Simplified Silver-Plating Stain for Flagella

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Rhodes' silver-plating technique for staining flagella was tested for its reliability and convenience as a routine procedure in the clinical laboratory. Modifications were made in the stain preparation and the procedure of staining and were tested with smears of known motile gram-negative nonfermentative bacilli. The stain has proved to be accurate and reliable and can be easily utilized with a minimum of training.

Determination of flagellar arrangement by staining has long been advocated as a tool in the identification of bacteria, especially with regard to certain gram-negative nonfermentative bacilli (1, 6, 11, 13). The most well-known method for flagella staining is based on Leifson's stain (7), a fuchsin-tannic acid preparation. Variations of Leifson's method have been published recently (5, 9, 12, 15) but are still time consuming and require careful preparation. It is usually recommended that slides be scrupulously cleaned in potassium dichromate solution and flamed just before use and that a precise area be ringed with a wax pencil. The stain must be carefully prepared and adjusted to a certain pH and left to stand overnight before it can be used. The method for growing and harvesting the bacteria for the best production and preservation of flagella has been simplified, but an initial dilution step is still recommended. A measured amount of stain must be added to each slide, and the optimum staining time must first be determined on trial smears before the actual unknown can be stained. Because of these drawbacks, many diagnostic laboratories have chosen not to use the flagella stain as a routine procedure, thereby forfeiting what could be a valuable tool for bacterial identification.

An alternate method for flagella staining has been published by Rhodes (10), who modified Fontana's silver-plating stain for spirochetes (3). It essentially consists of a ferric-tannate mordant followed by a heated ammoniacal silver nitrate solution, which forms a dark precipitate on the bacteria and flagella. We further refined Rhodes' procedure by eliminating the precleaning of slides, the dilution step of the organism into distilled water, and the use of a saturated aqueous solution of aniline as a portion of the mordant. The purpose of this study was to eval-

uate these modifications by rating the stain quality of smears made from known cultures of gram-negative nonfermentative bacilli. The ability to stain flagella of organisms grown on various media, from Formalin-treated cultures, and without heating the silver nitrate solution was also studied. To determine the stain's reliability as an aid in the identification of bacteria in the clinical laboratory, smears of 43 known cultures of gram-negative nonfermentative bacilli were rated for flagellar arrangement and stain quality.

MATERIALS AND METHODS

Preparation of stain. Solution 1 (mordant) is made by mixing 25 ml of a saturated aqueous solution of aluminum potassium sulfate, 50 ml of a 10% tannic acid solution, and 5 ml of a 5% ferric chloride solution. The resulting mixture turns black and is stored in the dark at 5°C.

Solution 2 (stain) is made by slowly adding concentrated ammonium hydroxide (ca. 2 to 4 ml) to 90 ml of a 5% silver nitrate solution until the brown precipitate formed just redissolves. More 5% silver nitrate is then added dropwise to the solution until a faint cloudiness persists; this takes from 2 to 20 ml. The stain is stored in the dark at 5°C.

Preparation of bacteria for staining. Two stock cultures of each type of flagellation were used: peritrichous, *Alcaligenes faecalis* and *Bordetella bronchiseptica*; monotrichous, *Pseudomonas aeruginosa* and *Pseudomonas stutzeri*; and multitrichous, *Pseudomonas maltophilia* and *Pseudomonas putida*. Each culture was inoculated into a tube of semisolid motility medium, onto a sheep blood agar plate, and onto a Trypticase soy agar (TSA) slant. The motility medium and TSA slant were incubated at 25°C, and the blood agar plate was incubated at 35°C, for 18 to 24 h. Smears were made on slides precleaned only by the manufacturer and wiped with lens paper before use. Four inoculating loopfuls of sterile distilled water were put on each slide. An inoculating needle was used to collect the organisms from the original stab line in the motility medium and gently used, so as not to

break off flagella, to spread the suspension over the slide surface. Another smear from the motility medium was made in the same manner, but 0.5% Formalin-treated distilled water was used on the slide. Smears were also made from isolated colonies taken from blood agar plates. For TSA slants, one inoculating loopful of organism suspension was taken from the liquid that forms at the base of the slant and mixed with the water on the slide. Rhode's method (10) of a dilution step was also used, in which 2 loopfuls of organism suspension from the TSA slant were added to 5 ml of sterile distilled water and left for 1 h at room temperature. Two loopfuls of this dilution were transferred to a slide. All slides were air dried.

Staining procedure. Each smear was covered with solution 1 (mordant) for 4 min. The slide was then rinsed gently with distilled water and covered with solution 2 (stain). Heat was applied until steam formed, and the stain was allowed to remain on the slide for 4 min. The slide was rinsed gently with distilled water while held horizontally to float off any loose precipitate and then air dried.

Staining known cultures. Forty-three known cultures of gram-negative nonfermentative bacilli representing 17 species were used: 20 organisms with monotrichous flagella; 13 with multitrichous flagella; and 10 with peritrichous flagella. Flagellar arrangement for each culture was based on the pattern listed in the *Manual of Clinical Microbiology* (6, 13). Cultures were coded so that the identification of the organisms was unknown throughout the testing procedure. Each culture was inoculated into motility medium and incubated at 25°C for 18 to 24 h. Smears were then prepared and stained as described above. A duplicate smear was made from each culture and stained without the application of heat.

Quality rating of stained smears. All stained smears were rated by a numbering system of 1 to 5: 1, bacteria but no flagella seen; 2, few flagellated forms seen and flagella morphology poor; 3, few flagellated forms seen but flagella morphology good; 4, many flagellated forms with good morphology seen but only on isolated portions of the smear; 5, many flagellated forms with good morphology seen over the entire smear.

RESULTS

Table 1 shows the flagella staining results of organisms grown on different media, diluted with distilled water, or fixed with Formalin. Organisms growing in motility medium or on TSA slants had the highest ratings, both with a mean of 4.8, for quality of stained flagella. The flagella and their position on the bacteria were easily discernible (Fig. 1, 2, and 3). Formalin-treated smears from motility medium had similar stain quality ratings, with a mean of 4.3. Growth from blood agar plates resulted in a mean stain quality of 3.3, whereas that of the dilution step was 2.8.

The results of staining 43 known gram-negative nonfermentative bacilli are recorded in Table 2; ratings of the stain quality of duplicate smears with and without the application of heat to the stain and the type of flagellar arrangement seen on each smear are listed. The smears stained with the use of heat had a mean stain quality of 4.2, and flagellar arrangement was correctly determined in all smears but one culture of *Pseudomonas putrefaciens*, in which stained bacteria were seen but no flagella were discernible. Those smears stained without the use of heat had a mean of 3.2, and flagellar arrangement was correctly determined 37 times. In the other six smears the stain quality was 1 (bacteria stained but no flagella seen); the organisms involved were two cultures of *P. putrefaciens*, two cultures of *Pseudomonas diminuta*, and one culture each of *P. maltophilia* and *B. bronchiseptica*.

DISCUSSION

Since the publication of Rhodes' modification of Fontana's silver-plating stain, several reports (2, 8, 14) have indicated that a silver staining method is reliable for the detection of bacterial flagella. Nevertheless, in our experience only a

TABLE 1. Comparison of flagella stain quality^a based on growth from different media,^b a dilution step,^c and Formalin treatment^d of the culture

Organism	Motility medium	Blood agar plate	TSA slant	Dilution	Formalin treatment
<i>A. faecalis</i>	5	5	5	5	5
<i>B. bronchiseptica</i>	5	4	4	3	4
<i>P. aeruginosa</i>	5	4	5	3	4
<i>P. stutzeri</i>	5	5	5	2	5
<i>P. maltophilia</i>	4	1	5	2	4
<i>P. putida</i>	5	1	5	2	4
Mean	4.8	3.3	4.8	2.8	4.3

^a For quality rating, see Materials and Methods.

^b All the cultures were incubated for 18 to 24 h, the motility medium and TSA slants were incubated at 25°C, and the blood agar plates were incubated at 35°C.

^c Growth from the TSA slant was added to sterile distilled water, and smears were made from this suspension.

^d Growth from the motility medium was inoculated into 0.5% Formalin-treated water on the slide.

FIG. 1. *P. aeruginosa* with monotrichous flagella stained by the modified silver-plating technique.

FIG. 2. *P. putida* with multitrichous flagella stained by the modified silver-plating technique.

few of the laboratories we have contacted in the past several years have routinely used this or any procedure for flagella staining, although it can be an important aid in the identification of bacteria. With further refinements, we found the silver-plating stain to be an easy and reliable method for flagella staining. Preparation of the mordant and stain is relatively fast and simple compared with Leifson's method (7), since pH adjustment and overnight standing to dissolve the stain are not necessary. Furthermore, both staining solutions are stable for at least 6 months if kept in the dark at 5°C. The stained slides have also shown no signs of deterioration even

with repeated applications of immersion oil and subsequent cleaning with xylene. Precleaning of slides was found to be unnecessary, although both Leifson's (7) and Rhodes' (10) procedures include this step. Any slides reasonably cleaned by the manufacturer were adequate and needed only to be wiped with lens paper before use. Also, an initial dilution step, recommended by both Leifson (7) and Rhodes (10), in which the organisms are suspended in sterile distilled water was found to be unnecessary and was eliminated. Instead, the dilution obtained by adding bacteria collected with an inoculating needle to several loopfuls of sterile distilled water on a slide pro-

FIG. 3. *A. faecalis* with peritrichous flagella stained by the modified silver-plating technique.

vided a light enough smear so that individual organisms could be observed. Leifson's method (7) requires that the optimum staining time must first be determined on trial smears before the actual unknown can be stained, whereas Rhodes' method (10) does not require trial staining and each solution is applied for 3 to 5 min. Before beginning this study, smears stained for 3, 4, and 5 min were examined, and no difference in stain quality was noted; therefore, 4 min was used throughout the study. Also, Rhodes added a saturated solution of aniline to the mordant to act as a stain accentuator (4). We have eliminated the aniline from our formula without affecting the staining ability of the silver nitrate solution.

The most convenient media for growing organisms to be stained for flagella may vary depending on laboratory preference. Growth from semisolid motility medium or TSA slants incubated at 25°C for 18 to 24 h proved most effective in this study, producing quality ratings of 4 or 5 for each culture tested. Growth from blood agar plates incubated at 35°C produced poor staining results; although the mean was 3.3, no flagella were discernible in smears from *P. maltophilia* and *P. putida*. We therefore feel that bacteria taken from a primary isolation medium, such as blood agar, generally incubated at 35 to 37°C, may not always be suitable for flagella staining. The dilution step produced ratings of less than 3 in half of the organisms tested, indicating that not only was this step unnecessary, but it reduced the effectiveness of the stain. Formalin treatment of the organisms did not seem to aid in preservation of the flagella or adversely affect

the stain quality and could be used if preferred.

To determine if heat was necessary in the staining process, duplicate smears from each of the 43 known cultures of gram-negative nonfermentative bacilli were stained with and without the application of heat to the silver nitrate solution. In all instances except one, a culture of *P. stutzeri*, the quality ratings of the smears stained with the use of heat were higher than or equal to the ratings of the smears stained without the use of heat. Also, stain ratings of less than 3 were given to 10 (23%) of the smears stained without heat, whereas only 4 (9%) of the smears stained with the use of heat had ratings of 1 or 2, indicating that the silver nitrate solution should be heated to steaming to maximize the silver-plating effect.

When testing the reliability of the stain, the correct flagellar arrangement was determined for all cultures with the exception of one culture of *P. putrefaciens*. Since this was a very mucoid organism and was given a stain rating of 1, the dilution step was used to disperse the mucoid material so that the flagella could be stained, but again flagella were not seen. Three other cultures produced stain ratings of 2: *P. aeruginosa*, *P. diminuta*, and another culture of *P. putrefaciens*. Although this rating of 2 (few flagellated forms seen and flagella morphology poor) connotes an unreliable stain, the correct flagellar arrangement was still determined in each case. Therefore, correct flagellar arrangement in smears stained with the use of heat was determined in 42 of the 43 known cultures, and 39 smears, or 91%, had stain ratings of 3 or above. This indicates that the modified silver-plating

TABLE 2. Results of the silver-plating flagella stain with 43 known cultures^a

Organism	Stain quality with heat	Type of flagella seen	Stain quality without heat	Type of flagella seen
Monotrichous flagellates (M)				
<i>P. aeruginosa</i>	4	M	4	M
<i>P. aeruginosa</i>	3	M	3	M
<i>P. aeruginosa</i>	5	M	3	M
<i>P. aeruginosa</i>	5	M	3	M
<i>P. aeruginosa</i>	5	M	3	M
<i>P. aeruginosa</i>	5	M	3	M
<i>P. aeruginosa</i>	4	M	3	M
<i>P. aeruginosa</i>	4	M	3	M
<i>P. aeruginosa</i>	2	M	2	M
<i>P. stutzeri</i>	5	M	4	M
<i>P. stutzeri</i>	4	M	5	M
<i>P. stutzeri</i>	5	M	3	M
<i>P. diminuta</i>	5	M	2	M
<i>P. diminuta</i>	2	M	1	—
<i>P. diminuta</i>	3	M	1	—
<i>P. alcaligenes</i>	3	M	3	M
<i>P. pseudoalcaligenes</i>	4	M	4	M
<i>P. mendocina</i>	5	M	3	M
<i>P. putrefaciens</i>	1	—	1	—
<i>P. putrefaciens</i>	2	M	1	—
Multitrichous flagellates (MM)				
<i>P. maltophilia</i>	4	MM	4	MM
<i>P. maltophilia</i>	4	MM	4	MM
<i>P. maltophilia</i>	4	MM	1	—
<i>P. putida</i>	5	MM	5	MM
<i>P. putida</i>	3	MM	3	MM
<i>P. putida</i>	5	MM	4	MM
<i>C. terrigena</i>	5	MM	4	MM
<i>C. terrigena</i>	5	MM	4	MM
<i>C. terrigena</i>	5	MM	4	MM
<i>P. cepacia</i>	5	MM	2	MM
<i>P. cepacia</i>	3	MM	3	MM
<i>P. cepacia</i>	3	MM	3	MM
<i>P. fluorescens</i>	5	MM	3	MM
Peritrichous flagellates (P)				
<i>A. faecalis</i>	5	P	5	P
<i>A. faecalis</i>	5	P	5	P
<i>A. odorans</i>	3	P	2	P
<i>A. denitrificans</i>	5	P	5	P
<i>Achromobacter</i>	5	P	5	P
<i>Achromobacter</i>	5	P	4	P
<i>Achromobacter</i>	5	P	4	P
<i>B. bronchiseptica</i>	5	P	4	P
<i>B. bronchiseptica</i>	5	P	4	P
<i>B. bronchiseptica</i>	4	P	1	—
Mean	4.2		3.2	

^a The cultures were coded and the organism identification was unknown. One smear from each culture was stained with heat applied to the stain until steam formed, and one smear was stained without heat application; each was rated according to flagellar arrangement and stain quality (see Materials and Methods for quality rating).

stain for flagella is a reliable and practical procedure for use in the clinical laboratory.

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