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Actinomyces naeslundii as an Agent of Human Actinomycosis

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The repeated isolation of *Actinomyces naeslundii* from clinical materials associated with disease led to a comparison of isolates from the normal mouth with isolates from pathological clinical materials not from the mouth area. No important differences were observed between the isolates from these two sources. A human case of empyema of the gall bladder, apparently due to *A. naeslundii*, is described.

Actinomyces naeslundii is generally considered to be a saprophytic component of the normal flora of the human mouth. It was probably first observed by Naeslund who described a group of "facultative *Actinomyces*-like organisms" from human dental tartar in 1925 (4). Thompson and Lovestedt described similar isolates from the oral cavity in 1951, and proposed the name *Actinomyces naeslundii* (6). Further characterization and comparison to *A. israelii*, which it resembles, were made by Howell et al. in 1959 (2). All of these workers considered *A. naeslundii* to be a harmless saprophyte that occurred commonly in the oral cavity of man. Neither isolation from pathological clinical materials nor evidence for an etiologic relationship to disease was reported by any of these workers.

The Mycology Section of the National Communicable Disease Center (NCDC) has identified cultures of *A. naeslundii* isolated from a wide variety of human clinical materials, as well as from the mouth or sputum. This study was undertaken to completely characterize seven isolates from pathological materials, and five from the apparently normal mouth. Included, also, is a case report that presents evidence that *A. naeslundii* may be an agent of human actinomycosis, in this case, the causative agent of empyema of the gall bladder.

MATERIALS AND METHODS

Source of isolates. The cultures used were from lyophilized stock of the NCDC Mycology Culture Collection. The original sources and pertinent clinical data are listed in Table 1.

Oxygen requirements. Measured inoculum (usually one small drop from the tip of a capillary pipette) from actively growing cultures in Thioglycollate Broth (1.5 g of dehydrated Trypticase Soy Broth and 1.25 g of Tryptose Broth per liter of Thioglycollate Broth, BBL) was streaked with a single stroke from the base to the top of each of eight Brain Heart Infusion Agar (BHIA) slants. Pairs of inoculated slants were incubated at 37 C under the following conditions: aerobically (cotton plugs only), aerobically with a CO_2 seal, under anaerobic seal, and anaerobically with a CO_2 seal. The various seals were prepared by placing pledgets of absorbent cotton in the necks of the tubes above the nonabsorbent plugs. The pledgets were soaked with five drops each of the following combinations: 10% Na_2 Co_3 and 1 M KH_2PO_4 (CO_2 seal), KOH and a saturated solution of pyrogallic acid (anaerobic seal), and 10% Na_2 CO_3 and pyrogallic acid (anaerobic + CO_2 seal). After the solutions were added to the cotton pledgets, the tubes were closed with rubber stoppers. Growth was recorded as 0 to 4+ after 7 to 14 days by gross comparison. The pair of slants showing heaviest growth was considered 4+. If replicate tubes did not give similar readings, the test was repeated.

Morphology. Inoculum from 3 to 5 day thioglycollate broth cultures was streaked on BHIA plates. They were incubated in a Brewer anaerobe jar using the BBL gaspak (no. 70304, hydrogen + CO_2) to maintain anaerobic conditions. Microcolony development was observed microscopically (100 ×) after 18 to 24 hr of incubation. Macrocolonies were examined with a dissecting microscope (40 ×) after 7 days of incubation. Cellular morphology was determined by examining (970 ×) Gram-stained smears of various culture media.

Biochemical tests. The following biochemical tests were done: catalase, nitrate reduction, indole, reactions in iron milk, gelatin liquefaction, esculin hydrolysis, urease production, hydrogen sulfide production, and acid production from carbohydrates.

Catalase production was determined by the production of a stream of bubbles after flooding one of the BHIA slants showing maximal growth with fresh 3% H_2O_2. Indole-Nitrite Broth (BBL) was inoculated in duplicate, and standard tests for nitrate reduction and indole production were done after 7 days. Gelatin liquefaction was determined using Thiogel (BBL).

TABLE 1. *A. naeslundii cultures studied*

NCDC[a] strain no.	NCDC diagnostic no.	Other identification	Clinical source
W826		Thompson, Mayo Clinic, Rochester, Minn., ATCC 12104[b], *A. naeslundii*, type strain	Sinus after tooth extraction
X569	45-756-62 (received 10/16/62)	Conn. State Dept. of Health #L50-794A	Blood (postmortem)
X600		Howell, NIH[c]-C5-1752	Dental calculus
W752	45-1103-64 (received 12/15/64)	Calif. Dept. of Public Health #6751-64	Postoperative thyroidectomy wound abscess
W821	44-330-65 (received 12/28/65)	Calif. Dept. of Public Health #7925-65	Aspirate from lesion on leg
W833	44-7-66 (received 1/20/66)	Long Island College Hospital, Brooklyn, N.Y., S. L. Cohen strain	Apex of tooth
W869	44-96-66 (received 5/20/66)	Gerencser, WVA-398a West Virginia Univ., Morgantown, W.Va.	Dental calculus
W953	44-234-66 (received 12/12/66)	Minn. State Dept. of Health #3040 B	Sputum
W1003	44-79-67 (received 4/17/67)	Arkansas State Bd. of Health	Blood culture
W1048	44-156-67 (received 8/11/67)	Ind. State Bd. of Health #687-654 Myc.	Postoperative abdominal wound abscess
W0196	44-213-67 (received 12/15/67)	Ark. State Bd. of Health #5522	Empyema of gall bladder
W1105	44-2-68 (received 1/10/68)	Ind. State Bd. of Health #1102-1197 Myc.	Blood culture from a case of leukemia and pyelonephritis

[a] NCDC, National Communicable Disease Center, Atlanta, Ga.
[b] ATCC, American Type Culture Collection, Rockville, Md.
[c] NIH, National Institutes of Health, Bethesda, Md.

Triple Sugar Iron (TSI) agar and Heart Infusion (HI) agar slants with lead acetate papers were used to detect H_2S production. Infusion broth with 0.1% agar and 0.1% esculin was used to test for esculin hydrolysis. This was determined by adding a drop of 1.0% ferric citrate (aq) to samples of the culture after 3 to 10 days of incubation. The development of a brown-black color indicates hydrolysis, Whole, nonhomogenized milk to which had been added a few iron filings was used to observe reactions in milk.

The basal medium for fermentation studies consisted of thioglycollate broth (without dextrose or indicator) with added yeast extract (0.2%) and bromothymol blue indicator (0.1%). The final carbohydrate concentrations used were 1.0%, except for starch which was 0.4%.

The inoculum for all biochemical tests was taken from actively growing enriched thioglycollate broth cultures. All readings were made after 14 days of incubation, except as indicated.

Serology. Fluorescent-antibody (FA) tests were performed on all cultures utilizing procedures described by Lambert et al. (3). *A. naeslundii* (NCDC X454 and X600)- and *A. israelii* (NCDC A601)-labeled antiglobulins were used to test all cultures.

RESULTS

Oxygen requirements. Results of the tests for oxygen requirements are shown in Table 2. Four of the 12 strains could be considered facultative. These strains grew as well aerobically as under conditions of reduced oxygen tension, and growth did not appear to be stimulated by the addition of CO_2. However, eight of the 12 strains grew best microaerophilically or anaerobically in the presence of CO_2. One strain (W1105) grew only under anaerobic conditions.

Growth in broth. In thioglycollate broth, *A. naeslundii* grows slightly below the surface, diffusing throughout the medium with small clumps of organisms (Fig. 1a).

Morphology. Stains revealed gram-positive organisms consisting of diphtheroidal cells and branched filaments. Filamentous forms were of uneven diameter and frequently showed clubbed ends. Very long forms were often clumped together in tangled colonies (Fig. 1b). Diphtheroidal forms, also of varying lengths, showed

uneven staining, knobbed ends, and were frequently in V and Y arrangements (Fig. 1c). Most strains regularly showed a mixture of filamentous and diphtheroidal forms (Fig. 1d).

Microcolonies. All cultures showed highly filamentous spider-like formations on the surface of the agar after 18 to 20 hr; however, they soon showed dense tangled masses at their centers and long radiating, branched filaments spreading out in all directions (Fig. 2a and b).

Macrocolonies. Mature colonies of the various strains presented a variety of morphological

TABLE 2. *Growth of A. naeslundii under varying O_2 tensions with and without added CO_2*

Condition	Strains											
	W826[a]	X569	X600	W752	W821	W833	W869	W953	W1003	W1048	W1096	W1105
Aerobic	2+	3+	2+	4+	4+	4+	1+	4+	1+	3+	1+	0
Aerobic + CO_2	3+	4+	4+	4+	4+	4+	4+	4+	3+	4+	4+	0
Anaerobic	3+	3+	3+	4+	4+	3+	4+	3+	2+	3+	4+	4+
Anaerobic + CO_2	4+[b]	3+	3+	4+	4+	3+	4+	4+	4+	3+	4+	4+

[a] ATCC 12104, type strain of species.
[b] Best growth for each strain was recorded as 4+; growth under other conditions was compared to this standard.

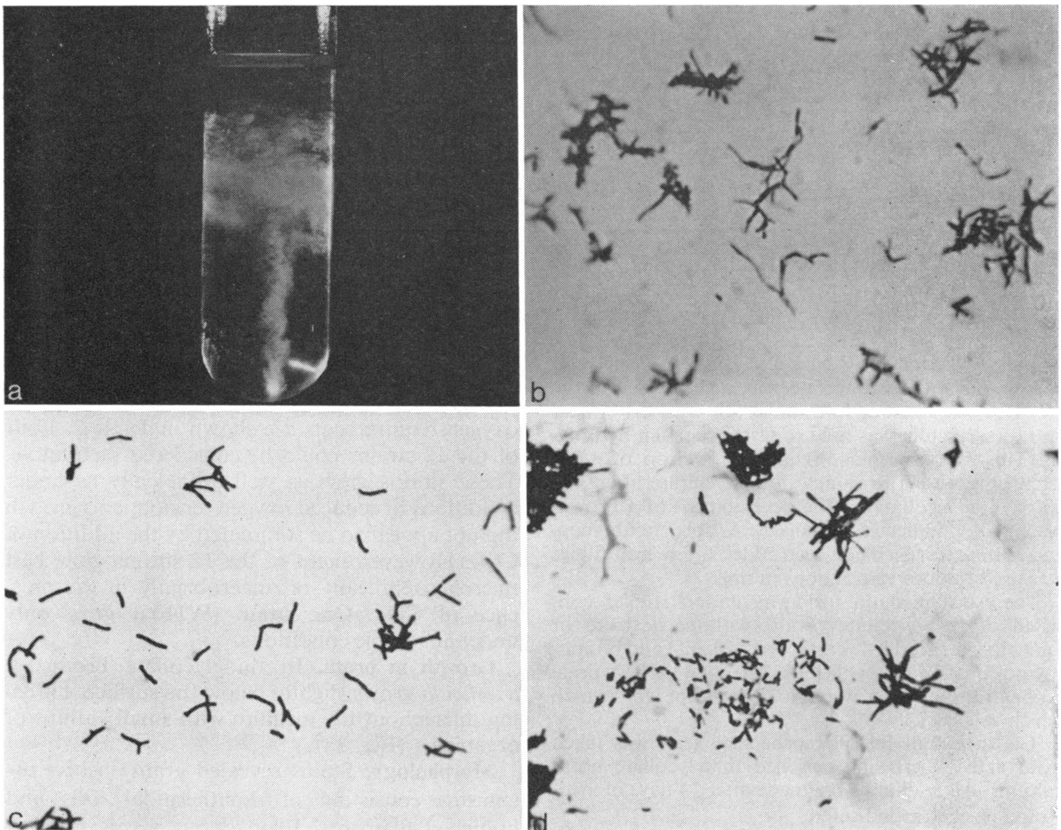

FIG. 1. *(a) Characteristic growth of A. naeslundii W826 in thioglycollate broth. (b) Clumped, filamentous forms of A. naeslundii W1003; Gram stain, 1150 ×. (c) Diphtheroidal forms of A. naeslundii W1105 with simple Y-type branching and bifid ends; 1150 ×. (d) Mixture of filamentous and diphtheroidal forms of A. naeslundii W821; 1150 ×.*

forms. In spite of the highly filamentous character of the very young colony, the mature colonies were usually fairly flat and presented smooth surfaces and entire borders (Fig. 3a). Some strains, however, developed colonies with varying degrees of surface irregularities. Colonies of W1096 (Fig. 3b) were raised in their centers and showed irregularly lumpy surfaces and scalloped borders. Strain W826 (Fig. 3c) regularly produced colonies with raised centers which were characteristically triangular or star shaped. Strain W821 (Fig. 3d) produced mature colonies with raised, highly folded, and scalloped borders.

Biochemical tests. Biochemical reactions of the various strains are shown in Table 3. All were catalase negative, and none produced urease or indole, or liquefied gelatin.

Esculin was hydrolyzed by all strains except W752, W833, and W1003. The only strain which did not reduce nitrate to nitrite was W1003. All cultures produced a change in the iron milk except strain X600. Four of the 12 strains produced H_2S.

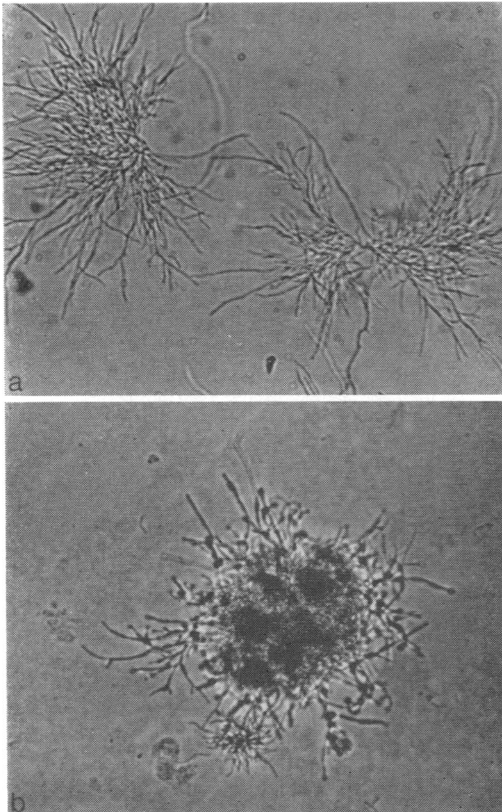

FIG. 2. *(a) Spider-type microcolonies of A. naeslundii* W826 *at 24 hr;* 400 ×. *(b) Microcolony of A. naeslundii* W1096 *showing dense center at 48 hr. 400 ×.*

Results of the fermentation tests are seen in Table 4. In all instances, the carbohydrates were fermented with the production of acid and no gas. The following carbohydrates were fermented by all cultures tested: glucose, lactose, sucrose, and maltose. Glycerol was fermented by all strains with the exception of W1003. The following carbohydrates were fermented by a majority of the strains: salicin, starch, raffinose, inulin, trehalose, and inositol. Rhamnose was fermented by X600 and W1096, and arabinose was fermented by X569 and W833. Mannitol, dulcitol, xylose, and adonitol were not fermented by any of the strains tested.

Serology. All of the strains reacted with NCDC X454 and NCDC X600 (*A. naeslundii*) conjugates at diagnostic titer, except strain W1105 which reacted only with NCDC X600. None of the strains tested reacted with NCDC A601 (*A. israelii*) conjugate.

Evidence for pathogenicity of A. naeslundii. The human mouth is recognized as a normal habitat for *A. naeslundii*; however, of the 12 strains included in this report, seven were isolated from sources other than the mouth. These seven strains were isolated from pathological clinical materials: three from the blood stream, three from wound abscesses, and one from empyema of the gall bladder. Of the remaining five strains, one was isolated from cervico-facial actinomycosis, one from a patient with periodontoclasia, and three strains were isolated from apparently normal areas of the mouth. Except for the case of empyema of the gall bladder (W1096), no information was obtained, indicating that organisms suggestive of *Actinomyces* were seen by direct examination of the clinical materials.

For the isolate from empyema of the gall bladder (W1096), convincing evidence was obtained for the pathogenic potentialities of *A. naeslundii*. An abstract of the case history and a description of the tissue reactions are given below.

A. naeslundii as the probable causative agent in a case of empyema of the gall bladder. A 65-year-old female from Brinkley, Ark., with a 3-day history of abdominal pain and fever was admitted to St. Vincent's Infirmary, Little Rock, Ark. She was known to have gallstones and had previous gall bladder attacks. The diagnosis was empyema of the gall bladder.

On 5 October 1967, a cholecystectomy was performed. The omentum was found to be densely adherent to the gall bladder. The gall bladder was described as gangrenous, but not ruptured. About half of its visible surface was mottled with a green color; the rest appeared normal. Dark puslike

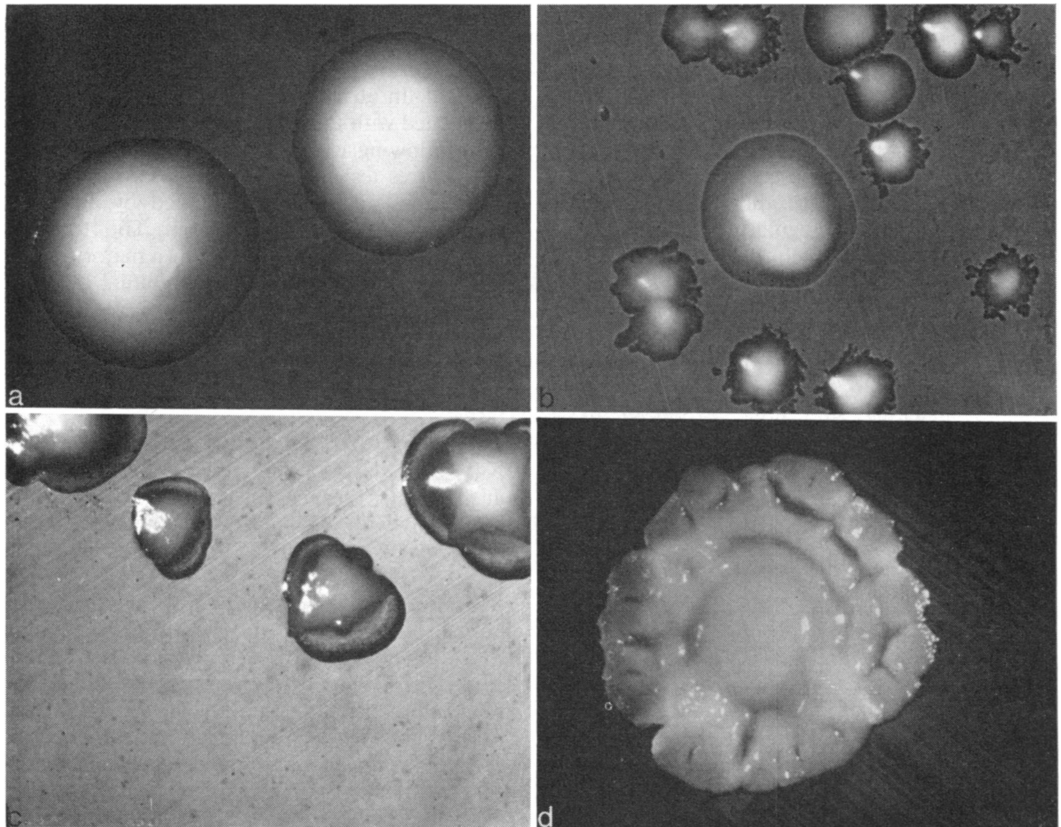

FIG. 3. (*a*) *Mature colonies of A. naeslundii W1003; smooth, 7 to 10 days.* (*b*) *Mature colonies of A. naeslundi W1096, smooth with irregular borders.* (*c*) *Mature colonies of A. naeslundii W826 with raised triangular or star-shaped centers.* (*d*) *Mature colony of A. naeslundii W821 with raised, ruffled borders.*

TABLE 3. *Biochemical reactions of A. naeslundii*

Test	Strains												No. of positive strains[b]	Percentage of positive strains
	W826[a]	X569	X600	W752	W821	W833	W869	W953	W1003	W1048	W1096	W1105		
Catalase	0	0	0	0	0	0	0	0	0	0	0	0	0/12	0
Nitrate reduction	+	+	+	+	+	+	+	+	0	+	+	+	11/12	91
Indole	0	0	0	0	0	0	0	0	0	0	0	0	0/12	0
Iron milk[c]	A	A + c	0	A	A + d	A	A	A	A + d	A	A + d	A	11/12	91
Gelatin liquefaction	0	0	0	0	0	0	0	0	0	0	0	0	0/12	0
Esculin hydrolysis	+	+	+	0	+	0	+	+	0	+	+	+	9/12	75
Urease production	0	0	0	0	0	0	0	0	0	0	0	0	0/12	0
H_2S production	0	0	0	0	+	0	+	+	+	0	0	0	4/12	33

[a] ATCC 12104, type strain of this species.
[b] Numerator, no. of strains giving positive reactions; denominator, total no. tested.
[c] Symbols: A, acid produced in 1 to 7 days; c, soft clot; d, digestion; 0, no acid produced.

TABLE 4. *Fermentation of carbohydrates by A. naeslundii*

Carbohydrate	Strains												No. of strains producing acid[b]	Percentage of strains producing acid
	W826[a]	X569	X600	W752	W821	W833	W869	W953	W1003	W1048	W1096	W1105		
Glucose	A[c]	A	A	A	A	A	A	A	A	A	A	A	12/12	100
Mannitol	0	0	0	0	0	0	0	0	0	0	0	0	0/12	0
Lactose	a	A	A	a	A	A	A	A	A	A	A	A	12/12	100
Sucrose	A	A	A	A	A	A	A	A	A	A	A	A	12/12	100
Maltose	A	A	A	A	A	A	A	A	A	A	A	A	12/12	100
Salicin	A	0	A	0	a	0	A	a	0	0	A	0	6/12	50
Glycerol	a	a	A	a	a	A	A	A	0	a	A	A	11/12	91
Starch	a	0	0	a	a	0	a	A	a	a	a	A	9/12	75
Arabinose	0	a	0	0	0	a	0	0	0	0	0	0	2/12	16
Xylose	0	0	0	0	0	0	0	0	0	0	0	0	0/12	0
Raffinose	A	0	A	0	A	A	A	A	A	0	A	A	9/12	75
Rhamnose	0	0	A	0	0	0	0	0	0	0	A	0	2/12	16
Dulcitol	0	0	0	0	0	0	0	0	0	0	0	0	0/12	0
Inulin	A	0	0	A	A	A	A	A	a	A	A	A	10/12	83
Trehalose	A	A	0	A	A	0	A	A	A	A	0	A	9/12	75
Inositol	A	A	A	A	A	A	A	a	0	A	A	A	11/12	91
Adonitol	0	0	0	0	0	0	0	0	0	0	0	0	0/12	0

[a] ATCC-12104, type strain for this species.
[b] Numerator, number of strains producing acid; denominator, total number tested.
[c] Symbols: A, acid produced in 1 to 7 days; a, acid produced in 7 to 14 days; 0, no acid produced.

fluid (3 oz) was aspirated under pressure. The remainder of the patient's hospital course was uneventful.

Macroscopic examination of tissue sections revealed that the serosa of the gall bladder was very irregular; where it was coarsely granular, the serosa contained foci of yellow-green discoloration. In other areas, it was markedly hemorrhagic and softened. The wall of the viscus was thickened and edematous. The mucosal surface was covered by a green-tan hemorrhagic mucoid material.

On direct Gram stain of the pus taken at the time of cholecystectomy, numerous filamentous, branched, pleomorphic, and gram-positive organisms were seen. Microscopic examination of the gall bladder tissues revealed an extensive erosion of the gall bladder wall with necrosis, inflammation, and focal infiltration of the hemorrhage throughout. Gram-positive, highly pleomorphic filamentous organisms were seen in sections of the tissue stained by the method of Brown and Brenn (Fig. 4a and b). All organisms observed appeared to be of the same general morphological form. They occurred singly or in loose clumps in the exudate. No granules were observed.

A pure culture of a gram-positive, filamentous, branched organism grew on blood and brain heart infusion media under aerobic conditions and, also, in thioglycollate broth that had been inoculated with the gall bladder exudate obtained at surgery. The culture was subsequently identified at the NCDC Mycology Section as *A. naeslundii* (W1096). No other organisms were isolated. It appears that this organism was responsible, at least in part, for the pathology observed at the time of cholecystectomy.

DISCUSSION

The results of comparative studies showed considerable range in gross colony characteristics among the 12 isolates; however, at 24 to 48 hr, all showed characteristic microcolonies (described as spider colonies with dense center), mixtures of diphtheroidal and highly pleomorphic, branched, filamentous forms in liquid or on solid media. The growth of most isolates was definitely stimulated by CO_2, and all could be considered facultative (in the presence of CO_2), except strain W1105. This strain did not grow aerobically with or without CO_2. It would appear that the ability to grow aerobically cannot be relied upon as a species characteristic in all instances.

The results of our fermentation studies are in agreement with Howell (2), except for the fermentation of arabinose by two strains (W833 and X569), and by the fermentation of glycerol by 11 of the 12 strains tested. However, the base for testing carbohydrate fermentation was not identical in both studies.

It has been shown by previous studies (1, 3, 5) that *A. naeslundii* shares an antigen with *A. israelii*, and, therefore, cross-reactions occur between

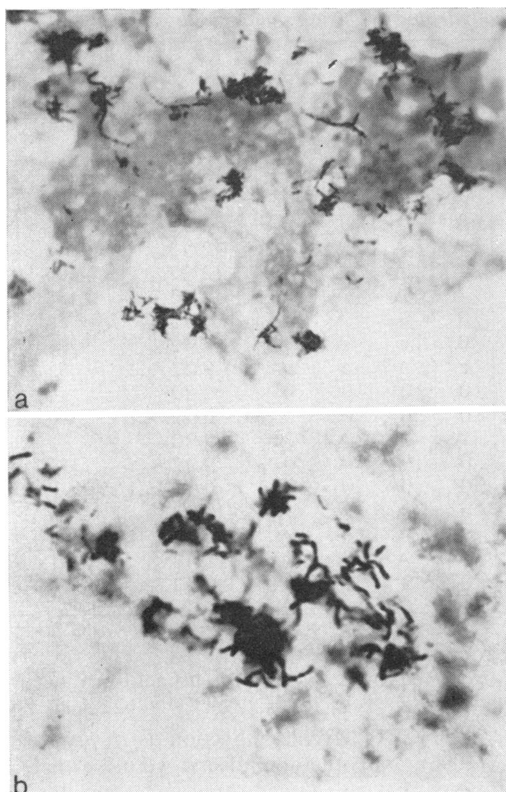

FIG. 4. (*a*) *Section from gall bladder; Brown and Brenn stain, 1000 ×.* (*b*) *Clusters of organisms in exudate of gall bladder, 2000 ×.*

these two species. However, specific staining of *A. naeslundii* may be obtained using properly diluted or adsorbed FA conjugates. All 12 *A. naeslundii* strains in the present study gave positive staining reactions with our *A. naeslundii* conjugates and did not show cross-reactions with our specific *A. israelii* conjugates (*A. israelii* conjugate adsorbed with *A. naeslundii* cells).

No important morphological, biochemical, or serological differences were observed between the *A. naeslundii* isolates from the oral cavity and the isolates from pathological materials from various areas of the body.

The ability of *A. naeslundii* to produce lesions was demonstrated by the pathological response seen in the patient with empyema of the gall bladder. On the basis of the materials studied, it appears that *A. naeslundii* does not form granules readily, as does *A. israelii*, but the organism may develop freely in the tissues.

It was believed that the pathogenicity of *A. naeslundii* could be further validated by attempting to produce experimental infections in laboratory animals. The results of these studies are given in a second report.

ACKNOWLEDGMENT

We acknowledge the work of John P. Gust of the National Medical Audiovisual Center, Atlanta, Ga., for the preparation of the photographs.

ADDENDUM IN PROOF

Since completion of this study, we have had an opportunity to study and confirm the identification of an isolate of *A. naeslundii* from a case of lacrimal canaliculitis. This organism was originally isolated in pure culture and identified by Geraldine W. Brown, bacteriologist at the Adelaide Children's Hospital, North Adelaide, Australia.

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