JOURNAL OF CLINICAL MICROBIOLOGY, July 1984, p. 84–88
0095-1137/84/070084-05$02.00/0

Vol. 20, No. 1

Differentiation of the Major Flagellar Antigens of *Pseudomonas aeruginosa* by the Slide Coagglutination Technique

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Received 25 January 1984/Accepted 30 March 1984

Antisera against the two major flagellar antigens of *Pseudomonas aeruginosa* were obtained by immunization of rabbits with isolated flagella and absorption of contaminating antisomatic antibodies. In the conventional slide agglutination test, the pure H antisera did not agglutinate the flagellated cells of the homologous strains. The addition of protein A-bearing staphylococci to H antiserum and homologous flagellated cells, the so-called slide coagglutination, results in a rapid development of flaky clumps. H coagglutination tests of reference strains, which formerly have been H typed by long-term tube agglutination and by the indirect fluorescent-antibody technique, yielded exactly the same subdivision of the strains in H type a and H type b as the more laborious and time-consuming methods. O grouping and H typing of 181 isolates from clinical specimens revealed a free combination of the somatic and flagellar antigens. 25 OH serovars were found. The simple and rapid coagglutination technique can promote the serovar determination of *P. aeruginosa*, particularly for the purpose of hospital infection control.

Pseudomonas aeruginosa possesses two major antigenic types of flagella (1, 7, 8). One type is characterized by a serologically uniform antigen, and the other type shows a complex antigen structure with one common factor and several partial factors. Because each partial factor is an independent determinant, highly discriminating flagellar antigenic schemata which consist of 8 (8) and 17 (1) H types have been devised. On the basis of the combination of O grouping and H typing, 53 (7) and 99 (1) OH serovars of *P. aeruginosa* have been found. Although the determination of H antigens in addition to O grouping allows a much more detailed epidemiological analysis of the dissemination of the microbe than O grouping alone, H typing is not even used routinely in laboratories in which H antisera are available (8). This is partly due to the fact that the methods of H typing—long-term tube agglutination after selecting motile cells by means of U-tube cultures (7, 8), indirect fluorescent-antibody technique with soft agar cells (1), and migration inhibition in soft agar tubes (8)—are laborious and time consuming. Unlike O grouping, the slide agglutination is unsuitable for H typing (7) due to the monotrichous flagellation of cells and the predominant immunoglobulin G (IgG) class of antiflagellar antibodies (3).

In the coagglutination technique, which was first introduced for the serotyping of pneumococci (6), *Staphylococcus aureus* cells are coated with IgG by fixation of the Fc portion of the antibody proteins on protein A, leaving the Fab fragments free to complex with the homologous antigen (11). Whereas immunoglobulin M is predominantly involved in the ordinary agglutination reaction, IgG can be additionally recruited for the agglutinating process by introducing staphylococci. It has recently been shown that, when compared to the conventional slide agglutination, the slide coagglutination facilitates the O grouping of *P. aeruginosa* by increasing the sensitivity of O antigen determination and the rapidity of the reaction (R. Ansorg and M. Knoche, Eur. J. Microbiol., in press).

The purpose of the present study is to investigate whether the slide coagglutination is suitable for H typing of *P. aeruginosa* as well.

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MATERIALS AND METHODS

***P. aeruginosa* strains.** The reference strains of the O groups 1 through 12 according to Habs (no. 5933, 5934, 5935, 5936, 5937, 5939, 5938, 5940, 5941, 5943, 5944, 5945) were from the Collection de l'Institut Pasteur, Paris. The reference strains of the H types (no. 170001, 170002, 170012, 170016, 170018, 170021) were obtained from B. Lányi, Budapest, Hungary. The other strains were freshly isolated from clinical material.

O antisera. The test sera for O grouping of *P. aeruginosa* according to the extended schema of Habs were obtained from the Institut Pasteur Production, Paris.

Isolation of flagellar filaments. Flagella of the strains no. 170001 (H type b) and 170002 (H type a) were isolated by shearing the flagella in a rotation homogenizer, followed by differential centrifugation as described previously (2). The purity of the preparations was controlled by electron microscopic examination after staining with 1% phosphotungstic acid adjusted to pH 6.4 with KOH. The flagellar pellets were stored at −20°C.

Preparation of antisera against isolated flagella (F antisera). One hundred milligrams (wet weight) of the flagellar preparations in 0.5 ml of 0.9% NaCl solution was emulsified with 0.5 ml of Freund complete adjuvant. Rabbits were injected subcutaneously with four 0.25-ml aliquots at four different sites. Twenty-one days later, the same amount was injected subcutaneously; on day 35, the rabbits were bled.

Preparation of H antisera by absorption. One milliliter of F antiserum diluted with 9 ml of 0.9% NaCl containing 0.01% NaN_3 was mixed with 1 g (wet weight) of homologous culture which had been heated at 100°C for 2.5 h. The mixture was incubated at 37°C for 18 h in an overhead rotator. Thereafter, the absorbed serum was separated by centrifugation.

***S. aureus* preparation.** The protein A-bearing strain, *S. aureus* ATCC 12598, Cowan serotype 1, was cultivated in Iso-Sensitest broth (Oxoid Ltd., Hampshire, England) for 48 h at 37°C with an aeration of 5 liters/min in a 10-liter fermentor (Braun, Melsungen, Federal Republic of Germany). The harvested bacteria were washed twice in phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M phosphate [pH 7.0]). One

TABLE 1. Flagellar specificity of *P. aeruginosa* immune sera[a]

Strain	Method	Antigen	Titers for:[b]			
			F antiserum		H antiserum	
			a	b	a	b
170002 O:10, H:a	IF	Native somatic	320	0	0	0
		Native flagellar	640	0	320	0
	AGG	Heated	80	0	0	0
		Native	80	0	0	0
	Co-AGG	Heated	640	0	0	0
		Native	>2,560	0	1,280	0
170001 O:3, H:b	IF	Native somatic	40	320	0	0
		Native flagellar	0	2560	0	1,280
	AGG	Heated	0	80	0	0
		Native	0	80	0	0
	Co-AGG	Heated	0	320	0	0
		Native	0	>2,560	0	1,280

[a] Comparison of the indirect fluorescent-antibody test (IF), conventional slide agglutination (AGG), and slide coagglutination (Co-AGG) for the detection of flagellar reactions. Immunization was with isolated flagella of strains 170001 and 170002. The F antisera are not absorbed, the H antisera are absorbed with heated cultures of the homologous strain.

[b] Reciprocal titers are indicated. IF, 0 = 1:≤20; AGG and Co-AGG, 0 = 1:<10.

gram of bacteria (wet weight) was suspended in 100 ml of 50% ethanol in PBS and agitated slowly for 2 h at room temperature. The ethanol-treated suspension was thereafter washed three times in PBS and finally adjusted to a concentration of 5% (vol/vol) in PBS containing 0.01% thimerosal. The ethanol-treated and thimerosal-preserved cells showed no self-aggregation or loss of antibody binding capacity for months when stored at 4°C.

Slide coagglutination. On a glass slide with a marked area 2 cm in diameter, 40 μl of O antiserum, F antiserum, or H antiserum was mixed with 10 μl of *Staphylococcus* suspension. Thereafter 10 μl of *P. aeruginosa* antigen suspension was added. The glass slide was then rocked on a reeling apparatus (Heidolph, Kehlheim, Federal Republic of Germany) at 30 rpm until visible agglutinates developed or for maximally 10 min when the reaction was negative. The following antigen preparations were used: cells from blood agar plates grown overnight at 37°C (native antigen) and cells from nutrient agar plates grown overnight at 37°C, which were harvested with 0.9% NaCl solution, heated at 100°C for 2.5 h to destroy the flagella (7), and finally washed twice in 0.9% NaCl solution (heated antigen). The native as well as the heated cells were suspended in 0.42% NaCl solution to a density which corresponds to McFarland nephelometer standard no. 10. Serum from nonimmunized rabbits plus staphylococci plus antigen suspension served as a negative control.

Slide agglutination. The procedure was the same as in the coagglutination test except that the staphylococci were replaced by 10 μl of 0.9% NaCl solution.

Indirect fluorescent-antibody test. For the examination of the content of antisomatic and antiflagellar antibodies in F antisera and H antisera, cells from blood agar grown overnight at 37°C were used as the antigen, and fluorescein-conjugated anti-rabbit γ-globulin serum was used as the second antibody. The procedure has been previously described in detail (1, 4).

RESULTS

Serological reactivity of flagella in the immunofluorescence test, slide agglutination, and slide coagglutination. The F antisera contain antiflagellar and antisomatic antibodies, as shown in the immunofluorescence reactions (Table 1). Although the flagellar preparations used in the immunization are electron microscopically pure (Fig. 1), there are apparently traces of material which induce the formation of antisomatic antibodies. Therefore, the F antisera are in serological terms OH antisera. They react in the agglutination and coagglutination tests with native as well as heated flagellum-free cells of the homologous strain. After absorption of the F antisera with heated cultures (O antigen), specific H antisera are obtained. They only stain the flagella of native cells in the immunofluorescence test; in the agglutination test, however, they show no reaction, and they only react with flagellum-bearing native cells in the coagglutination test (Table 1).

In comparing the results of the immunofluorescence test, agglutination, and coagglutination with F antisera (OH antisera) and flagella-specific H antisera, the applicability of the different methods for the detection and analysis of flagellar antigens is shown (Table 1). In the usual slide agglutination, reactions only take place with F antisera containing antiso-

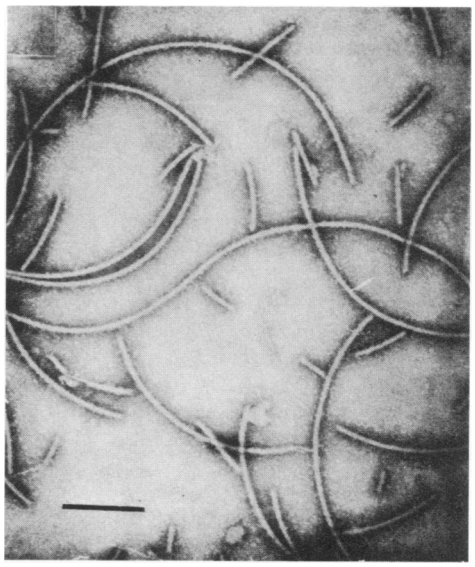

FIG. 1. Electron microscopic photograph of isolated flagella from *P. aeruginosa* strain 170001. Bar, 0.25 μm.

matic antibodies. Because heated and native cells yield the same agglutination titer, the flagella of native cells are obviously not involved in the reaction. In the slide coagglutination, however, the F antisera react with native cells at much higher titers than with heated cells, indicating that the coagglutination includes the flagella of the cells in addition to the somata. The results with H antisera confirm that the ordinary slide agglutination does not identify flagellar antigens. In contrast, the coagglutination shows a strong reaction between flagellated cells and H antisera and no reaction between cells without flagella and H antisera. Thus, the coagglutination resembles the indirect fluorescent-antibody technique in identifying serological reactions of the flagella.

Morphology of flagellar reactions in the slide coagglutination. In the slide coagglutination test, native *P. aeruginosa* cells from blood agar plates form flaky clumps in homologous H antiserum blended with staphylococci, and the homogeneous milky suspension of the original mixture clears (Fig. 2). The reaction is easily distinguished with the naked eye from the permanently milky suspension of native cells in heterologous H antiserum plus staphylococci. In comparison, no clumping of native cells develops in the ordinary agglutination test with either homologous or heterologous H antiserum.

Native cells of mucoid strains may arrange into threads during rotation on the glass slide. In such cases, the mixture becomes only partially clear, which indicates that the antibody-coated staphylococci are not included in the aggregates. Because the negative control shows the same arrangements, misinterpretation as true coagglutination is avoidable. The self-aggregation of the cells can be abolished by letting the suspension stand for 1 to 2 h at room temperature or by careful washing with an 0.42% NaCl solution.

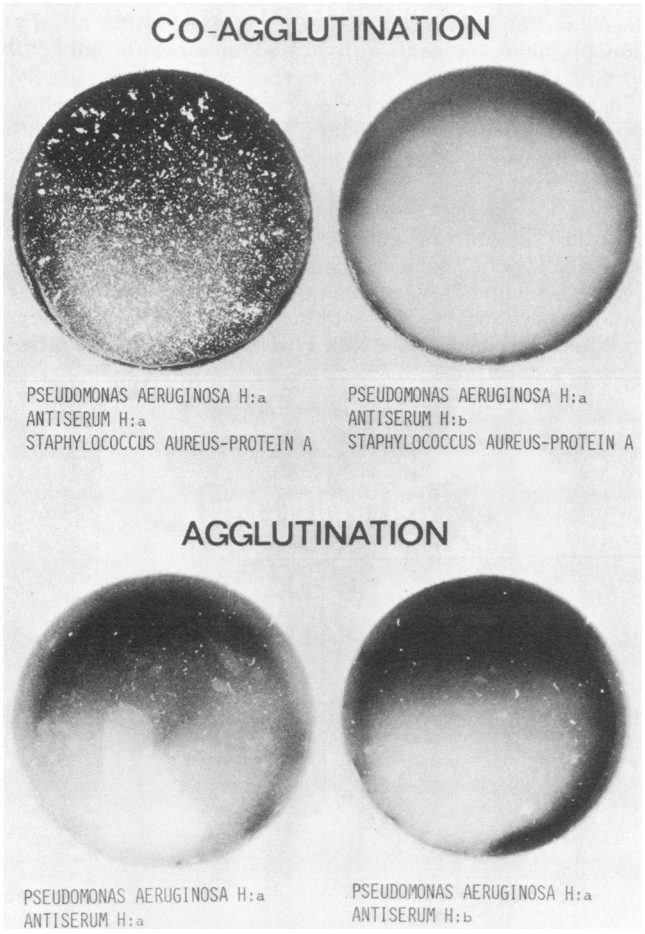

FIG. 2. Slide coagglutination and conventional slide agglutination reactions of native cells of *P. aeruginosa* with homologous (left side) and heterologous (right side) flagella-specific H antisera.

TABLE 2. H coagglutination of reference strains of *P. aeruginosa* with antisera specific for the flagellar antigen types a and b

P. aeruginosa strain	H type by long-term tube agglutination according to Lányi (7)	H type by immunofluorescence according to Ansorg (1)	Coagglutination reaction	
			anti-H:a	anti-H:b
5933	2	a	+	−
5939	2	a	+	−
5940	2	a	+	−
5941	2	a	+	−
170002	2	a	+	−
170012	2	a	+	−
170016	2	a	+	−
170018	2	a	+	−
170021	2	a	+	−
5934	1	b	−	+
5935	1	b	−	+
5936	1	b	−	+
5937	1	b	−	+
5938	1	b	−	+
5943	1	b	−	+
5944	1	b	−	+
5945	1	b	−	+
170001	1	b	−	+

Comparison of H typing by long-term tube agglutination, immunofluorescence, and coagglutination. To confirm that the coagglutination test is equivalent to the more laborious H typing methods, strains which formerly have been H typed by indirect fluorescence technique and long-term tube agglutination technique were examined by the H coagglutination test. The results (Table 2) show that the coagglutination with antisera, which are proved to contain only antiflagellar antibodies, distinctly differentiates the H types of *P. aeruginosa* and, therefore, is able to replace the other methods, at least on the level of the two major flagellar antigens.

Combination of O groups and H types. Nonfecal *P. aeruginosa* isolates from patients of a coherent epidemiological area were O grouped and H typed by coagglutination (Table 3). Of the strains 98.9% were O groupable and predominantly belong to the serogroups O:1, O:4, and O:6. Other serogroups showed an incidence of no more than 10%. One of the 181 strains was not H typable. H type a and H type b are almost evenly distributed among the strains. Because both types are present in all O groups of considerable incidence, O antigens and H antigens obviously combine freely. Of the 32 theoretically possible OH serovars, 25 OH serovars were found.

DISCUSSION

The definite identification of flagellar and nonflagellar reactions or the absolute exclusion of nonflagellar reactions is a crucial prerequisite for the H typing of *P. aeruginosa*. The indirect fluorescent-antibody technique allows the identification of flagellum and other cell surface structures at

TABLE 3. Distribution of H types a and b in O groups of *P. aeruginosa* isolates from miscellaneous nonfecal human specimens[a]

O group	No. of isolates	H type		
		a	b	NT[b]
1	26 (14.4%)	21	5	—[c]
2	4	1	3	—
3	10	2	8	—
4	56 (30.9%)	34	21	1
5	3	3	—	—
6	22 (12.2%)	11	11	—
7	1	—	1	—
8	3	3	—	—
9	12	7	5	—
10	10	3	7	—
11	13	10	3	—
12	—	—	—	—
13	7	5	2	—
14	2	—	2	—
15	3	3	—	—
16	7	2	5	—
NG[d]	2 (1.1%)	1	1	—
Total	181 (100%)	106 (58.5%)	74 (40.9%)	1 (0.6%)

[a] OH serovar determined by coagglutination.
[b] NT, Nontypable.
[c] —, No isolates.
[d] NG, Nongroupable.

the morphological and serological level simultaneously, and thus the specific analysis of flagellar antigens, even with OH antisera (1). Serological methods, however, which are based on the observation of a secondary phenomenon of the antigen-antibody reaction, i.e., agglutination, immobilization, precipitation, or lysis, are in themselves not appropriate for the determination of the topical position of the reacting antigen. H typing with such methods requires antisera which have been verified to contain only antibodies against flagella.

Antisera against thermolabile surface antigens of *P. aeruginosa* (7) are not automatically specific antiflagellar antisera because heat-labile antigens are also found on fimbriae (10). The reactions of F antisera show that the use of isolated flagellar filaments as a vaccine does not yield flagella-specific antisera either. It has been shown that flagellar preparations obtained by a similar method always contain some lipopolysaccharide (9) which induces O antibodies. The O antibodies of F antisera must be removed by absorption to obtain specific H antisera. Whatever the method of immunization, however, the cellulotopographic specificity of the antiflagellar antisera must be verified before any secondary phenomenon method can be applied. The indirect fluorescent-antibody technique, which renders the reactions of the cellular surface structures visible, is obviously a reliable method for the examination of the flagellar specificity of antisera.

Pure H antisera tested in this manner show no agglutination of native *P. aeruginosa* cells in the ordinary slide agglutination test. This confirms the finding that macroscopic H agglutination of *P. aeruginosa* cells is a slow procedure, and therefore slide agglutination has not been found suitable for the purpose (7). Antisera from rabbits which agglutinate native cells also react with heated cells, indicating that nonflagellar reactions are responsible for the ordinary slide agglutination. It has been reported, however, that antisera obtained by immunization of mice with isolated flagella agglutinate live cells but not heat-killed cells of *P. aeruginosa*, which is interpreted as a sign of antiflagellar antibodies (5). If one may exclude reactions with other thermolabile cell surface substances, these discrepancies in flagellar agglutination reactions could be attributed to differences in the immunoglobulin classes of the antiflagellar antibodies. In antisera from rabbits, the antiflagellar antibodies predominantly belong to IgG (3). The low agglutinating capability of IgG in combination with the monotrichous flagellation of the cells could explain the lack of reactivity in the conventional slide agglutination.

By introducing protein A-bearing staphylococci to the agglutination process, the IgG antiflagellar antibodies are fixed on staphylococcal particles providing a structure resembling the large immunoglobulin M molecules with regard to the distance between antigen binding sites. Thus, IgG is equipped to reach the flagellar antigens of neighboring cells and to form an agglutination lattice. The slide H coagglutination technique is specific for the differentiation of the major H types of *P. aeruginosa*, just as is the indirect fluorescent-antibody technique, but it is much simpler and more rapid. An additional valuable trait of the coagglutination is that the same method can be used for O grouping (Ansorg and Knoche, in press) and H typing of *P. aeruginosa*.

With the extended schema of Habs, 16 O groups of *P. aeruginosa* can be identified. In general, however, isolates from clinical specimens are concentrated in three to four serogroups (8). For the purpose of hospital infection control, the source and the dissemination of a strain with a rare serogroup are quite easy to elucidate. For the majority of strains, however, the serogrouping is not discriminating enough for epidemiological tracing. By H subdivision of the serogroups, the epidemiology of strains belonging to frequent serogroups can be followed more accurately. Further refinement of H typing by including the partial factors of flagellar antigens (1, 7) in the slide H coagglutination may result in a very discriminating and practicable system of the serovar determination of *P. aeruginosa*.

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