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NOTES

A Simplified Leifson Flagella Stain

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Flagella of bacteria taken directly from 24- and 48-h blood agar plates can be stained by using a simple, reliable procedure.

Laboratories engaged in bacterial identification frequently shy away from flagella staining because the procedure commonly used is time consuming. However, determining flagellar morphology is helpful and often necessary in identifying bacteria. Flagella of bacteria taken directly from 24- and 48-h blood agar plates can be stained by a simple and reliable procedure, based on Leifson's method (1).

A 1.2% solution of basic fuchsin, special for flagella staining (Matheson, Coleman and Bell, Norwood, Ohio, or Eastman Kodak Co., Rochester, N.Y.), prepared in 95% ethyl alcohol and left overnight at ambient temperature (about 25 C), was mixed with an equal volume of a solution of 0.75% NaCl and 1.5% tannic acid (J. T. Baker "Analyzed Reagent," Phillipsburg, N.J.) prepared in distilled water. The final pH of the stain (Matheson, Colman and Bell) was found to be below the optimal (1), so it was adjusted carefully (to minimize addition of ions) with 1 N NaOH to pH 5.0. Fifty-milliliter portions of the stain were stored frozen in tubes with the caps tightened. Once thawed and remixed (the water and alcohol separate during freezing) the stain was kept at 4 C until used. Freshly prepared stain works better if kept 2 to 3 days at 4 C. Frozen stain is stable indefinitely; thawed stain is stable for about 1 month at 4 C.

The flagella stain will not work unless slides are scrupulously clean. New "precleaned" slides were soaked for 4 days at ambient temperature in acid dichromate solution (dissolve 60 g of $K_2Cr_2O_7$ in 300 ml of water; add 252 ml of concentrated H_2SO_4 cautiously, with mixing, holding Pyrex glass mixing container in cold water to dissipate heat); for soaking, they were placed in glass racks in covered staining jars (cleaner turns green when spent). Slides were rinsed in 10 changes of tap water and then in two changes of distilled water and were then air-dried. They were handled with forceps and stored in a covered container.

Slides also could be cleaned satisfactorily by substituting 3% concentrated HCl in 95% ethyl alcohol for acid dichromate solution in the above procedure.

Cultures were plated on Columbia agar (Baltimore Biological Laboratory) containing 5% rabbit blood. Aerobes were incubated in candle jars at 35 C for 18 to 24 h. Anaerobes were grown in GasPak anaerobic jars (BioQuest, Cockeysville, Md.) for 24 to 48 h. A small amount of growth, equivalent to a 1-mm colony, was picked with a needle, avoiding the agar, and transferred to a test tube containing 3 ml of distilled water. The growth was emulsified in a droplet of water against the side of the tube. The droplet of suspension then was mixed with the water to produce a faint opalescence.

The plate cultures were kept for an additional 24 h at 25 C in air, and the procedure was repeated.

For controls, a longer procedure known to be reliable was followed. Colonies from blood agar were subcultured to motility agar (motility medium [Difco], 16 g; nutrient broth (Difco), 4 g; NaCl, 1 g; distilled water, 1 liter) and incubated for 18 to 24 h at 25 C. Then motile subcultures were selected from the periphery of the growth, transferred to flagella broth (tryptose [Difco], 10 g; K_2HPO_4, 1 g; NaCl, 2.5 g; distilled water, 1 liter, pH 7), and incubated for 18 to 24 h at 25 C. The flagella broth cultures were formalinized by adding 0.05 ml of 37% formaldehyde solution per 1 ml of culture, centrifuged, washed with distilled water, recentrifuged, and then suspended in sufficient distilled water to give a faint opalescence.

In preparing the slide, the side for the smear was passed slowly back and forth through the tip of a blue Bunsen flame until extremely hot, thus burning off any insoluble residue from cleaning. (A yellow flame will deposit carbon on the slide, rendering it unusable.) The slide was cooled, flamed side up, on a paper towel. A thick wax pencil line was drawn across the width of the slide to contain stain in an area 1 by 1 3/4 inches (ca. 2.5 by 4.5 cm).

A large loopful of culture suspension was

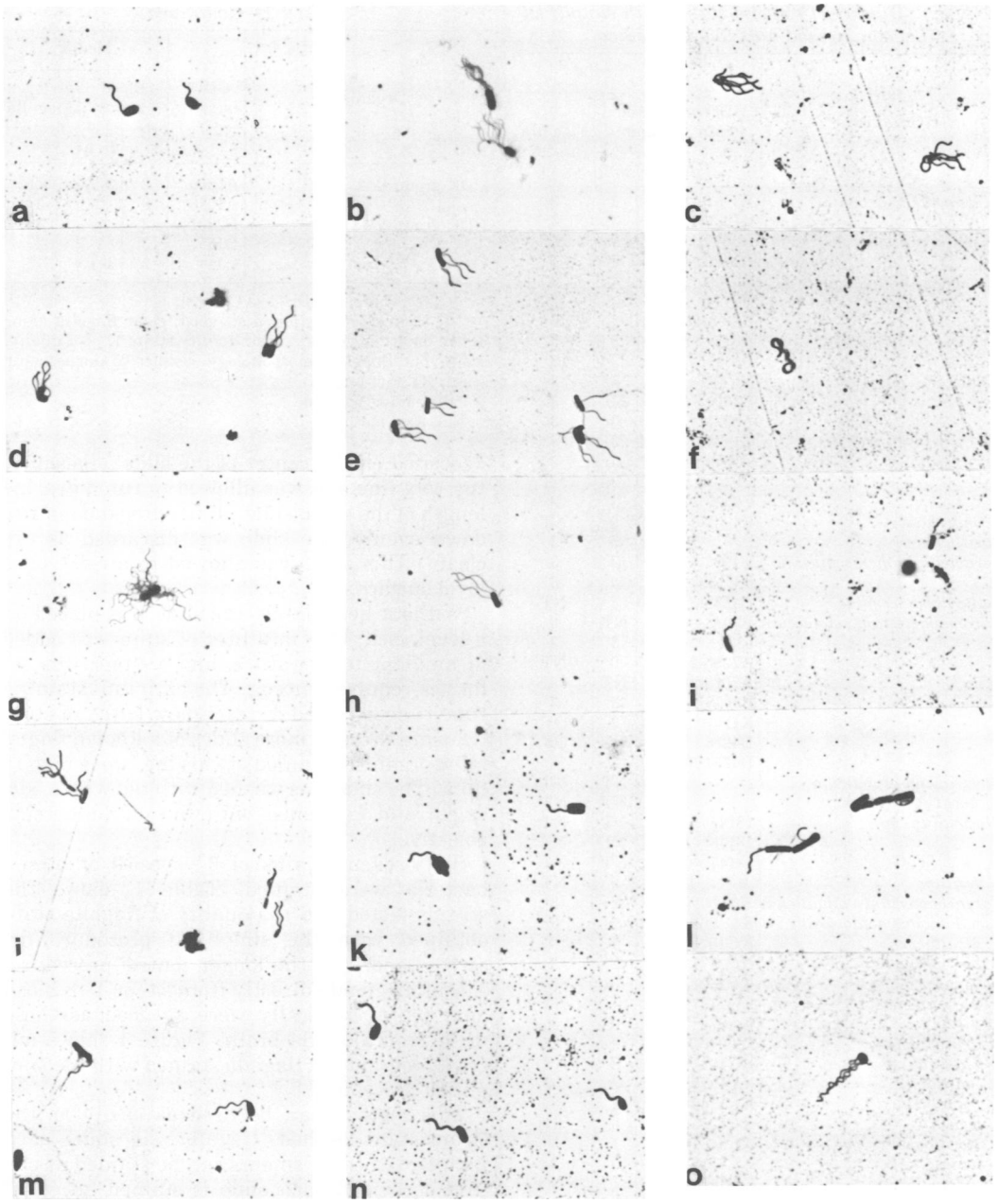

FIG. 1. *Microphotographs (×1,150) of bacteria stained with simplified Leifson method: (a) Aeromonas hydrophila; (b) Plesiomonas shigelloides; (c) Alcaligenes denitrificans; (d) A. faecalis; (e) Bordetella bronchiseptica; (f) Campylobacter foetus; (g) Clostridium novyi; (h) C. tertium; (i) Pseudomonas diminuta; (j) P. maltophilia; (k) P. mendocina; (l) P. pseudoalcaligenes; (m) P. stutzeri; (n) Vibrio cholerae; (o) V. parahaemolyticus.*

TABLE 1. *Quality of flagella stains with simplified Leifson method and control method*

Organism	CDC[a] strain	Media[b]		
		Blood agar[c]		Flagella broth[d]
		24 h	48 h	
Aeromonas hydrophila	B3932	3	3	3
	KC1343	3	3	3
	A8647	3	3	NT
	A1170	3	3	3
Plesiomonas shigelloides	B3196	3	3	3
	A9774	3	3	3
	KC1342	2	3	3
	A712	3	3	3
	B5331	3	3	3
Alcaligenes denitrificans	KC367	2	3	3
	B3500	2	NT	3
	A6224	2	3	3
	C1139	3	3	3
A. faecalis	C1549	3	3	2
	B6353	3	3	NT
	C2027	3	3	2
A. marshallii	KC1339	2	0	NT
Bacillus sp.		3	2	3
Bordetella bronchiseptica	D1977	3	3	3
	KC1390	3	3	3
Campylobacter fetus	D4533	2	3	NT
Chromobacterium violaceum	B3033-2	2	NT	3
	9334	3	3	3
	A7296-2	0	0	NT
	A7019-2	3	NT	3
	B3033-1	3	3	3
	KC531	3	3	0
	D4286	3	2	0
	D4754	3	3	3
Clostridium glycolicum		2	NT	NT
C. novyi A		3	NT	NT
Clostridium sp.	18145	0	NT	NT
	18146	1	NT	NT
	18147	1	NT	NT
C. tertium		3	3	NT
Corynebacterium aquaticum	KC680	2	2	3
Pseudomonas acidovorans	KC1069	0	2	3
P. alcaligenes	KC1075	3	1	3
P. cannabina	KC1177	3	3	3
P. cepacia	KC1371	2	0	3
P. coronafaciens	KC1187	2	1	NT
P. diminuta	KC679	3	0	3
P. denitrificans	D2993	3	3	3
P. maltophilia	KC1078	3	3	NT
P. marginata	KC1370	2	2	2
P. mendocina	KC1140	3	3	3
P. pickettii	KC1295	1	0	1
P. pseudoalcaligenes	KC945	3	1	3
P. pseudomallei	KC871	3	3	3
Pseudomonas sp.	C1700	2	1	3
P. stutzeri	KC1077	3	2	3
P. testosteroni	KC1071	3	1	3
P. vesicularis	KC944	1	2	3
Vibrio alginolyticus	D2276	3	2	1
V. cholerae	A3835	3	3	3
	A1258	2	3	3
	D927	2	3	2
V. extorquens	B8126	3	1	0
	D1911	0	2	NT
	B4139	0	0	NT
	C3740	0	NT	NT
V. parahaemolyticus	D3942	3	2	3
CDC group IV-C (2)	A5366	2	NT	NT
	A1036	3	2	3
CDC group V-D	D2701	2	2	3
	D1857	3	2	2
	C349	2	NT	1

[a] CDC, Center for Disease Control.

[b] 3, High quality stain, i.e., good, clear flagella along entire length of smear; 2, fair quality stain; 1, poor quality stain, but flagellar morpohology visible; 0, unsuccessful stain; NT, not tested.

[c] Simplified method.

[d] Control method.

deposited on the center of the slide adjacent to the wax line and was allowed to run down the length of the tilted slide. (If the drop did not run down evenly, the slide was discarded as unclean.) This slide was allowed to air-dry on a level surface.

Without heat-fixing, the slide was placed on a level rack. One milliliter of stain was added at ambient temperature, and a timer was set for the required timing. The optimum staining time is determined for each new batch of stain by using three or more slides of a known flagellated organism stained at varying times (5 to 15 min). The slide was washed with tap water, air-dried, and examined with the oil immersion objective.

Sixty-seven strains of 37 species of motile bacteria were stained. Table 1 shows the strains tested and the quality of flagella stain obtained with the simplified procedure described and with the longer control procedure. The stains made directly from 24- or 48-h blood agar plates generally were as good as those made from flagella broth. Figure 1 shows microphotographs of flagella stained with the simplified procedure.

This method can be used as easily as the Gram stain method. Usually, six slides each with two or three smears can be stained at one time, provided each slide is stained an equal amount of time. Thus, satisfactory flagella stains can be prepared directly from 24- to 48-h plates.

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LITERATURE CITED

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