Reproducibility of an Indirect Immunofluorescent-Antibody Technique for Capsular Serotyping of *Klebsiella pneumoniae*

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Reproducibility of capsular serotypes of 55 consecutive clinical isolates of *Klebsiella pneumoniae* was evaluated by an indirect immunofluorescent-antibody technique previously described by Riser et al. (J. Clin. Pathol. **29**:296-304, 1976). Five colonies per specimen were examined for colony-to-colony variation, day-to-day variation, and reader-to-reader variation. Seventy-two reference strains were tested with each of 18 pools and 72 specific antisera prior to the clinical specimens to determine antiserum specificity and cross-reaction patterns. Lot-to-lot variation was examined with the reference strains. There was minimal lot-to-lot variation among the antisera tested. Ten antiserum pools required supplementation with individual antisera. The patterns of supplementation may vary from lot to lot. Colony-to-colony variation in intensity of immunofluorescence occurred, but there was no variation in serotype. These findings differ from previously reported colonial variation which occurred when API 20E biotypes were determined for individual colonies of *K. pneumoniae* directly from clinical specimens. Eighteen percent of clinical isolates studied gave cross-reactions when tested with the indicated specific antiserum. All but one of the cross-reactions were resolved with further dilutions. Day-to-day and reader-to-reader variations were minimal. The immunofluorescent-antibody technique is a reliable and reproducible method for capsular serotype determination. Capsular serotypes are less variable than API biotypes since colony-to-colony variation of serotype does not occur.

Klebsiella pneumoniae is one of the more common pathogens among the gram-negative bacteria and is an important cause of community-acquired as well as nosocomial infections (4, 6, 8, 13). Oropharyngeal colonization with gram-negative organisms has been documented in institutionalized patients (7), and a recent study revealed that the incidence of colonization in elderly persons increases with the level of care. Only 9% of those in independent apartments were colonized, whereas 60% of those in an acute hospital ward were colonized; *Klebsiella* species was the most common colonizer (14).

The ability to identify a hospital outbreak and perform the appropriate epidemiological studies rests on the availability of accurate and easy-to-perform identification of isolates below the species level. Traditionally, capsular serotype determination has been used for subdividing *K. pneumoniae*. However, the quellung test is very subjective and often difficult to read. It is also expensive and requires a highly skilled observer (10, 11).

Other typing methods of *K. pneumoniae* have been of limited success. Subtyping by bacteriocin sensitivity has been attempted, but only 67% of clinical isolates could be typed and reproduc-

ibility is only 80 to 90% (2). There are reports of the usefulness of biotyping for typing bacterial species and, in particular, *K. pneumoniae* (9). Traditional biotyping, using a separate tube of medium for each reaction, is time consuming and expensive. The API 20E system (Analytab Products Inc.) was evaluated in our laboratory for biotyping *K. pneumoniae* (5). We found more than one biotype among colonies tested from single specimens. Although some of the multiple biotypes appeared to be stable, some of the observed variation seemed due to variability in the system itself. Therefore, it was desirable to examine clinical specimens of *K. pneumoniae* for the existence of more than one subtype by another method.

Recently, Riser et al. (11) described an indirect fluorescent-antibody (IFA) test for capsular typing of *K. pneumoniae*. This procedure was reported as easy to perform, inexpensive, and rapid. Therefore, we examined the reproducibility of this system and tested for the presence of possible colony-to-colony variation similar to that which we observed with the API biotypes.

MATERIALS AND METHODS

Organisms and media. A set of the 72 reference

capsular type strains of *K. pneumoniae* was provided by I. Ørskov of the Statens Seruminstitut, Copenhagen. Fifty-five consecutive specimens containing more than five well-isolated colonies of *K. pneumoniae* which were received in the clinical microbiology laboratory of Saint Francis Hospital over a period of 2 months were examined. Worfel-Ferguson agar (Difco) was used to subculture *K. pneumoniae* before serotyping.

Antisera. Antisera for each of 17 pools and 72 individual serotypes were obtained from Difco. Pool 12 was not commercially available and was prepared from individual antisera 45, 46, and 47. Antisera were diluted in Britton-Robinson buffer, pH 9.0 (1). Fluorescein-conjugated anti-sheep serum was used (Wellcome).

Slides. Slides with 5-mm wells were prepared by dropping glycerol on the slides, spraying with Fluoro Glide (Chemplast), rinsing with tap water, and air drying. Bacterial suspensions were made by emulsifying single colonies from Worfel-Ferguson agar medium in 5 ml of phosphate-buffered saline, pH 7.3 (Microbiological Associates). A 20- μ l MLA precision pipette was used to drop the bacterial suspension on each well. This method consistently gives about 100 bacteria per $\times 43$ field. The slides were air dried and heat fixed.

Antisera and conjugate dilutions. Antiserum pools were diluted in Britton-Robinson buffer (pH 9.0) to a final dilution of 1:40. Individual antisera were diluted to a final dilution of 1:32. Changes in dilution to enhance fluorescence or resolve cross-reactions were made as necessary. The fluorescent conjugate was diluted in phosphate-buffered saline (pH 7.3) to 1:40. This dilution gave the best fluorescence with the two lots of conjugate used.

IFA procedure. The procedure of Riser et al. (10, 11) was followed. The slides were covered with 0.02 ml of the antisera to be tested and incubated at room temperature for 20 min. The slides were rinsed by placing them on a staining rack and flooding with running tap water every 2 min for 20 min. After air drying, the conjugate was added (0.02 ml/well), and the slides were incubated for 20 min. Slides were rinsed with running tap water, air dried, mounted, and examined with a $\times 43$ objective on an AO vertical fluorescent microscope with a BG12 exciter filter, an OG1 barrier filter, and a 50-W mercury vapor lamp as the excitation source. Fluorescence was graded as negative, 1+, 2+, 3+, or 4+. The strong reactions (3+ to 4+) were clearly distinguishable from weak reactions. The slides were read by two independent observers.

RESULTS

Fluorescence patterns of known serotypes. The 72 reference serotypes of *K. pneumoniae* were tested with 18 antiserum pools. Ten of the pools gave weak reactions with their corresponding serotypes and required supplementation with specific antisera to increase the reactions to 3+ to 4+ (Table 1). Cross-reactions occurred with both pools and individual antisera but were especially common with the antiserum

TABLE 1. *Specific antiserum supplementation of antiserum pools*^a

Antiserum pool	Specific antisera added
1	3
2	5
3	— ^b
4	—
5	18, 19
6	21
7	26
8	40
9	—
10	—
11	39
12	— ^c
13	49
14	55
15	—
16	62, 63
17	—
18	—

^a Each antiserum was added at a concentration of 1/40 in a 1/40 dilution of the pool.

^b —, No supplementation required.

^c Prepared with type-specific antisera 45, 46, and 47.

TABLE 2. *Cross-reactions of supplemented antisera pools with 72 reference serotypes of K. pneumoniae*

Antiserum pool	<i>K. pneumoniae</i> serotype	
	Homologous reactions	Heterologous reactions
1	1, 2, 3, 24	(68, 69) ^a
2	4, 5, 6, 7	(8)
3	8, 9, 10, 25	(7, 18, 19)
4	13, 14, 15, 16	(9, 22)
5	17, 18, 19, 20	40, 45 (12, 22, 41, 44)
6	11, 21, 22, 23	37 (24, 25, 39)
7	26, 27, 28, 30	(22, 24)
8	12, 29, 40, 41	(22, 24, 50, 63)
9	31, 32, 43, 44	(24, 25, 39)
10	33, 34, 35, 36	42 (9, 10, 11, 12, 20, 45, 49, 50)
11	37, 38, 39, 42	12, 22 (10, 20, 24, 45)
12	45, 46, 47	(12, 19, 37, 41)
13	48, 49, 50, 51	
14	52, 53, 54, 55	(39, 47)
15	56, 57, 58, 59	(24, 25, 43)
16	60, 61, 62, 63	(22, 24, 41, 45)
17	64, 65, 66, 67, 68, 69	36 (14, 18, 24)
18	70, 71, 72	(45, 46)

^a Reactions in parentheses are $\leq 2+$ fluorescence.

pools. As shown in Tables 2 and 3, the majority of these were weak reactions ($\leq 2+$). No attempt was made to resolve strong cross-reactions among the pools by dilution since none of the clinical isolates gave strong reactions to more than two pools.

Twenty-seven (37.5%) of the known serotypes gave cross-reactions with the type-specific antisera (Table 3). All but four of these were resolved by using a higher antiserum dilution (1:

TABLE 3. *Specific antisera reactions with 72 reference serotypes of K. pneumoniae*

Capsular type	Specific antisera giving positive reactions	Dilution to resolve cross-reactions	Remaining antisera giving positive reactions
1	1 ^a		1
2	2, (13, 30, 63, 69) ^b	1:64	2
3	3, 68, (63)	1:256	3, (68)
4	4		4
5	5 ^c		5
6	6 ^c		6
7	7, (1)	1:64	7
8	8		8
9	9		9
10	10		10
11	11, (33)	1:64	11
12	12, (29, 41)	1:128	12
13	13, (30)	1:64	13
14	14, (64, 66)	1:64	14
15	15		15
16	16		16
17	17		17
18	18		18
19	19		19
20	20, (19, 37)	1:256	20, (19, 37)
21	21 ^c		21
22	22, (37, 41)	1:64	22
23	23, 22	1:64	23
24	24		24
25	25		25
26	26 ^c		26
27	(28, 46)	1:256	(28, 46)
28	28, (27, 8)	1:128	28
29	29, (12, 24, 42)	1:128	29
30	30		30
31	31		31
32	32		32
33	33, (11)	1:64	33
34	34		34
35	35, (33, 34)	1:128	35
36	36		36
37	37, 22	1:64	37
38	38		38
39	39		39
40	40		40
41	41, (12)	1:64	41
42	42		42
43	43		43
44	44, (18, 20)	1:64	44
45	45, (46)	1:256	45, (46)
46	46, (27, 28)	1:64	46
47	47, (53)	1:64	47
48	48		48
49	49, (48)	1:64	49
50	50		50
51	51		51
52	52		52
53	53 ^c		53
54	54		54
55	55		55
56	56		56
57	57		57
58	58		58
59	59		59
60	60		60
61	61		61
62	62, (63)	1:256	62
63	63		63
64	64, 65	1:128	64
65	65		65

TABLE 3—Continued.

Capsular type	Specific antisera giving positive reactions	Dilution to resolve cross-reactions	Remaining antisera giving positive reactions
66	66, (64)	1:64	66
67	67		67
68	68		68
69	69, (63)	1:64	69
70	70, (72, 69)	1:64	70
71	71		71
72	72		72

^a Reaction $\geq 3+$ at an antiserum dilution of 1:32.^b Reactions in parentheses are $\leq 2+$ fluorescence at an antiserum dilution of 1:32.^c Dilution of 1:16.

64, 1:128, or 1:256). The cross-reactions of the remaining four were all $\leq 2+$. Five antisera required a lower dilution (1:16) to achieve a 3+ to 4+ reaction with their homologous type. The antiserum to type 27 did not react with its homologous type.

Fluorescence patterns of clinical isolates—colony-to-colony variation. Five well-isolated colonies were picked from each of 55 primary plates (MacConkey agar) of the clinical isolates and plated on Worfel-Ferguson agar. Reactions to the 18 pools and the indicated specific antisera were determined. Although colony-to-colony variation in the intensity of fluorescence was quite common, there was no variation in serotype. In some cases the variation was quite marked, ranging from 1+ to 4+ among the five colonies tested.

The reactions of the clinical isolates are listed in Table 4. Five (10%) of the clinical isolates had some colonies which gave strong reactions in two pools (isolates 12, 13, 44, 45, and 56). Ten (18%) clinical isolates gave cross-reactions with the specific antisera tested. Six of these were weak reactions and four were strong. Three of the four strong cross-reactions with the specific antisera were resolved by further dilution. Isolate 56 gave equal reactions in antisera to types 62 and 63 at all dilutions until no fluorescence was seen (1:512). Five (10%) isolates were non-typable.

Reproducibility. Lot-to-lot, day-to-day, and reader-to-reader variations were examined. Two antiserum pools and nine specific antisera of different lot numbers than the antisera described above were tested against the 72 known serotype strains. The patterns of cross-reactions as well as the required antiserum supplements in the pools varied from the original results and are listed in Table 5. Most of these variations were minor; however, the requirements for antiserum supplementation of the pools were different.

TABLE 4. Capsular serotyping of clinical isolates of *K. pneumoniae*

Isolate	Antiserum pool	Specific antisera giving positive reactions	Dilution to resolve cross-reactions	Remaining antisera giving positive reactions
1	1, ^a (5) ^b	3		3
3	6	23		23
4	12	46		46
7	11	38		38
8	6	21, (11)	1:128	21
9	1	2		2
10	14	55		55
11	17, (18)	65, 69	1:128	65
12	17, 12	68, (66)	1:128	68
13	17, 18	70, 72	1:128	72
14	(18)	71 ^c		71
15	4	13		13
16	11	38		38
17	12, (7)	46		46
18	1, (17)	3		3
19	11	38		38
20	11	39		39
21	(5, 13)		Nontypable	
22	10	33, (35)	1:64	33
23	11	42	1:128	42
24	16	60, (63)		60
26	9, (1)	31, (32, 44)		31
27	11	38		38
28	17, (1)	68		68
30	3	8		8
31	14	55		55
32	16	62		62
33	6 (5)	11		11
34	4	15		15
35	16, (2)	6 ^c		6
36	4	15		15
37	7	26		26
38	1	1		1
39	6	23		23
40	7	30		30
41	14, (1, 2)	54		54
42	16, (7)	60		60
43	Nontypable			
44	12, 14	47		47
45	12, 14	54, 47	1:128	47
46	16, (5)	60, (61, 62, 63)	1:64	60
47	3	9		9
48	Nontypable			
49	15	58		58
50	Nontypable			
51	18	70		70
52	(1, 13)	24		24
53	16	60		60
54	5	19		19
55	Nontypable			
56	1, 16	62, 63	1:256	62, 63
57	16	62		62
58	9	31		31
59	4	14		14
60	4	14		14

^a Reaction $\geq 3+$ at an antiserum dilution of 1:32.^b Reactions in parentheses are $\leq 2+$ fluorescence at an antiserum dilution of 1:32.^c Antiserum dilution of 1:16.

Also, two of the original specific antisera gave 3+ to 4+ fluorescence only when used at a 1:16 dilution, whereas the second lot of these antisera

tested could be used at the standard dilution of 1:32.

At the conclusion of the study, five clinical isolates were picked randomly and retested. The same serotype was obtained as previously. All slides throughout the study were read independently by both of us. There was 98% agreement between the two sets of readings.

Twelve strains were sent to the Connecticut State Health Department for serotyping. This reference laboratory screens *K. pneumoniae* isolates by slide agglutination, using antiserum pools 1 through 6 (Difco) and type-specific antiserum 12. Specific types are determined by the quellung test. Six of the 12 isolates (no. 1, 15, 16, 36, 57, and 14) reacted with from three to six of the pools and were not tested further. Three did not react with the antiserum pools tested. Antisera to two of these would not be in the pools used by the Connecticut State Health Department, but one (no. 59) was type 14, which is contained in pool 4. The other three isolates reacted in just one pool by agglutination, and the same serotype was obtained by quellung as by IFA.

Nine patients in this study had *K. pneumoniae* isolated on more than one occasion from the same or a different site (Table 6). Isolates of different serotypes were present in at least two cases (22%). In two other cases the second isolate was nontypable, so they may also have been different serotypes. In case 1, the specimens were from different sites, and in case 7 the patient was in two different areas of the hospital at the time each specimen was collected.

DISCUSSION

Fluorescent-antibody techniques have been useful for identification of organisms when cross-

TABLE 5. Lot-to-lot variation with *K. pneumoniae* antisera

Pool/type	Variable reactions among 72 reference strains of <i>K. pneumoniae</i> ^a	
	Lot 1	Lot 2
Pool		
1	(3) ^b	(43)
5	18, 19, 40, 45, (22)	(18, 19), (40, 45)
Type		
3	68	(68, 69)
5	5 ^c	5
6	6 ^c	6
7	(1)	(10)
10		(61)
1, 4, 8, 9	— ^d	—

^a Variation in intensity of fluorescence or dilution of antiserum.^b Reactions in parentheses are $\leq 2+$ fluorescence at an antiserum dilution of 1:32.^c Antiserum dilution of 1:16.^d —, No lot-to-lot variation.

reactions can be minimized. Riser et al. (11), by finding an appropriate pH (pH 9.0), were able to decrease the cross-reactions in the capsular serotyping of *K. pneumoniae* by IFA. They showed that types determined by IFA correlated well with those determined by traditional capsular swelling (10).

We were able to reproduce the technique satisfactorily with minimal variations in procedure. We found that a final dilution of conjugate of 1:400 was much too dilute. With the two lots of conjugate used, a dilution of 1:40 gave optimum fluorescence. This variation may be due to a difference in equipment. Riser et al. (10) used an inverted microscope with a tungsten halogen lamp, whereas we used a vertical fluorescent microscope with a mercury vapor lamp. The variables studied were found to have no significant effect in the reproducibility of the capsular serotypes.

Although colonial variation in intensity of fluorescence did occur, there was no serotype variation from colony to colony. Because of this variation and since all colonies from each specimen were the same type, several colonies should be selected for testing rather than isolated colonies.

TABLE 6. Serotypes of *K. pneumoniae* isolated more than once from the same patient

Patient	Isolate no.	Source	Time between cultures	Capsular serotype
1	4	Sputum	48 h	46
	12	Blood		68
2	16	Urine	48 h	38
	19	Urine		38
3	10	Sputum	2 weeks	55
	43	Sputum		Nontypable
4	21	Urine	48 h	Nontypable
	25	Urine		Nontypable
5	44	Wound	24 h	47
	45	Wound		47
6	42	Sputum	48 h	60
	48	Sputum		Nontypable
7	11	Sputum	4 days	65 ^a
	26	Sputum		31
8	56	Urine	48 h	62, 63 ^b
	57	Urine		62
9	59	Urine	48 h	14
	60	Urine		14

^a Patient 7 was moved after the first culture to another area of the hospital.

^b The cross-reaction between isolate 56 and antisera 62 and 63 could not be resolved by dilution.

Variation in the cross-reaction patterns of antisera from lot to lot occurred. Also, the supplementation pattern of the antiserum pools differed from those tested by Riser et al. (11). These results reinforce the necessity of testing each new lot of antiserum with antigen reference strains.

Twelve clinical isolates were submitted to the Connecticut State Health Department for typing, but clear-cut results were obtained with only three. The types determined for these three isolates were identical by both procedures. The cross-reactions they reported did not occur with IFA or were weak reactions ($\leq 2+$). The cross-reactions with the quellung reaction among the 72 reference strains are greatly decreased by IFA. Using the Difco antisera, Casewell (3) found 72 cross-reactions with the quellung test. Riser et al. found 26 with the IFA, and we found only four strong cross-reactions. There were an additional 41 weak heterologous reactions.

The variation of serotypes from repeat specimens from the same patient is not surprising. Rennie and Duncan (9) reported similar results with 8 of 37 (22%) patients with repeat specimens containing more than one serotype of *K. pneumoniae*. We reported that at least two of seven patients with repeat isolates of *Serratia marcescens* had different serotypes (12). It was suggested that these variations were due to either more than one serotype in an infection or a secondary infection or colonization with different subtypes of the same species. The lack of colony-to-colony variation in serotype of the 55 clinical isolates in this study tends to rule out infection with multiple types. We are presently examining the latter suggestion.

Unlike the results obtained with the API biotypes, only a single serotype was found among the colonies tested in each specimen. Thus, serotyping of *K. pneumoniae* is less variable than API biotypes and, therefore, more useful in epidemiological studies. It is still not clear whether the multiple biotypes in single specimens are due to true colony-to-colony differences or merely to variations in the technique. Preliminary data in our laboratory indicate that at least for *S. marcescens* the latter is true.

The IFA for capsular typing of *K. pneumoniae* showed only minimal variation from day to day and reader to reader and variation only in intensity of fluorescence from colony to colony, indicating that the procedure is quite reproducible. Although the IFA requires about the same amount of time as serotyping by capsular swelling, it is easier to perform and interpret and is about one-fourth the cost (10). The ease of performance, the low cost, and the excellent repro-

ducibility of the IFA makes this the method of choice for subtyping *K. pneumoniae*.

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