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Yersinia pseudotuberculosis: Use of Cold-Temperature Enrichment for Isolation

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The successful isolation of *Yersinia pseudotuberculosis* from the stool of an asymptomatic family member of a patient with yersinia septicemia is presented. Cold enrichment permitted the isolation after 4 weeks of refrigerator incubation.

The cold-enrichment technique has been used successfully for the recovery of *Yersinia enterocolitica* from stool specimens (4, 5, 9). The recovery of yersiniae at refrigeration temperature (4°C) is a result of their marked increase in numbers after regular multiplication, whereas other enteric bacteria fail to grow or even decline in numbers at this temperature (4, 10). Human disease caused by *Y. pseudotuberculosis* has rarely been recognized in North America, with less than two dozen cases reported in the literature (6, 8, 13). Perhaps this is because *Y. pseudotuberculosis* can produce a variety of clinical manifestations which may be confused with other diseases, or that clinical microbiology laboratories fail to recover this slow-growing organism when using standard culture techniques to examine stool specimens. This report documents the recovery of three isolates of *Y. pseudotuberculosis* from clinical specimens over an 11-month period. Two isolates were from blood cultures, and one was from the stool of an asymptomatic contact. The importance of extended incubation of cold-enrichment cultures beyond the usually recommended 3-week period is emphasized.

The first isolate was from a 65-year-old white male who was diagnosed as having well-differentiated lymphocytic lymphoma. He underwent an exploratory laparotomy with splenectomy 3 years earlier and was on intermittent chemotherapy. A week before admission he was noted to have an enlarging left inguinal lymph node, increasing leukocyte count and anemia. The patient also had a low-grade fever for 1 week and subsequently developed a fever of 102°C on the morning of admission. At that time, he also was noted to be leukopenic and was hospitalized with suspected sepsis. Blood, urine, and sputum were obtained before treatment with intravenous cephalothin and gentamicin. On hospital day 2, a Gram stain of the blood culture revealed a small gram-negative rod that was identified as *Y. pseudotuberculosis* by using the methods described below.

Since the patient lived on a farm, stool samples were collected from the patient, and multiple samples were collected from his dogs and cats, as well as from the dog and cat of his neighbor. Environmental samples including residual food and well water were also examined. All specimens were inoculated directly on plated media and put into isotonic saline for cold enrichment, and all were negative for yersiniae. Stool cultures from the asymptomatic wife of the patient were examined directly and with cold enrichment. A single stool specimen yielded *Y. pseudotuberculosis* only with the aid of cold enrichment.

The third isolate of *Y. pseudotuberculosis* was from a 66-year-old white male with a long history of chronic obstructive pulmonary disease with emphysema. The patient had a 2-week history of increasing shortness of breath, recurrent fever of 103°C with shaking chills, and white sputum production noted 6 days before admission. No apparent source of infection was noted on physical examination. Antibiotics were withheld and cultures of blood, urine, and sputum were obtained at the time of hospitalization. On hospital day 4, five of eight blood cultures yielded a small, gram-negative rod that was identified as *Y. pseudotuberculosis*. Stool cultures from the patient and from his wife, granddaughter, and family dog were negative for yersiniae after direct plating and cold-enrichment techniques.

The blood culture isolates from the two septicemic patients were detected by the BACTEC method (Johnston Laboratories, Inc., Cockeysville, Md.) within 1 and 2 days after receipt of the respective blood specimens. Samples from each positive blood culture bottle were Gram stained and subcultured to sheep blood agar, chocolate agar, and MacConkey agar plates for isolation. The plates were incubated overnight

at 35°C and examined for growth. Stool and environmental samples were processed by direct culture and by cold enrichment. A 10 to 20% suspension of each stool or food specimen was prepared in 30 ml of saline, emulsified, and incubated at 4°C. A 5-ml water sample was added to the saline. Each stool, environmental, and water sample also was added to routine media used for isolation of fecal pathogens, namely, MacConkey, salmonella-shigella and Hektoen-enteric agars and gram-negative broth, and incubated at 35°C for 24 and 48 h. Cold-enrichment cultures were held for 6 weeks at 4°C and were subcultured once weekly on the aforementioned enteric media. All plated media were incubated at 35°C and examined after 24 h, followed by overnight incubation at room temperature before being discarded as negative. The single stool specimen which yielded *Y. pseudotuberculosis* did so on the fourth isolation attempt from the cold-enrichment saline culture.

On MacConkey agar the isolates appeared almost as pinpoint colonies after 18 to 24 h of incubation, with an increase in size after continued incubation. Each organism was identified by using the API 20E system and confirmed by standard biochemical procedures (3). Inoculum for the standard biochemical tests consisted of a 4- to 5-h broth culture of selected colonies taken from MacConkey agar. Inocula for the API tests were saline suspensions of the organisms. Each isolate was serotyped as type 1A by the Plague Branch, Center for Disease Control, Ft. Collins, Colo. Antibiotic susceptibilities were determined by the standardized disk diffusion method (7). Minimal inhibitory concentrations (MICs) were determined by using commercially prepared antibiotics trays (Prepared Media Laboratories, Tualatin, Oreg.).

Table 1 shows the results of standard biochemical tests routinely employed in our laboratory. The reactions at 35°C incubation were uniform and conformed to the predicted findings for *Y. pseudotuberculosis*. Since the organisms fermented neither sucrose nor lactose in triple sugar iron agar, and motility in semisolid media at 35°C was lacking, the resemblance of the organisms to shigellae was remarkable. One of the three strains demonstrated a delayed urease response as well. The motility of the yersiniae was difficult to demonstrate since overnight incubation at room temperature was not adequate to manifest a positive test in semisolid medium. Continuous passage at room temperature was necessary to demonstrate motility in one of the isolates.

The antibiograms of the organisms are shown in Table 2. All three isolates were very susceptible to all antibiotics tested, except colistin sulfate (Coly-Mycin S). The MICs correlated well with those of the disk diffusion test except those of chloramphenicol. The zones of inhibition for chloramphenicol were quite large, although one isolate had an MIC of 4.0 μg/ml. Because of slow growth in broth at 35°C, MIC plates had to be incubated at room temperature for 6 h before the control wells contained satisfactory growth for reading purposes. Disk diffusion plates could be read after overnight incubation.

The isolation and subsequent identification of

TABLE 1. *Biochemical characteristics of three isolates of Y. pseudotuberculosis*

Test or substrate[a]	Positive in 1 to 2 days[b]		% Positive predicted for *Y. pseudotuberculosis*[c]
	No.	%	
TSI	K/A[d]		K/A
Indole	0	0	0
Methyl red	3	100	100w[e]
Voges-Proskauer	0	0	0
Citrate	0	0	0
Motility; 22°C	1 (1)	33 (33)	40 (45)
35°C	0	0	0
Urease	3	100	100
Phenylalanine deaminase	0	0	0
Lysine decarboxylase	0	0	0
Arginine dihydrolase	0	0	0
Ornithine decarboxylase	0	0	0
Nitrate reduction	3	100	—[f]
Gelatin liquefaction	0	0	—
Malonate utilization	0	0	—
Oxidase	0	0	—
DNase	0	0	—
Esculin hydrolysis	3	100	100
Starch hydrolysis	0	0	—
ONPG	3	100	70
Glucose	3	100	100
Lactose	0	0	0
Sucrose	0	0	0
Maltose	3	100	90
Mannitol	3	100	100
Xylose	3	100	100
Fructose	3	100	—
Salicin	2 (1)	67 (33)	5 (95)
Dulcitol	0	0	—
Adonitol	0	0	0
Inositol	0	0	—
Arabinose	1 (2)	33 (67)	50 (45)
Raffinose	0	0	20
Rhamnose	3	100	100
Sorbitol	0	0	0
Trehalose	1 (2)	33 (67)	100
Cellobiose	0	0	0

[a] TSI, Triple sugar iron; DNase, deoxyribonuclease; ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

[b] Each value in parentheses indicates percentage of delayed reactions (3 or more days).

[c] See reference 2.

[d] K/A, Alkaline/acid reaction.

[e] w, Weak reaction.

[f] —, No data available.

TABLE 2. *Susceptibility of three strains of Y. pseudotuberculosis to 12 antibiotics*

Antibiotic	Zone size (range, mm)	MIC (μg/ml)
Carbenicillin	39–42	≤8.0
Colistin sulfate	6	≥4.0
Ampicillin	34–36	<0.25
Kanamycin	28–30	≤1.0
Tetracycline	25–26	1.0
Chloramphenicol	31–38	≤0.50–4.0
Gentamycin	26	≤0.25
Cephalothin	38–41	≤1.0
Cefoxitin	36–40	NT[a]
Streptomycin	22–25	NT
Tobramycin	26–28	≤0.25
Amikacin	24–26	≤1.00

[a] NT, Not tested.

Y. pseudotuberculosis recovered from blood cultures has proven not to be difficult. The low rate of recovery from stool specimens, however, exemplifies the difficulty and extensive procedures which are necessary to isolate the organism from this source. This is not surprising, since standardized measures for isolation of yersiniae have not been agreed upon. For example, stool and rectal cultures usually are not reliable specimens for the recovery of yersiniae in asymptomatic patients and in infected patients without gastroenteritis (9). Also, incubation of primary isolation plates at 22°C for 5 days had been chosen arbitrarily by Daniels (1), whereas the practice of incubation for 2 days at room temperature after incubation at 35°C was discontinued by Pai and associates (9) because of ineffectual recovery. Room-temperature incubation of plates after cold-temperature incubation was proven effective for *Y. enterocolitica* by several investigators (4, 5). Our test plates were incubated for 24 h at 35°C and then held at room temperature for an additional 24 h.

Nutrient broth (4) and phosphate-buffered saline (5) have been used for cold-enrichment isolation of *Y. enterocolitica*, whereas saline (8, 12) and phosphate-buffered saline (10) have been used for *Y. pseudotuberculosis*. Paterson and Cook (10) found, and Tsubokura and associates (11) confirmed, that *Y. pseudotuberculosis* multiplied gradually in 0.067 M phosphate-buffered saline to reach a maximum level of growth after 20 days of incubation. The same phenomenon was observed with both *Yersinia* species in broth in 5 days (4). Tsubokura and co-workers (11) showed, however, that phosphate-buffered saline offered little advantage over use of regular saline, although the choice of isolation medium (MacConkey agar) was critical, in contrast to findings with salmonella-shigella agar (4, 5). We recommend, based upon our experience in isolating *Y. pseudotuberculosis* from an asymptomatic human patient after 4 weeks of cold enrichment, that enrichment cultures should be held beyond the customary 3 weeks. This appears as equally important as the temperature selected for primary isolation on solid media, and certainly more so than the choice of suspending fluid medium used for cold enrichment.

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