS

Cultural characteristics. B. refringens is an anaerobe, as evidenced by the absence of growth in an adequate medium under aerobic conditions. In the same medium under anaerobic conditions, the top level of growth is approximately 1 cm from the surface of the medium; this is similar to the cultural characteristics encountered with the anaerobic cultivatable members of the genus *Treponema*.

The organism grows poorly or not at all in the absence of a small concentration of agar. Growth is very slow at 20 to 30 C, maximal growth occurs at 34 to 36 C, and the organism fails to grow at temperatures above 39 C. Cultures maintain viability for 1 week without transfer when maintained at 20 to 36 C.

Morphology. In young, active cultures, many cells are found with a length of 8 to 20 μ and a width of 0.4 to 0.75 μ. The motility of the organism is serpentine with rotation, marked flexion, and translation. The spirals are coarse and deep in relation to those of the genus *Treponema*. They are regular in nature, tapering toward the end to a fine projection. These same general morphological characteristics are observed in clinical material. In older cultures, the length of a cell is from 3 to 5 μ with many granules or what appear to be cysts. The cysts have a diameter of 1 to 1.5 μ and are highly refractile. The organism has been observed to reverse its rotation to free itself from an entanglement. The organisms stain well with the ordinary aniline dyes.

Pathogenicity. B. refringens is nonpathogenic for rabbits, white mice, and guinea pigs. Viable organisms were not recovered from the peritoneal cavity, viscera, or blood stream 48 hr after intraperitoneal inoculation of large numbers of motile organisms.

DISCUSSION

B. refringens was successfully isolated from five of ten specimens collected from patients with condylomata acuminata and cultivated in a medium consisting of 45% spirolate broth, 45% Brain Heart Infusion broth, 0.05% sodium thioglycolate, 0.025% asparagine, 0.025% Tryptone, 0.1% agar, and 10% normal inactivated rabbit serum. Morphological characteristics similar to those found in clinical material and also described in *Bergey's Manual* (Breed et al., 1957)

have been retained in this medium for more than 12 months of cultivation.

During the late stationary-growth phase, this organism develops granules that may be a cystic form, as postulated for certain other cultivatable Treponemataceae.

The two primary isolation media used proved to be of equal value for isolation. The coagulated serum medium was superior in maintaining the viability of *B. refringens* in mixed cultures for greater lengths of time without transfer—up to 3 weeks. This medium is not as effective in cultivating the organism in pure culture, a possible indication that the serum is acting in a dual role—a nutrient supplement and a detoxifying agent.

Sparse growth occurred in the absence of agar, suggesting that the agar may act to detoxify the medium or that *B. refringens* needs impurities in the agar, as Bachman (1955) found in studies with *Cytophaga fermentans*.

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