Evaluation of a *Proteus rettgeri* O-Serotyping System For Epidemiological Investigation

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An evaluation of the serotyping system for *Proteus rettgeri* has been performed through its application to 561 human isolates of this species. Of these, 545 were typable and could be separated into 58 O serotypes. A group of isolates from one hospital, and clinically implicated in cross-infections, was found to belong to the same O serotype, confirming the clinical findings. Isolates with more than one specificity of thermostable antigen, in most cases, were found to have combinations of such specificities restricted to combinations previously defined for the O-type strains. Furthermore, the use of pooled antisera was investigated and a system of pooling was devised to retain the specificity of the system and to increase the facility of the typing procedure.

Recent reports implicating *Proteus rettgeri* as an infectious agent of the human urinary tract and its involvement in nosocomial disease (4, 7, 11-13) have indicated the necessity for epidemiological investigations. The development of procedures for serotyping on the basis of somatic (O) antigens has been in progress in our laboratory (9). In this paper we report on the details of the procedure and on an assessment of its use for epidemiological studies by examining the results of typing 561 human isolates.

MATERIALS AND METHODS

Bacterial isolates. The bacterial isolates in this study were obtained solely from human sources from contributors in hospital laboratories previously cited (9). The biochemical reactions have been described (10).

Serological tests. The passive hemagglutination technique, the slide agglutination reaction, and the methods of preparing somatic O antigens for passive hemagglutination (PHA) and slide agglutination (SA) reactions have been described in detail (9).

Antisera. Antisera were prepared against formalin-treated cell suspensions of the O-type strains according to methods and immunization schedules described in an earlier report (9). In addition to the 72 typing antisera against 72 O-type strains described previously (9), 12 more were prepared against recently defined O-type strains designated 73 to 84. Antisera that were directed against O-type strains that showed interstrain relations with O types were cross-absorbed to remove cross-reacting antibodies if the cross-reactions they caused were demonstrated by both PHA and SA techniques.

RESULTS

Application of 84 antisera in typing human hospital isolates of *P. rettgeri*. Isolates were serotyped by testing cell suspensions auto-claved for 2.5 h in each of the 84 antisera raised against the O-type strains. In the first step unabsorbed antisera at a dilution of 1:5 were used. Some isolates agglutinated in only one antiserum, but others agglutinated in two or more. The latter were retested in antisera which were absorbed with cell suspensions of the O-type strains with which they had shown relations caused by the cross-reacting "a" components indicated in Table 1. Agglutination in the absorbed antiserum identified the O component specific for the O-type strain. Single factor anti-"a" antisera were not available and, therefore, the presence of "a" components on the isolate was demonstrated indirectly by observing agglutination in two or more unabsorbed antisera which contained antibody against both the specific O components and the "a" components and by observing the absence of agglutination in the absorbed antisera from which anti-"a" antibody had been removed.

Of considerable significance was the finding that, when an isolate agglutinated in only one antiserum, it was one raised against an O-type strain for which no interstrain relations had been recognized (see Table 1). When an isolate agglutinated in two or more antisera, the antisera belonged to those known to have been raised against related O-type strains. Moreover, when absorbed antisera were applied

against an isolate of the latter kind, it was found to agglutinate, like the O-type strain, in only one of the absorbed antisera. The exceptions to these observations were so few that it became evident that the great majority of iso-

lates had an arrangement of antigenic components in combinations like those of the O-type strains. Although the number of O specificities within the species has been shown to be large, 84 having been defined to date, a random distribution of these antigens among the strains was not indicated from our results. Rather, the combinations of the antigenic components appeared to be restricted to a limited number well represented among the O-type strains. On the basis of these findings the decision was made to designate the typed isolate according to the O-type strain with which it corresponded in its antigenic structure. In a few cases, isolates agglutinated in two or more antisera against unrelated O-type strains. These are described in a later section.

Approximately 70% of the 561 isolates were typed by this method of applying, first, each of the unabsorbed antisera and, then, if necessary, the appropriate absorbed antisera. However, with the realization that associations of thermostable antigens were limited to complexes already known for the O-type strains and with the increasing demands for serotyping, investigations of the applicability of pooled antisera were initiated.

Use of pooled antisera in typing. To facilitate the typing routine and to reduce the demand for typing antisera, a system of pooling was devised. The pools were prepared by adding together 10 undiluted, unabsorbed antisera selected so that a pair or group of cross-reacting antisera was allocated to the same pool. Four antisera against recently defined O-type strains 81, 82, 83, and 84 were not included in the pools but were used separately against each isolate submitted for typing. The compositions of the pools are shown in Table 2 and the cross-reacting antisera are indicated by underscor-

TABLE 1. Extended^a O antigen typing scheme for *P. rettgeri* showing the reciprocal relations among the O-type strains

O-type strain ^b	Related strains ^c	O-type strain	Related strains	O-type strain	Related strains
1	39	29	47	57	
2		30		58	
3		31		59	
4		32	47	60	
5	64	33	63	61	14, 15, 46
6		34		62	
7	67	35		63	33
8		36		64	5
9		37		65	66
10		38		66	65
11	54	39	1	67	7
12		40		68	
13		41	24	69	
14	15, 52, 61, 70	42		70	14, 52
15	14, 61	43	76	71	
16		44		72	
17		45		73	
18	48, 53	46	61	74	
19		47	29, 32	75	
20		48	18, 53	76	43
21		49		77	
22		50		78	
23		51		79	
24	41	52	14, 70	80	
25		53	18, 48	81	
26		54	11	82	
27		55		83	
28		56		84	

^a Extended and with minor modifications from previous report (9).

^b The numerical designation of each O-type strain indicates its O antigen which is serologically distinct from all others in the list.

^c Strains related through an "a,b-a,c" type relation with O-type strain on the left.

TABLE 2. Composition of antiserum pools^a

Pool	Antisera in pool									
1	<u>1</u>	<u>39</u>	2	3	4	<u>5</u>	<u>64</u>	<u>7</u>	<u>67</u>	80
2	6	8	9	10	<u>11</u>	<u>54</u>	12	13	16	17
3	<u>14</u>	<u>15</u>	<u>52</u>	<u>61</u>	<u>70</u>	57	38	46	19	20
4	<u>18</u>	<u>48</u>	<u>53</u>	21	22	23	<u>24</u>	<u>41</u>	25	67
5	26	28	<u>29</u>	<u>32</u>	<u>47</u>	30	31	34	35	36
6	<u>33</u>	<u>63</u>	37	40	42	43	44	45	49	50
7	51	55	56	58	76	59	60	<u>65</u>	<u>66</u>	69
8	62	68	71	72	73	74	75	<u>77</u>	<u>78</u>	79
Unpooled antisera	81									
	82									
	83									
	84									

^a Underscoring indicates antisera against related O-type strains according to scheme in Table 1.

ing. Because the pools were prepared in this way, the antibodies specific for O antigens exclusive to each O-type strain suffered a dilution factor of 1:10, but the antibodies against "a" components equalled a dilution of 1:10 of the total "a" antibody contributed by each cross-reacting antiserum. Since the "a" antibodies of the same specificity were not separated into different pools, it was expected that isolates with such components would agglutinate in only one pool. Because the antisera in the pools were at low dilution it was felt necessary to demonstrate that this would occur in practice and, therefore, the pools were checked for specificity by testing each O-type strain in each pool. It was shown first that each pool agglutinated only strains homologous for antisera included in the pool. Further assessment of the specificity of the pools and their application in serotyping is illustrated in Table 3 for pool 1. The results showed that each O type against which antisera was included in pool 1 agglutinated, and when the strains were tested in the 10 unabsorbed antisera each agglutinated either in the homologous antiserum only or in an antiserum against an O-type strain with which it was related, as well as in its homologous antiserum. Finally, only homologous strains agglutinated in antisera cross-absorbed to remove the "a" antibodies. Similar results demonstrated specificity for pools 2 to 8.

Approximately 30% of the isolates were typed after the introduction of the pooled antisera. Each was tested first in the eight pools and in the four unpooled antisera. The observation of agglutination in a pool was followed by testing in each of the 10 unabsorbed antisera that were used to prepare the pool. If an isolate agglutinated in two or more of these it was retested in the corresponding absorbed antisera.

In all but three cases (described in the follow-

ing section) each isolate agglutinated in only one pool. This attested to the specificity of the pools and was considered consistent with the finding (described in the previous section) that most complexes of associated thermostable antigens among the species occurred in combinations characteristic of the O-type strains.

Isolates with combinations of specificities apparently unlike those of the O-type strains.

In contrast to the majority of the isolates, three agglutinated strongly in two pools and, in individual antisera, against O-types 2 and 61, 56 and 73, and 56, 72, and 75. During the study of the type strain O antigens no relation had been observed between 2 and 61, only a unilateral reaction had been observed between 56 and 73, and low-titered reactions considered insignificant had been observed among 65, 72, and 75. In the latter two cases, the relations among the O-type strains need to be reassessed before the agglutination reactions of these isolates can be attributed to an association of specificities unlike ones found in the O-type strains. On the other hand, the occurrence of the isolate O2, 61 indicated an association of factors unlike any other strain in our collection. Similar to the latter were six isolates that agglutinated in antisera against O-types 64 and 80. No relation was known between these types, but, in a further investigation, it was shown that they agglutinated in antisera 64 and 80 even after each has been cross-absorbed. Like the O2, 61 isolate, these six bore O specificities in combination unlike either O-type strains 64 or 80 or any other in the scheme. Antisera are being prepared against each of the four different types to investigate further their antigenic complexes.

An evaluation of the applicability of the serotyping scheme for epidemiological investigations. To evaluate the applicability of serotyping as an investigative procedure in epide-

TABLE 3. Examination of the specificity of antiserum pool no. 1

Cell suspensions of O-type strains	Reaction in pool no. 1	Reaction in unabsorbed antisera of pool no. 1										Reaction in absorbed antisera					
		1	39	2	3	4	5	64	7	67	80	1 (39) ^a	39 (1)	5 (64)	64 (5)	7 (67)	67 (7)
1	+	+	+	-	-	-	-	-	-	-	-	+	-				
39	+	+	+	-	-	-	-	-	-	-	-	-	+				
2	+	-	-	+	-	-	-	-	-	-	-						
3	+	-	-	-	+	-	-	-	-	-	-						
4	+	-	-	-	-	+	-	-	-	-	-						
5	+	-	-	-	-	-	+	+	-	-	-			+	-		
64	+	-	-	-	-	-	±	+	-	-	-			-	+		
7	+	-	-	-	-	-	-	-	+	+	-					+	-
67	+	-	-	-	-	-	-	-	+	+	-					-	+
80	+	-	-	-	-	-	-	-	-	-	+						

^a Antigen of strain used for absorption indicated in parentheses.

miological studies, 561 isolates of *P. rettgeri* were serotyped on the basis of their O antigens. All isolates were from hospitals and, in one hospital, clinical evidence for *P. rettgeri* cross-infection was reported and in some of the others investigations of suspected cross-infections were in progress. All but 16 could be typed and the remaining 545 could be separated into 58 different O serotypes shown in Table 4. The 10 serotypes listed first included 391 isolates constituting 69% of the total collection from the hospitals. The remaining 170 were distributed over 48 serotypes, 20 of which included only one isolate. Seventy-three of the 75 isolates of serotype O26 were received from one hospital reporting *P. rettgeri* cross-infection and these were interpreted to be duplicates of the cross-infecting strain, consistent with the clinical findings (13). On the basis of these results, it was concluded that serotyping on the basis of O antigens could provide considerable resolution among strains of *P. rettgeri* and its application would offer a substantial contribution in the studies of cross-infection caused by this species.

DISCUSSION

Since *P. rettgeri* has been recognized as a cross-infectious agent in hospitals (4, 7, 11-13) and often as refractory to antimicrobial agents (1-4, 12) it has become increasingly more apparent that epidemiological investigations are necessary. Such investigations require facilities for typing isolates. A scheme based on somatic (O), flagellar (H), and capsular (K) antigens was reported in 1958 but the scheme was not widely used in epidemiological studies (5).

TABLE 4. Classification of *P. rettgeri* isolates according to O-serotype

O sero-type	No. of isolates	O sero-type	No. of isolates	O serotype	No. of isolates
26	75	64, 80 ^a	6	25	1
42	63	4	5	29	1
1	54	9	5	31	1
6	50	16	5	35	1
2	39	76	5	40	1
18	39	37	4	44	1
15	33	38	4	45	1
10	15	46	4	48	1
17	13	8	3	49	1
7	10	24	3	54	1
5	9	64	3	68	1
3	8	13	2	69	1
12	8	19	2	73	1
21	8	41	2	74	1
83	8	47	2	75	1
84	8	53	2	56, 73 ^a	1
11	6	57	2	56, 72, 75 ^a	1
30	6	80	2	2, 61 ^a	1
33	6	14	1		
36	6	23	1	Untypable	16

^a O antigenic structure under further investigation.

Within the latter scheme, complex and unilateral reactions were observed. Unilateral reactions were, to some extent, accounted for by the observation of an antigen, thermolabile at 121 C and common to strains with different O antigens (8). Subsequently, cell suspensions that were autoclaved were used to define more clearly the O specificities and serotyping on the basis of thermostable antigens was advocated (9). In this paper serotyping on the basis of these antigens has been assessed by testing 561 isolates collected from patients in different hospitals in both Canada and the United States. The ability of the procedure to separate all but 16 isolates into 58 serotypes established confidence in advocating the system for further use in epidemiological studies.

Furthermore, the examination of this collection of isolates led to a clearer understanding of the antigenic structure of the species. The number of thermostable antigens has been demonstrated to be large. Eighty-four have been defined by specific antisera and the untypable isolates possibly include more. Numerous cross-reacting "a" components have been defined but, significantly, in almost all cases a particular "a" specificity was found to be carried by only one pair of O types and isolates bearing the O specificity of one of the O types also carried the "a" specificity of the O-type strain. Therefore, it became clear that O specificities (defined by specific antisera) and the "a" components (defined indirectly) were restricted in their associations to a limited number of combinations, nearly all of which have, so far, been recognized among the O-type strains initially selected for the purpose of studying the O antigenic structure of the species.

The limitation in the number of antigenic complexes made the species less heterogeneous than would have been the case if an unrestricted or random association of these thermostable specificities had occurred among the strains. Since most complexes were represented among the O-type strains, a system was devised for pooling antisera so that isolates would agglutinate in only one pool. This was accomplished by not separating cross-reacting antisera into different pools but by including antisera against a cross-reacting pair or group in the same pool. It should be noted that specificity in typing was obtained without diluting antisera to obscure nonspecific reactions. Antisera, when used singly, were diluted 1:5 and pools were prepared so that specific O antibody was diluted only 1:10. The subsequent observations that isolates generally agglutinated in only one pool led to increased confidence in the use of the pools and in the specificity of the

typing procedure. Epidemiological studies involving the serotyping procedure are now in progress.

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