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Characterization and Identification of 95 Diphtheroid (Group JK) Cultures Isolated from Clinical Specimens

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Ninety-five cultures of group JK bacteria isolated from clinical specimens were characterized morphologically and biochemically. The microorganisms were isolated primarily from blood cultures. The bacterial cultures produced positive reactions when tested for catalase, Tween hydrolysis, and carbohydrate fermentation. Glucose and galactose were fermented by more than 90% of the organisms. Gas-liquid chromatography of trimethylsilyl derivatives of whole-cell hydrolysates of some of the group JK cultures yielded nearly identical elution profiles. The group JK microorganisms were susceptible to vancomycin but were resistant to most of the other 17 antimicrobial agents tested. A method is presented for differentiating the group JK microorganisms from other similar bacteria encountered in clinical specimens. Although these bacteria rarely occur in clinical specimens, they are capable of producing fatal infections (endocarditis and sepsis) in humans.

Aerobic diphtheroids, which are gram-positive rods morphologically similar to *Corynebacterium diphtheriae*, are widely dispersed throughout nature (1, 13, 29, 31) and have been observed in clinical specimens of human origin (4-6, 10-12, 14-16, 18-21, 23-25, 30, 32, 35). In the latter case, the detection of the coryneforms is usually considered to be a result of contamination. This is often true because these microorganisms are commonly present on normal skin and mucous membranes. Thus, many investigators who study human indigenous diphtheroids are concerned primarily with cutaneous isolates (6, 18, 23-25).

Some of these species, however, including *C. aquaticum* (35), *C. haemolyticum* (14), *C. xerosis* (10), *C. pyogenes* (4, 20), and *C. pseudodiphtheriticum* (15), have been reported to be responsible for serious human infections. In addition, the most serious infections, although quite rare, may be due to unidentified diphtheroids. These microorganisms have been implicated as the etiological agents in endocarditis (5, 10, 11, 15, 16, 19, 21, 32) and purulent meningitis (30). In most cases the blood stream infection occurred after prosthetic cardiac valve replacement surgery. A high mortality rate is associated with the prosthetic heart valve infections.

This study was performed to characterize a group of diphtheroids which have been isolated from clinical specimens and have been designated "group JK" by the Special Bacteriology Section (SBS) at the Center for Disease Control. During the past 15 years we have examined 95 group JK cultures. Fifty-six percent of these cultures were grown up from blood specimens (Table 1). Moreover, in some cases group JK microorganisms were repeatedly isolated from a patient's blood. Four cultures of the group JK bacteria were isolated by Davis et al. (5) from three patients with endocarditis that occurred after cardiopulmonary bypass surgery for repair of heart valve defects. Group JK cultures from three patients who had bacterial endocarditis affecting prosthetic valves were studied by Van Scoy et al. (32). Hande et al. (12) observed four patients with cases of "corynebacteria" sepsis, and organisms from these patients were examined by the SBS and found to be group JK bacteria.

In this report the morphological and physiological traits of the group JK bacteria are described.

MATERIALS AND METHODS

Bacteria. The bacterial cultures were secured from the stock culture collection maintained by the SBS. They were identified from clinical isolates submitted to this laboratory for additional examination (Table 1).

Media and procedures. The biochemical tests were those routinely used in the SBS for investigating microorganisms submitted for identification. The media preparation and the procedures used in determining biochemical characteristics have been described previously (7, 17). Esterase activity on several Tween compounds was determined by the method of Smith

(23). All agar slant media were inoculated with 1 to 2 drops of an 18- to 24-h heart infusion broth (Difco) culture.

Gas-liquid chromatography. The freeze-dried cells were hydrolyzed by the technique of Farshy and Moss (8). The dried hydrolysate was dissolved in 0.5 ml of TRI-SIL (Pierce Chemical Co.). The vial containing the sample was flushed with nitrogen, capped, and placed in a 65°C water bath for 15 min. After incubation, the sample was reduced to dryness under nitrogen. The dried residue was dissolved in 1 ml of hexane, and the final volume was reduced to 0.1 ml under nitrogen.

The samples were analyzed with a Hewlett-Packard 402-B gas chromatograph equipped with dual-flame ionization detectors and 1.2-m-long glass columns having an ID of 3 mm. The detector temperature was 250°C. Helium, with a flow rate of 40 ml/min, was used as the carrier gas. Samples were analyzed on columns consisting of 3% OV-1 on GasChrom Q, 100/120 mesh (Applied Science Labs, Inc.). The column temperature was held for 4 min at 130°C and then programmed to 230°C at the rate of 3°C/min. The peak areas and the percentages of each eluted component in the samples were determined with a Hewlett-Packard 3380A reporting integrator.

TABLE 1. *Sources of 95 strains of group JK bacteria*

Source	No. of isolates
Blood	53
Genitourinary specimens	11
Cerebrospinal fluid	9
Miscellaneous wounds	6
Lung lesions	3
Exudates (toe, breast, vagina)	3
Eye	2
Hip	2
Peritoneal fluid	2
Unknown	2
Pleural fluid	1
Placenta	1

Antimicrobial agent susceptibility testing. The agar dilution method (34) was used to determine the minimal inhibitory concentrations of 18 antimicrobial agents. Twofold dilutions of the following antibiotics were tested at concentrations of 128 to 1 μg/ml: amikacin, ampicillin, cephalothin, chloramphenicol, clindamycin, erythromycin, furadantin, gentamicin, kanamycin, methicillin, penicillin G, polymyxin B, streptomycin, tetracycline, tobramycin, and vancomycin. Sulfadiazine was tested at 1,024 to 4 μg/ml, and carbenicillin was tested at 512 to 4 μg/ml.

Cells from 18- to 24-h cultures were inoculated into 2 ml of Mueller-Hinton broth containing 0.5 ml of sterile rabbit serum and 0.02 ml of Tween 80 and incubated at 35°C for about 4 h. The culture suspensions were then diluted with sterile Mueller-Hinton broth to a final concentration of approximately 10^8 colony-forming units/ml. The Steers replicator (26) was used to inoculate approximately 0.001 ml (ca. 10^5 colony-forming units) onto the surface of the dried medium containing the antibiotic. The inoculated plates were incubated at 35°C for 24 h.

RESULTS

The group JK microorganisms were pleomorphic, nonmotile, nonsporulating, gram-positive rods (Fig. 1). None of the JK cultures was

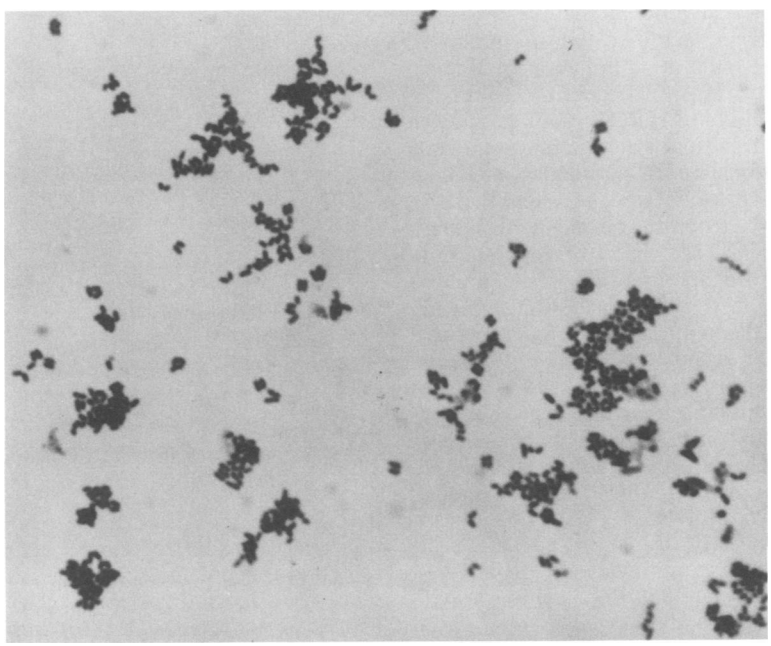

FIG. 1. *Illustration of a Gram-stained smear of the group JK bacteria (D2563).* ×*1,700.*

acid fast. The cells varied in size and shape from short coccobacilli to longer bacillary forms, some straight and some slightly club-shaped. There was no evidence of true branching.

After 18 to 24 h of incubation in a candle jar on heart infusion agar (Difco) supplemented with 5% rabbit blood, the bacterial growth was light to moderate, with no observable action on the blood. Colonies ranged from punctate to 0.5 mm and had entire edges and a low convex profile. The smooth, translucent bacterial colonies were butyrous in consistency and tended to become opaque upon continued incubation.

The group JK cultures were catalase-positive, oxidase-negative, fermentative microorganisms which did not reduce nitrate (Table 2). These nonpigmented bacteria did not alkalinize citrate, peptonize litmus milk, produce a halo on Tinsdale medium, or hydrolyze urea. Although the incorporation of Tween 80 into heart infusion agar medium enhanced the growth of these microorganisms, the group JK bacteria were not found to be "lipo-dependent." Except for Tween 80, all of the Tweens tested were hydrolyzed by at least 75% of the cultures.

Several carbohydrates were tested for acidification by these fermentative microorganisms (Table 3). Of 27 carbohydrates examined, only glucose was acidified by all of the group JK strains. Galactose was acidified by most (92%) of the bacterial cultures. Of the remaining substrates tested, only maltose (44%), fructose (35%), mannose (31%), dextrin (26%), and trehalose (23%) were acidified at significant levels. None of the strains acidified xylose, mannitol, lactose, sucrose, adonitol, dulcitol, rhamnose, raffinose, sorbitol, inositol, cellobiose, erythritol, or starch. Exclusive of the reactions in glucose and maltose, three major acidification patterns were noted. Fifty percent of the group JK cultures acidified only galactose; 16% acidified glycogen, galactose, fructose, mannose, trehalose, dextrin, and melezitose; and 10% acidified only galactose, fructose, and mannose.

The antimicrobial agent susceptibility testing indicated that these microorganisms are resistant to a number of antibiotics (Table 4). However, the 61 strains tested were susceptible to vancomycin. Bimodal distribution of the minimal inhibitory concentrations were evidenced on several antibiotics (amikacin, ampicillin, cephalothin, clindamycin, gentamicin, kanamycin, penicillin G, streptomycin, tetracycline, and tobramycin). Generally, the more resistant cultures were isolated from blood specimens.

Analysis of the gas-liquid chromatograms of the trimethylsilyl profiles revealed some variations among the strains tested (Table 5). The derivatives recorded did not vary considerably in either number of peaks or relative peak elution time. The elution time for each of the peaks recorded did not vary from strain to strain by more than 0.1 min. A total of 37 to 40 peaks (≥0.5% of the total peak area) were observed in the trimethylsilyl profiles of each of the bacterial strains tested. Each of the cultures examined

TABLE 2. *Some characteristics of the group JK bacteria*[a]

Test or substrate	Sign[b]	%[c]
Catalase	+	100
Oxidase	−	0
Motility	−	0
Nitrate reduction	−	0
Indole	−	0
Gelatin hydrolysis	−	0
Tinsdale reaction	−	0
Methyl red	−	0
Voges-Proskauer	−	0
Urea hydrolysis	−	0
Growth on:		
MacConkey agar	−	0
Cetrimide agar	−	0
6% NaCl broth	− or +	38
TSI agar (slant/butt)		
No change/no change	+ or −	80
Acid/acid	− or +	12
Hydrolysis of:		
Tween 20	+ or −	81
Tween 40	+ or −	89
Tween 60	+	97
Tween 80	+ or −	75
Tween 85	+ or −	56
Growth at:		
25°C	− or +	20
35°C	+	100
42°C	+ or −	58
Esculin hydrolysis	−	0
Chromogenesis	−	0
Citrate alkalinization	−	0
Litmus milk peptonization	−	0
Hydrogen sulfide production		
Lead acetate paper	+ or −	78
TSI butt	−	0
Acid fast	−	0
Gas production from glucose broth	−	0
ONPG hydrolysis	−	0[d]

[a] Abbreviations: TSI, triple sugar iron agar; ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

[b] Sign: + = 90% or more positive in 1 or 2 days; − = 90% or more negative; (+) or + = most reactions delayed 3 or more days, some occur within 1 or 2 days; + or (+) = most reactions occur within 1 or 2 days, some delayed 3 or more days; + or − = most strains positive, some negative; − or + = most strains negative, some positive; − or (+) = most strains negative, some delayed positive.

[c] Numbers indicate the percentage of cultures positive in 2 days.

[d] Fifty-five strains of group JK bacteria were tested.

TABLE 3. *Carbohydrate fermentation by 95 strains of group JK bacteria*

Substrate[a]	Sign[b]	%[c]
Glucose	(+) or +	(71) 29
Xylose	−	0
Mannitol	−	0
Lactose	−	0
Sucrose	−	0
Maltose	− or (+)	3 (41)
Glycerol	− or (+)	0 (16)
Salicin	−	2
L-Arabinose	− or (+)	(1)
Adonitol	−	0
Dulcitol	−	0
Galactose	+ or (+)	61 (31)
Fructose	− or (+)	28 (7)
Mannose	− or (+)	12 (19)
Rhamnose	−	0
Trehalose	− or (+)	18 (5)
Raffinose	−	0
Sorbitol	−	0
Inositol	−	0
Cellobiose	−	0
Inulin	− or (+)	1 (2)
Dextrin	− or (+)	5 (22)
Glycogen	− or (+)	0 (2)
Erythritol	−	0
Melibiose	−	2
Melezitose	− or (+)	3 (11)
Starch	−	0

[a] The filter-sterilized carbohydrates were added aseptically to the fermentation broth to a concentration of 1%. One drop of sterile rabbit serum was added to each tube after 24 h of incubation.

[b] Sign: − = 90% or more negative; (+) or + = most reactions delayed 3 or more days, some occur within 1 or 2 days; + or (+) = most reactions occur within 1 or 2 days, some delayed 3 or more days; − or (+) = most strains negative, some delayed positive.

[c] Numbers indicate the percentage of cultures positive in 2 days; those in parentheses indicate the percent positive after 3 or more days.

had six common peaks, eluting at 9.3, 11.4, 19.2, 21.5, 27.4, and 33.8 min, which accounted for 44 to 54% of the total peak area measured.

When the group JK bacteria are compared to some other well-documented corynebacteria, some characteristic similarities are observed (Table 6). The species most similar to group JK bacteria in morphological and biochemical characteristics is *C. bovis*. However, close comparative examination of cultures of *C. bovis* and group JK indicated that they are two separate groups (unpublished data).

Two organisms which may produce the same reactions as group JK in the indicated tests (Table 6) are *C. minutissimum* and *C. diphtheriae* (nitrate-negative strains). Both of the latter organisms, however, produce heavier growth on blood agar than does group JK. There is also a difference in the reactions of these organisms on triple sugar iron agar. Eighty percent of the group JK organisms did not produce an observable change in the pH of the slant in 18 to 24 h. Both *C. diphtheriae* and *C. minutissimum* produce alkaline or acid reactions on the slant after the same incubation period.

To obtain consistent reactions for the group JK bacteria in carbohydrate tests, it was necessary to supplement the fermentation broth with serum (Table 3). Supplementation with serum is not required for testing the reactions of *C. diphtheriae* and *C. minutissimum*.

DISCUSSION

Our primary purpose in examining the group JK bacteria was to obtain enough morphological and physiological characteristics to enable the clinical laboratorian to recognize this microor-

TABLE 4. *Minimal inhibitory concentrations (MICs) of 85 strains of corynebacteria-like organisms (group JK) to 18 antimicrobial agents as determined by agar dilution studies*

≥1,024															49			
≥512			58												8			
256			5												8			
≥128	39	43	5	51	2	53	6	11	40	41	50	48	6	28	26	20	36	
64	2	5	19	1	25	0	1	1	0	0	10	4	32	7	4	7	0	
32	1	1	2		18	2	4	12	0	0	13	0	47	1	1	5	0	
16	0	2	0	1	6	0	9	45	0	1	2	2	8	8	0	14	0	
8	1	6	7	5	22	7	6	28	1	1	1	2	6	7	4	5	0	
≤4	1	9	4	12	25	7	14	3	1	0	10	8	1	25	0	6	1	
2	5	6		5	2	5	1	0	1	27	6	8	0	14		19	1	
≤1	51	28		25	0	26	59	0	57	30	8	28	0	10		24	62	100

[a] Abbreviations: AN, amikacin; AM, ampicillin; CB, carbenicillin; CF, cephalothin; C, chloramphenicol; CC, clindamycin; E, erythromycin; F/M, furadantin; GM, gentamicin; K, kanamycin; DP, methicillin; P, penicillin G; PB, polymyxin B; S, streptomycin; Sd, sulfadiazine; Te, tetracycline; NN, tobramycin; VA, vancomycin.

[b] Forty-three strains were tested.

[c] Sixty-one strains were tested.

TABLE 5. *Relative percentage of peaks obtained from chromatograms of trimethylsilyl derivatives prepared from whole-cell hydrolysates*[a]

Retention time (min)	JK bacteria							
	C5389	D1076	D3734	C6317	C8456	D2563	D4967	C5609
2.2	0.6	0.8	T	T	T	0.7	0.5	1.5
2.7		0.6	0.9	1.2	1.9	0.7	0.6	
3.7	0.5	1.8	0.6	0.7	1.1	1.6	2.1	1.8
4.3	1.7	0.8	0.7	0.6	T	0.6	0.8	1.4
7.4	2.0	T	1.2	0.7	1.0	T	T	2.4
8.4	3.5	0.9	1.2	1.5	0.8	0.7	2.9	2.1
9.3	8.8	8.7	6.6	5.6	9.6	6.4	4.8	6.7
10.1	4.9	0.9	1.5	0.6	0.8	T	0.9	T
10.6	0.8		T	0.6		T		T
11.4	6.9	4.8	5.9	5.0	8.5	6.9	4.8	4.9
12.9	0.5	0.5	1.1	0.5	3.7	0.5	2.6	T
14.4	T	0.8	0.6	1.1	4.3	1.1	0.6	2.4
15.3	0.5	0.6	0.8	1.3	1.0	T	0.9	1.5
16.7	1.6	1.3	1.0	T	0.9	0.7	0.8	1.7
17.6	0.6	0.9	1.6	0.7	2.3	1.4	0.7	2.6
18.3	3.5	2.0	3.9	4.5	1.0	2.8	2.3	3.1
19.2	3.8	6.5	5.5	4.1	7.8	5.8	8.9	6.5
20.2	4.1	2.8	2.4	2.9	1.1	3.6	1.2	3.0
20.6	2.5	1.5	6.4	3.7	1.7	5.9	1.7	4.3
21.0	3.2	1.4	2.0	2.5	4.4	1.2	3.0	1.8
21.5	6.4	4.6	3.8	4.2	4.8	6.1	9.8	5.9
23.3	0.7	0.9	1.5	1.8	0.9	0.9	0.8	0.6
23.8	2.5	3.1	1.7	3.9	1.6	5.2	1.2	3.2
24.6	0.6	2.2	3.0	2.3	2.5	0.7	4.1	T
25.6	T	0.6	2.2	1.1	0.7	T	2.2	1.0
26.2		0.5	0.6	0.7				T
27.4	15.2	18.5	20.5	20.8	19.7	22.4	17.3	24.5
28.5	2.2	0.9	T	1.7	T	0.7	T	T
29.6	2.7	1.7	3.4	4.8	2.7	3.2	3.7	0.5
30.5	0.6	1.1	1.2	0.8	0.6	T	1.0	0.8
31.3	0.9	1.8	1.1	1.0	T	0.8	0.6	0.7
33.0	1.9	3.6	1.2	1.8	0.9	0.8	2.1	0.8
33.8	3.7	3.5	5.8	4.2	2.8	5.5	2.5	5.5
34.8	0.6	1.8	2.1	1.4	0.5	0.5	0.9	0.5
36.5	T	0.7	0.8	1.4	T	T	0.9	0.7
37.5	3.1	2.2	2.5	3.6	4.0	2.8	2.6	3.7
42.5		0.5	T		0.7	0.6	T	0.9
44.0	1.1	2.0	0.9	T	0.8	0.5		
45.0	2.1	3.2	1.5	0.6	T	1.5	0.9	0.9
46.0		1.0	T		T		T	
47.0	T	2.5		T	T	1.1	T	1.0

[a] Relative percentage reflects the amount of each component in the sample. T = relative percentage of less than 0.5%.

ganism when it is isolated from clinical specimens. In addition, the cultural characteristics were compared with those of various unidentified groups described in the literature (6, 18, 23, 25) as well as with those of documented named species (3). The group JK organisms appear to comprise a unique group which has not been previously described.

The JK group is a homogeneous group in that few variations were noted in morphological characteristics or biochemical patterns. The three carbohydrate acidification patterns observed did not justify separation into biotypes since other biochemical assays did not corroborate a difference in these cultures. Moreover, the gas-liquid chromatographic profiles of whole-cell hydrolysates were nearly identical. Intraspecies similarities in derivative patterns have been shown to be characteristic of some bacterial species (2, 8, 9, 22).

Catalase, motility, nitrate reduction, urease, *o*-nitrophenyl-β-D-galactopyranoside, glucose, maltose, and sucrose reactions (Table 6) combined with a triple sugar iron reaction and morphological and growth characteristics appear to provide sufficient characteristics for the identi-

TABLE 6. *Reactions obtained with the group JK bacteria and with some Corynebacterium species isolated from clinical specimens*[a]

Cultures	Catalase	Motility	Nitrate reduction	Urease	Carbohydrate fermentation	Acid from glucose	Acid from maltose	Acid from sucrose	ONPG[b]
Group JK	+	−	−	−	+	+	− (+)	−	−
C. diphtheriae	+	−	+ (−)	−	+	+	+	−	NT[c]
C. ulcerans	+	−	−	+	+	+	+	+ (−)	NT
C. xerosis	+	−	+	−	+	+	+	+	NT
C. bovis	+	−	−	−	+	+	−	−	+
C. minutissimum	+	−	−	−	+	+	+	− (+)	NT
C. haemolyticum	−	−	−	−	+	+	+	+	NT
C. renale	+	−	−	+	+	+	−	−	NT
C. striatum	+	−	+	−	+	+	−	+	NT
C. pseudotuberculosis	+	−	+ (−)	+	+	+	+	− (+)	NT
C. kutscheri	+	−	+	+	+	+	+	+	NT
C. pseudodiphtheriticum	+	−	+	+	−	−	−	−	NT
C. aquaticum	+	+	− (+)	−	−	+[d]	+[d]	+[d]	NT

[a] The reactions in parentheses indicate the reaction recorded by a minority of the cultures.
[b] ONPG, *o*-Nitrophenyl-β-D-galactopyranoside.
[c] NT, Not tested.
[d] Oxidative reaction.

fication of the JK group.

The diagnosis of a legitimate diphtheroid infection is difficult, because (i) blood cultures may require an incubation period of a week or more before positive cultures are observed (10, 16, 32) and (ii) these bacteria are inhabitants of the skin and mucous membranes and frequently may contaminate blood cultures (27, 33). In studying aerobic and anaerobic blood cultures, Washington (33) found that approximately 10% of the positive cultures also contained coryneform bacteria that had no apparent clinical significance.

The fact that corynebacteria other than *C. diptheriae* not only may be significant plant and animal pathogens but also may be pathogenic to humans has been documented (4, 10, 14, 15, 20, 35). Kaplan and Weinstein (16) reported cases in which the diphtheroids were recovered from blood and infected tissue from patients with pyogenic infections. They concluded that these microorganisms were the etiological agents. Johnson and Kaye (15) listed 52 cases of serious human infections caused by diphtheroid organisms. Thirty-one of the 52 patients had bacterial endocarditis. Moreover, 42% of these patients developed endocarditis after undergoing cardiac surgery with placement of prosthetic valves or leaflets. When diphtheroid endocarditis occurs in the presence of prosthetic heart valves, the outlook for patient recovery is much graver (11). Gerry and Greenough (11) surveyed 20 cases, and of these, 9 patients died. When diphtheroidal endocarditis develops after a heart valve operation, the infection can sometimes be overcome only by surgical removal of the septic focus in the heart (19).

At least 14 isolates of the group JK bacteria have been obtained from patients infected after heart valve replacement or cardiac surgery. Davis et al. (5) reported on four cases of endocarditis that occurred after surgery for repair of heart valve defects. They repeatedly isolated diphtheroids (identified by the SBS lab as group JK cultures) from the blood of these patients. Each of the cases was characterized by the development of congestive failure caused by destruction of the surgical repair. Van Scoy et al. (32) discussed three group JK bacterial cultures isolated from patients with endocarditis who had prosthetic valves. They found that these microorganisms produced multiple positive blood cultures and were very resistant to most of the antibiotics commonly used for gram-positive bacterial therapy. The severity of this type of endocarditis is evident, since of the seven cases reported in the literature some required a second operation and five of the seven died.

Moreover, 13 additional group JK cultures, referred to the SBS, were isolated repeatedly (4 to 12 times) from blood specimens taken at intervals from patients. Hande et al. (12) observed four cases of sepsis caused by a "corynebacterium species" (these cultures were identified by the SBS as group JK cultures). Clinical features of each case indicated infection, and multiple isolations were made from blood samples drawn at least 1 day apart. Furthermore, these bacteria were isolated from multiple abscesses noted in the liver, spleen, lungs, and

spine in one patient. Each of the patients was clinically comprised at the time of the infection. Three of the patients suffered from leukemia in relapse, and the fourth patient had a porencephalic cyst with a ventriculoatrial shunt in place.

The cases cited in the literature (5, 12, 32) that concern the group JK bacteria establish the fact that these microorganisms may produce serious and even fatal infections. They are particularly hazardous to patients suffering from terminal debilitating diseases (12; J. D. Meyers, personal communication). In addition, these microorganisms are difficult to detect in blood cultures. Blood samples initially drawn may be negative. Moreover, their antimicrobial agent resistance creates problems in successfully treating these infections. Vancomycin has been suggested as the treatment of choice for group JK diphtheroid endocarditis (12, 32). Our findings that all 61 strains tested were susceptible to vancomycin support this suggestion.

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