JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1976, p. 124-128

Vol. 4, No. 2
Printed in U.S.A.

Preparation of Typing Antisera Specific for O Antigens of *Pseudomonas aeruginosa*

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Received for publication 12 April 1976

Results of serotyping 966 clinical isolates of *Pseudomonas aeruginosa* showed that 72% agglutinated specifically in one or another of the 16 typing antisera, but 28% agglutinated in two or more and often in as many as 10 antisera; this polyagglutinability correlated with a high incidence of cross-reactivity among the antisera. Absorption of each typing antiserum with either cell suspensions of five O-type strains or with a suspension of a particular polyagglutinable strain (SMC 247) abolished cross-reactivity in the typing antisera without significantly reducing titers against the homologous strains. All but four of the polyagglutinable strains agglutinated specifically in one or another absorbed antisera. The cross-reactions of unabsorbed antisera were interpreted to have been caused by antibodies directed not against specific O antigens but against thermostable specificities that remain undefined.

Infection with *Pseudomonas aeruginosa* continues to be a major problem in nosocomial disease, particularly in the compromised patient. The recognition and investigation of episodes of cross-infection with this organism require a reproducible and discriminating typing scheme for the differentiation of isolates. Serotyping on the basis of O antigens is generally considered the most useful and reliable primary approach.

The preparation of suitable O-typing antisera is not, however, without problems. Although not specifically reported in the published literature, most laboratories seem to have experienced difficulty in the preparation of specific high-titer antisera, and various workers (5, 6) have reported that many of their strains could not be typed satisfactorily because they agglutinated in more than one typing antiserum.

In the past 2 years we have serotyped over 3,000 clinical isolates of *P. aeruginosa*. Among the first 966 isolates tested, 28% were untypable in that they agglutinated in two or more antisera. Because of this large number of strains whose O serotype did not appear to be clearly definable, we investigated methods to improve the specificity of the O-typing antisera.

(This paper contains material which forms part of a thesis submitted by N.H.D. to the Graduate Department of Microbiology and Parasitology of the University of Toronto in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Strains. Nine-hundred and sixty-six isolates of *P. aeruginosa* from 576 patients were isolated at Sunnybrook Medical Centre, Toronto, during 1968 to 1969. These had previously been pyocin typed (3) and therefore served as the basis for comparing the results of the serotyping and phage typing procedures which we were introducing in our laboratory. All isolates were stored at −70°C until serotyping was carried out. Then they were cultivated on either Trypticase soy agar (TSA; BBL) or in Trypticase soy broth (BBL) without dextrose (TSB).

Preparation of antisera. Prototype strains of 16 serotypes were obtained from M. T. Parker, Central Public Health Laboratory, Colindale, London, England. These were Habs' (4) original 12 O-serotypes 1 to 12, Véron's (11) subdivision of O-types 2 and 5, Sandvik's O-type S13 (9), and Lányi's O-type Ps11 (6). These include all but three of the provisional serotypes presently under investigation by members of the Subcommittee on Pseudomonadaceae of the International Committee on Systematic Bacteriology (8).

Antisera were raised in New Zealand white rabbits. Prototype strains were grown for 18 h on TSA plates and harvested into saline. Immunizing antigens were prepared by steaming the saline suspensions for 2.5 h. These antigens were then washed three times in sterile saline and resuspended, and the concentration was adjusted so that the optical density at 625 nm of a 1:200 dilution was 0.75. This corresponds to the concentration of 5×10^{10} cells/ml advocated by Mikkelsen (7). The rabbits received six intravenous injections of this dense suspension at 2-day intervals over a period of 2 weeks in doses of 0.25, 0.5, 1.0, 1.5, 2.0, and 2.0 ml. Four days after the last injection the animals were exsanguinated by

cardiac puncture. Preimmune sera were all negative when titrated against all antigens.

Preparation of O antigens for serological testing. For each strain to be typed, four TSA plates were heavily inoculated from a 6-h TSB culture and incubated at 37°C overnight. A suspension of the cells was made in 3 ml of saline per plate, and the pooled suspension was autoclaved for 2.5 h at 121°C. The antigen was then washed once and then resuspended in 2 ml of saline. This suspension was used directly in slide agglutination tests. Antigens for use in antiserum titrations were prepared similarly but were further diluted in saline so that the optical density at 625 nm was 0.75. Antigens for the absorption of antisera were prepared by the same method but were washed three times in saline. Two milliliters of the dense suspension was centrifuged to produce a discrete pellet, the supernatant was removed, and 10 ml of antiserum diluted 1:5 was added.

Titration of antisera. All titrations of the antisera were performed by agglutination in U-type microtiter plates. All sera were initially diluted 1:5, and serial twofold dilutions were prepared in a volume of 50 μl. An equal volume of autoclaved bacterial suspension was then added, giving a final dilution of antiserum in the first well of 1:20. The plates were incubated at 37°C for 2 h and refrigerated overnight to allow settling of the cells. The titer recorded was the highest dilution showing agglutination.

Typing by slide agglutination. Autoclaved suspensions were tested in each of the 16 typing antisera by slide agglutination. Black glass plates (115 mm by 95 mm) with 20 circular areas 12 mm in diameter outlined in ceramic ink were used for the tests. One drop of suspension was mixed with one drop of antiserum in each circle, and the plate was agitated on a variable speed rotator at one rotation per second for 8 min before examining for agglutination.

RESULTS

Reactions of unabsorbed and minimally absorbed antisera. Antisera raised against each of the prototype strains were titrated against cell suspensions of the type strains. The results showed that in all cases, with the exception of antiserum 5d and antigen O6, the homologous titer was equal to or higher than the heterologous titers (Table 1). The most notable observation was the wide range of cross-reactions. One group of these was the block of reactions between strains 2a, 2b, 5c, and 5d, which Véron (11) has shown to be related, and also Ps11, which M. T. Parker has informed us is related to strains 2a, 2b, 5c, and 5d. The other cross-reactions were among prototypes considered to be unrelated, particularly the agglutination of the O6 suspension in 13 of the 16 antisera. On the basis of this observation, all antisera, except the O6 antiserum, were absorbed with O6 cell suspensions, and this was followed by the cross-absorptions of antisera against types 2a, 2b, 5c, 5d, and Ps11 required to make them monospecific. These absorptions resulted in the elimination or reduction in titer to less than 1:160 not only of reactions of the O-antisera with antigen 6, but also of their reactions with other unrelated antigens. On testing these antisera by slide agglutination, reactions were observed only with homologous suspensions because slide agglutination is a less sensitive method that detects only those reactions corresponding to microtiter titers of 1:160 or more. The 16 antisera absorbed in this way constituted the set used in routine serotyping of *P. aeruginosa* isolates.

All 966 isolates were tested by slide agglutination in these antisera; 687 agglutinated only in a single antiserum and could be definitely assigned to one or another of the 16 serotypes; 8 were untypable due to autoagglutinability or failure to agglutinate in any antiserum; 271 (28%) agglutinated in two or more antisera, in some cases in as many as 10 of the antisera. Because these polyagglutinable (PA) strains were so numerous and were unclassifiable into specific O types, further investigation into the nature of this polyagglutinability seemed necessary. In addition, the success of absorption with strain O6 in reducing cross-reactions in antisera against unrelated strains indicated the need for further study of the antisera.

Absorption of antisera with five prototype strains. Because of their frequent occurrence in the polyagglutination reactions, suspensions of O-types 1, 6, 9, 10, and 5c were used to absorb another set of untreated antisera, omitting only the homologous antigen for each serum. When these serially absorbed antisera were titrated, all observable cross-reactions among O-unrelated strains were found to have been removed while the homologous reactions were retained (Table 2).

A number of the 966 isolates were retyped by slide agglutination using these multiply absorbed antisera. Representatives of the 687 single O-factor isolates agglutinated exactly as in the routine typing, but, in contrast, all but four of the 271 PA strains agglutinated in only one or another of the multiply absorbed antisera and could therefore now be assigned to specific O types (Table 3). On the basis of this observation, the occurrence of polyagglutinability with the routinely prepared antisera was interpreted to have been due to agglutinations arising through the involvement of antibodies that were directed not against the O factors specific for the prototype strains of the typing scheme but against other undefined antigenic specificities.

TABLE 1. *Cross-titrations of unabsorbed P. aeruginosa antisera*

Prototype O antigen	Titer of unabsorbed antiserum[a]																
	1	3	4	2a	2b	5c	5d	Ps11	6	7	8	9	10	11	12	S13	SMC 247
1	5,120	80	160	160	20	80	320	80	40	80	80	640	80	80	20	640	1,280
3	40	640	80		20		40	80	40	80	40	40	40	80		160	320
4		40	640		20				20	20				20		80	20
2a		20	20	5,120	1,280	80	80	80	20	20	20	20		20			40
2b	20		20	1,280	1,280	160	80	80	20	20	20			20			40
5c	20			2,560	640	1,280	320	160	20	20							160
5d	20		40	320	80	160	320	40	40	40	40	20	20	20	20	20	160
Ps11	20			640	160	320	80	320	20	20	20			40			20
6	640	320	320				640	320	1,280	160	160	640	320	320	40	1,280	10,240
7	160	80	160	80	40	40	40	80	80	2,560	1,280	160	40	160	80	40	160
8	20		40					80	20	320	2,560	20		80	20	20	80
9	160	40	80				160	40	20	160	40	640	80	80		320	640
10	160	40	160		20		80	40	40	80	40	160	2,560	80		160	640
11		40	40		20					80	20			10,240			40
12	20	40	40				20	80	20	80	160	40		320	640	20	80
S13		40	20		20			20	40	40	40	40	20	40		1,280	320
Isolate SMC 247	2,560	1,280	2,560		40		2,560	320	640	320	160	5,120	2,560	640	80	5,120	5,120

[a] Slide agglutination tests were positive when microtiter titers were ≥1:160.

TABLE 2. *Titers of typing antisera before and after absorption*

Strains for absorption	Homologous titers of typing antisera															
	1	3	4	2a	2b	5c	5d	Ps11	6	7	8	9	10	11	12	S13
None	5,120 (11)[a]	640 (10)	640 (13)	5,120 (6)	1,280 (11)	1,280 (6)	320 (11)	320 (13)	1,280 (14)	2,560 (15)	2,560 (13)	640 (10)	2,560 (7)	10,240 (14)	640 (5)	1,280 (10)
06	5,120 (1)	640	320	1,280 (3)	640 (1)	640 (4)	320	160 (1)	640 (5)	1,280 (3)	1,280 (1)	320 (1)	640 (2)	2,560 (1)	640	2,560 (5)
06, 1, 9, 10, 5c SMC 247	1,280 2,560	2,560 2,560	160 1,280	1,280 2,560	160 1,280	1,280 640	160 640	320 160	640 1,280	640 2,560	1,280 2,560	640 1,280	2,560 1,280	5,120 10,240	1,280 1,280	640 2,560

[a] Number of heterologous reactions shown in parentheses; where no figure in parentheses appears there were no cross-reactions.

TABLE 3. *Differentiation of 271 PA strains into O serotypes with multiply absorbed antisera*

O serotype	No. of isolates
1	11
3	8
5c	4
6	113
7	2
8	18
9	11
10	82
11	18
PA	4

Absorption of antisera with PA strain SMC 247. Microtiter titrations were performed with all 16 unabsorbed antisera and four selected PA strains. Although all four strains agglutinated in several antisera, one of the strains, SMC 247, agglutinated in a greater number of antisera and in higher titers (Table 1). An antiserum was therefore raised against this strain and it was titrated against all the prototype cell suspensions. This antiserum agglutinated all the suspensions, and nine reactions had titers of 1:160 or greater (Table 1). Because of the many high-titered reactions involving strain SMC 247, it was used to absorb fresh samples of all 16 unabsorbed antisera. One absorption with SMC 247 was sufficient to remove all cross-reactions in most of the antisera. Antisera against O-types 9 and S13 required two and three consecutive absorptions, respectively, and antisera 2a, 2b, 5c, 5d, and Ps11 still required the usual cross-absorptions to eliminate the known cross-reactions. Each of the antisera retained its capacity to agglutinate homologous cell suspensions (Table 2). Like the antisera absorbed with five prototype strains, but unlike the minimally absorbed antisera first used in routine typing, the antisera absorbed with SMC 247 resolved PA strains into specific O types. Thus, absorption with SMC 247 duplicated the results of absorption with five prototype strains and provided an easier means of preparing *P. aeruginosa* antisera with high titers of anti-O specificity.

Application of the modified serotyping scheme. The significance of the results of resolving PA strains to specific O types by using

either multiply absorbed antisera, or antisera absorbed with SMC 247, was evaluated by comparing the serotypes of repeat isolates from individual infections. In our series, there were 17 examples of two or more isolates of the same phage pattern and pyocin type being isolated from the same patient. In each group, at least one isolate demonstrated a specific O-type in minimally absorbed antisera, and the remainder were PA. When the PA strains were retyped either with multiply absorbed antisera or with antisera absorbed with SMC 247, they demonstrated the same O specificity as the isolates that had typed specifically in the minimally absorbed antisera. A typical example illustrates the findings for all 17 groups of isolates. *P. aeruginosa* was isolated on eight occasions from the patient over a 6-week period. Clinically, it seemed likely that it was the same strain on each occasion, and all proved to be of the same pyocin and phage type. With minimally absorbed antisera, two isolates typed specifically as O6 and the other six isolates were PA. With fully absorbed antisera all eight isolates typed specifically as O6.

The success of the highly absorbed antisera in abolishing polyagglutinability and consequently eliminating apparent differences among isolates of the same O type greatly facilitated our subsequent investigations on the epidemiology of nosocomial *P. aeruginosa* infections. This was particularly true with serotypes O6 and O10 because so many of them had typed as PA strains with our minimally absorbed antisera. Some measure of the extent to which use of the original antisera had biased our earlier results can be obtained from Table 4, which shows the distribution of the six serotypes most affected by the use of minimally absorbed antisera.

TABLE 4. *Effect of use of minimally and highly absorbed antisera in determining serotype distribution among 966 P. aeruginosa isolates*

O antiserum	Isolates (%) agglutinating in antisera	
	Minimally absorbed	Highly absorbed
10	15.8	24.4
11	11.5	13.5
1	7.6	8.8
6	6.5	18.3
9	2.1	3.2
Other	28.1	31.4
PA	28.0	0.4

DISCUSSION

Little detailed information has been published on methods of preparing fully specific O antisera for typing *P. aeruginosa*. There are, however, clear indications in several papers of the difficulties encountered in attempting to do so. Wahba (12) stated that dilution of typing sera was not satisfactory and that absorptions were necessary for the preparation of monospecific typing antisera. Bergan (1) commented on the many cross-reactions found in typing sera and also noted that these reactions varied from laboratory to laboratory. Lányi (6) found that 11% of his series were PA strains, and Homma et al. (5) quoted two figures, 16% and 26%, for PA strains in two groups of isolates.

When setting up a serotyping scheme in our own laboratory, we followed most other investigators in this field (4, 6, 7, 9, 10) in using steamed bacterial suspensions for immunizing rabbits. We decided to use autoclaved rather than steamed suspensions as antigens in agglutination tests in an attempt to exclude reactions associated with heat-labile antigens and thus to restrict typing to the definition of O antigens alone. When these reagents were used to type our collection of *P. aeruginosa*, 28% were found to be PA. Many cross-reactions were also observed in antisera against unrelated O types. We found that some of these were removed by absorption with strain O6, and all our original typing antisera were absorbed in this way. This partial success in improving the specificity of the antisera by absorption with a strain unrelated to their O type led to a method of producing monospecific typing antisera by absorption with five different type strains. Strains that had typed specifically in the antisera absorbed with O6 typed with the same specificity in these multiply absorbed antisera. However, all but four of the strains that had previously been PA were resolved into specific O types by the multiply absorbed antisera.

From these results it was concluded that agglutination in unabsorbed *P. aeruginosa* typing antisera could be attributed not only to anti-O antibodies reacting specifically with O antigens serologically distinct for each type strain, but also to antibodies against other uncharacterized, thermostable components. It is possible that heat treatment of the bacteria could result in an alteration of antigenic determinants or could expose common antigens which may or may not be associated with "core" polysaccharide described by other workers (2, 13). Furthermore, slight variations in cultural conditions have been reported to result in altered lipopolysaccharide composition (2), and this effect, in conjunction with small differences in the intensity of heat treatment from one prepa-

ration of cell suspension to the next, should be investigated as possible factors in contributing to the formation of cross-reacting components. Variations in the number of cross-reactions observed on testing suspensions prepared from fresh cultures of the same organism on different days support this view. Whether the cross-reactivity in the unabsorbed antisera reflected an altered antigenicity or common antigenic components or a combination of both remains unresolved. Each of the five strains used for preparation of multiply absorbed antisera possessed antibody-binding capacity sufficient to remove some but not all of the cross-reactive antibodies. After absorption with all five, residual antibody showed specificity only for homologous strains. In contrast, strain SMC 247, agglutinable in 12 of the 14 antisera, appeared to possess the cross-reactive components in both quantity and specificity, and this indicated its potential usefulness as an absorbing agent. This proved to be correct. Cross-reactivity in the typing antisera was totally removable from each typing antiserum when cell suspensions of strain SMC 247 were used for absorption. The antisera absorbed with strain SMC 247 showed the same specificity as the multiply absorbed antisera when numerous isolates were retested.

The phenomenon of polyagglutinability was provisionally interpreted to have been caused not by the occurrence in the strain of two or more O factors corresponding to the antisera in which the strain agglutinated, but rather by undefined cross-reacting components present on the cells in addition to the specific O factor.

Although the precise nature of the factors contributing to cross-reactivity have not been elucidated, it is nevertheless possible to prepare specific antisera against the O antigens of *P. aeruginosa* by absorbing out cross-reactive antibodies with strain SMC 247 or with other strains with similar properties. With the aid of such a strain for absorption, antisera may be prepared with which isolates can be typed according to their O specificities.

ACKNOWLEDGMENTS

We wish to acknowledge the excellent technical assistance of Donna-Marie Jackson.

This work was supported by National Health grant no. 605-7-784 awarded by the Department of Health and Welfare, Canada.

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