JOURNAL OF CLINICAL MICROBIOLOGY, July 1994, p. 1654–1657
0095-1137/94/$04.00+0

Vol. 32, No. 7

Biotypes of *Arcanobacterium haemolyticum*

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Received 3 January 1994/Returned for modification 22 February 1994/Accepted 8 April 1994

Colony morphology, beta hemolysis on horse blood agar, β-glucuronidase activity, and ability to ferment sucrose and/or trehalose defined two biotypes of *Arcanobacterium haemolyticum*. One, the smooth type, grew as smooth, beta-hemolytic colonies and was β-glucuronidase negative but often fermented sucrose and/or trehalose, while the other, the rough type, grew as rough colonies and was nonhemolytic, β-glucuronidase positive, and negative for sucrose and trehalose fermentation. About 75% of the *A. haemolyticum* strains studied (n = 138) were of the smooth type. The smooth type predominated in wound infections, while the rough type was isolated almost exclusively from respiratory tract specimens; thus, 84% of the smooth-type strains were derived from wounds and 91% of the rough-type strains were isolated from respiratory tracts.

The clinical importance of *Arcanobacterium haemolyticum*, an aerobic, slowly growing, catalase negative, gram-positive rod, has been recognized since the early 1940s (2, 11). Recently, it has been isolated not only from patients with tonsillitis but also from patients with other infections (13).

The criteria used to identify *A. haemolyticum* are well established (1, 7). A report on the effects of media, atmosphere, and incubation time on *A. haemolyticum* colony morphology has recently been published (3). We found that colony morphology and beta hemolysis on horse blood agar varied with individual *A. haemolyticum* isolates, and the colonies were of either the smooth or the rough type. The colony types were found to correlate with two biotypes. The two biotypes are described in this report.

MATERIALS AND METHODS

Bacterial strains. The *A. haemolyticum* strains studied (n = 138) were clinical isolates collected by our laboratory and other Finnish clinical microbiology laboratories between 1989 and 1992. The strains were isolated from blood agar plates, identified as described by Krech and Hollis (7), and preserved in skim milk at −70°C until used. Thirty-six of the isolates were from throats, 6 were from peritonsillar abscesses, 3 were from maxillary sinuses, 92 were from wounds, and 1 was from a blood culture. Patients with tonsillar infections from whom *A. haemolyticum* was isolated were usually young adults (mean age, 20.0 ± 0.9 years; range, 3.4 to 37.1 years), while patients with wound infections were older than the patients with tonsillar infections. As a control strain, *A. haemolyticum* ATCC 9345 was used.

Culture. *A. haemolyticum* strains were cultured on horse blood agar plates (4% Trypticase Soy Agar II [BBL Microbiology Systems, Cockeysville, Md.] with 6% defibrinated horse blood) for 48 h at 35°C in a humidified atmosphere of 5% CO_2 in air.

Identification and biotyping methods. Colony morphology and beta hemolysis were recorded after 48 h of growth on horse blood agar plates. Identification of *A. haemolyticum* was based on criteria described by Krech and Hollis (7). The tests used were Gram stain, catalase activity, nitrate reduction, urease activity, gelatin hydrolysis, motility, reverse CAMP, and fermentation of glucose, maltose, sucrose, mannitol, and xylose. In addition, API Staph (BioMerieux, Marcy-l'Etoile, France) identification strips were used. The following tests not included in those suggested by Krech and Hollis are included in API Staph: fructose, mannose, lactose, trehalose, xylitol, melibiose, and raffinose fermentation; α-methyl-D-glucoside and *N*-acetylglucosamine utilization; nitrate reduction; alkaline phosphatase activity; Voges-Proskauer reaction; and arginine dihydrolase and urease activities. For API Staph, bacterial suspensions with McFarland no. 4 turbidity were used. The results were read after overnight incubation at 35°C in ambient air. In addition, a β-glucuronidase test with a β-glucuronidase diagnostic tablet (*p*-nitrophenyl-β-D-glucuronic acid; final concentration, 1.4 mg/ml; Rosco Diagnostica, Taastrup, Denmark) added to 0.25 ml of a bacterial suspension with MacFarland no. 4 turbidity was performed. The tubes were incubated for 4 h in air at 35°C. A distinct yellow color was interpreted as a positive reaction in the β-glucuronidase test. Some strains (n = 51) were also tested with API Coryne (BioMerieux) identification strips.

Electron microscopy. For electron microscopy, bacteria obtained from horse blood agar plates after 48 h of incubation were prefixed with 2.5% buffered glutaraldehyde for 2 h at room temperature and then centrifuged at room temperature (10,000 × *g*, 30 s; Eppendorf 5412 centrifuge; Eppendorf Gerätebau, Hamburg, Germany). The supernatant was discarded, and the samples were stained with tannic acid, washed twice with phosphate buffer (pH 7.3), and sliced into thin sections as previously described (9). Electron micrographs of thin-sectioned specimens were taken with a JEM-1200EX transmission electron microscope (JEOL, Tokyo, Japan) at 60 kV. Negative staining was done with 2% phosphotungstic acid, and the cells were studied with a JEM-100CX transmission electron microscope (JEOL) at 60 kV.

RESULTS

Culture characteristics. Smooth and rough colony types were found on horse blood agar plates after 48 h of incubation. Each smooth colony had a smooth, glistening surface and an entire edge and was surrounded by a zone of moderate-to-strong beta hemolysis. Each rough colony had a rough surface

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TABLE 1. Properties of the two biotypes of *A. haemolyticum*[a]

Biotype	No. of strains	β-hemolysis	No. (%) of strains β-glucuronidase positive[b]	No. (%) with following API Staph reactivity:		
				Sucrose positive	Trehalose positive	Sucrose and/or trehalose positive
Smooth	106	Moderate to strong	0 (0)	43 (41)	51 (48)	67 (63)
Rough	32	Absent to weak	31 (97)	0 (0)	0 (0)	0 (0)

[a] In tests given by Krech and Hollis (7), the percentages of the smooth/rough types positive were as follows: nitrate reduction, 0/0; urease activity 0/0; gelatin hydrolysis, 0/0; motility, 0/0; reverse CAMP, 100/100; glucose fermentation, 100/100; maltose fermentation, 99/100; sucrose fermentation, 53/0; mannitol fermentation, 0/0; xylose fermentation, 0/0. In API Staph, the positive percentages were as follows: glucose fermentation, 100/100; fructose fermentation, 99/100; mannose fermentation, 99/88; maltose fermentation, 100/100; lactose fermentation, 100/100; mannitol fermentation, 0/0; xylitol fermentation, 0/0; melibiose fermentation, 0/0; nitrate reduction, 0/0; alkaline phosphatase activity, 76/47; Voges-Proskauer reaction, 0/0; raffinose fermentation, 3/0; xylose fermentation, 0/0; α-methyl-D-glucoside utilization, 0/0; *N*-acetylglucosamine utilization, 94/100; arginine dihydrolase activity, 0/0; urease activity 0/0.

[b] By diagnostic tablets.

and an irregular edge. Beta hemolysis was either absent or very weak, and a dark discoloration around the colony was frequently noted. Of the clinical isolates, 106 (77%) were of the smooth type and 32 (23%) were of the rough type.

Biochemical differencies between strains of the smooth and rough colony types. The biochemical reactivities of smooth and rough colony type strains in the tests described by Krech and Hollis (7) were similar (see Table 1, footnote a), except that about half of the smooth colonies but none of the rough colonies fermented sucrose. All of the strains growing as smooth colonies were β-glucuronidase negative, while 31 (97%) of 32 of the strains growing as rough colonies were β-glucuronidase positive. Neither sucrose nor trehalose was fermented by the strains with rough colonies, while most of the smooth-type strains fermented sucrose and/or trehalose in API Staph (Table 1). The API Staph sucrose fermentation test was easier to interpret than the Krech and Hollis tube test but gave a lower percentage of positives, 41% (43 of 106) versus 53%

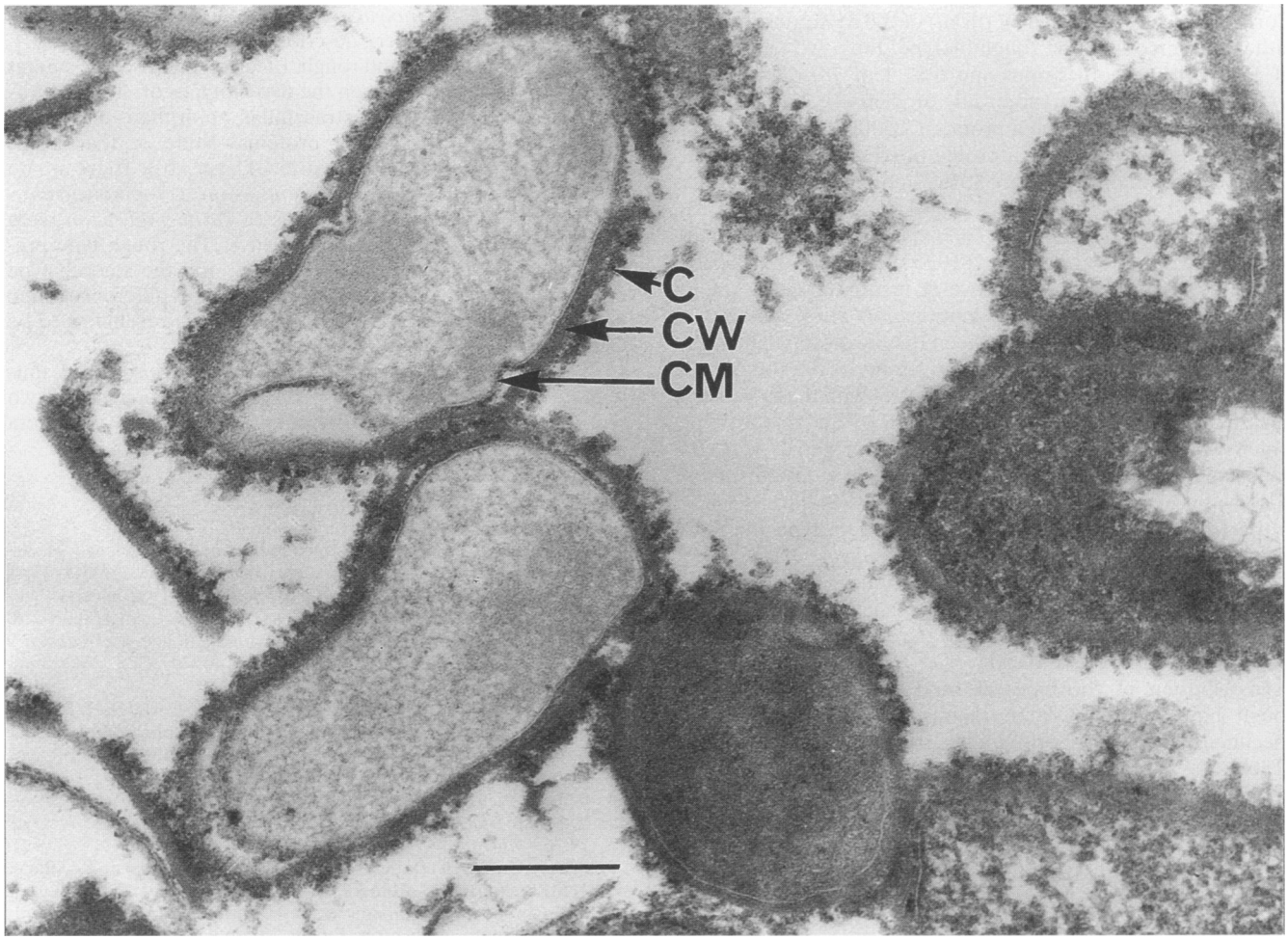

FIG. 1. Transmission electron micrograph of thin-sectioned cells of *A. haemolyticum* isolated from blood. CM, cytoplasmic membrane; CW, cell wall; C, extracellular (capsular) material. Bar, 0.2 μm.

TABLE 2. Frequencies of *A. haemolyticum* biotypes in different infections

Type of infection	Total no. of isolates	No. (%) with biotype	
		Smooth	Rough
Respiratory			
All	45	16 (36)	29 (64)
Tonsillitis	36	13 (36)	23 (64)
Peritonsillar abscess	6	1 (17)	5 (83)
Maxillary sinusitis	3	2 (67)	1 (33)
Wounds	92	89 (97)	3 (3)
Septicemia	1	1 (100)	0 (0)
Total	138	106 (77)	32 (23)

(56 of 106) (no significant difference by the chi-square test). The ability to grow as the smooth or rough colony type did not change upon subculture and storage (a minimum of five subcultures per strain). Likewise, β-glucuronidase activity and the ability to ferment sucrose did not change. The colonies formed by the type strain *A. haemolyticum* ATCC 9345 were smooth and surrounded by beta hemolysis. The strain was β-glucuronidase negative, fermented trehalose, but failed to ferment sucrose. It thus belonged to the smooth type. API Coryne correctly identified 47 (92%) of the 51 strains tested as either the smooth or the rough biotype. Fifty-two percent (17 of 33) of the smooth strains and 6% (1 of 18) of the rough strains gave an API Coryne profile of 2530360; 27% (9 of 33) and 0%, respectively, gave a profile of 2530361; 15% (5 of 33) and 0%, respectively, gave a profile of 6530361; 3% (1 of 33) versus 0% gave a profile of 6530360 and 3% (1 of 33) versus 6% (1 of 18) gave a profile of 2730361; 0 and 72% (13 of 18), respectively, gave a profile of 2730360; and 0 and 6% (1 of 18), respectively, gave a profile of 2510360, 2710360, or 3730360. The β-glucuronidase and sucrose fermentation tests are included in the API Coryne identification strip. Discrepant β-glucuronidase test results between the diagnostic tablets and API Coryne were obtained with two strains of the rough type, which both tested β-glucuronidase positive with the tablets and negative by API Coryne, and one strain of the smooth type, which was positive by API Coryne but negative with the diagnostic tablet. One strain of the rough type tested sucrose positive by API Coryne but negative by API Staph.

Electron microscopic studies. Three smooth-type and three rough-type strains, as well as *A. haemolyticum* ATCC 9345, showed similar extracellular material surrounding the cell walls (Fig. 1). The biotypes could not be distinguished on the basis of conventional thin-sectioning and negative-staining techniques. None of the strains had an S layer.

Distribution of the biotypes in different infections. The rough biotype was isolated mainly from respiratory tract specimens, and the smooth biotype came mainly from wounds (Table 2). Of the 32 rough-type isolates, 29 (91%) were derived from respiratory tract specimens while 89 (84%) of the 106 smooth-type isolates were from wounds. The blood culture isolate was of the smooth type. The blood culture strain was from a 45-year-old diabetic male with an amputation of a gangrenous foot. *Streptococcus agalactiae* was simultaneously isolated from the blood, and both organisms were cultured from a tissue specimen from the affected foot, together with *Staphylococcus aureus* and *Bacteroides fragilis*.

DISCUSSION

Colony morphology and beta hemolysis on horse blood agar divided clinical *A. haemolyticum* isolates into smooth and rough colony types. *A. haemolyticum* has been isolated from both sheep blood agar plates and horse blood agar plates (3, 4, 13). In this study, the isolates were from horse blood agar plates and were easily recognized after 48 h of incubation in a CO_2-enriched atmosphere. The growth of *A. haemolyticum* on sheep blood agar plates and horse blood agar plates was equally good upon subcultures, but the differences in colony morphology and hemolysis of the two biotypes were easier to interpret with horse blood agar plates. The smooth and rough colony morphologies correlated with two biotypes. The Krech and Hollis criteria did not differentiate between the two types of *A. haemolyticum*, but the two biotypes could be defined by β-glucuronidase activity and the ability to ferment sucrose and/or trehalose. β-Glucuronidase activity alone correctly identified 99% of the *A. haemolyticum* strains as the smooth or rough biotype. *A. haemolyticum* has previously been reported to be β-glucuronidase negative. The discrepancy may be due to the different substrates, such as 7-bromo-3-hydroxy-2-naphtoic-*o*-aniside-β-D-glucuronide rather than *p*-nitrophenyl-β-D-glucuronic acid, used to test β-glucuronidase activity (8, 12). The API Coryne system, which has been shown to identify *A. haemolyticum* correctly (5, 6), seems also to be able to differentiate between the two biotypes.

Biotypes of *A. haemolyticum* have not been reported before. Fell et al. (4), however, described two colony types of *A. haemolyticum*, smooth and rough. Electron microscopic studies did not differentiate between the two biotypes of *A. haemolyticum*: both biotypes had extracellular amorphous polymeric material containing at least proteins. None of the strains possessed a paracrystalline S (surface) layer (10). There are no previous electronic microscopic studies on *A. haemolyticum*.

The smooth and rough biotypes of *A. haemolyticum* were isolated from different anatomical sites. The rough type predominated in respiratory tract isolates, and the smooth type predominated in wound isolates. Whether this difference is due to, for instance, different adherence properties remains to be established.

The smooth and rough biotypes of *A. haemolyticum* thus seemed to preferentially cause different infections. The two biotypes differed not only by colony morphology and beta hemolysis but also by β-glucuronidase activity.

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