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New Method of Serotyping *Escherichia coli*: Implementation and Verification

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This paper describes the implementation and verification of a new method of serotyping *Escherichia coli* based on a mathematical analysis of the results of agglutination reactions between standard O antigens of *E. coli* and rabbit antisera raised against them. Details of the new method, which uses on average one-fifth of the serum used for the old method and is now in routine use in New Zealand, are given.

The problems associated with the serotyping of *Escherichia coli* since it was realized that very large numbers of different O groups are found in this species have been formidable. Thus, while there is a great demand for serotyping these organisms, not many laboratories are able to provide this service. Detailed analyses of the sera and the cross-reactions involved have been published in the past (4, 6) and have provided some guidance to serotyping. Despite these analyses, the problems of serotyping strains of *E. coli* remain formidable even when antisera to all currently described O types have been prepared. Semi-automatic equipment has been described (1). While it removes some tedium, it does not help to solve the major problem of analysis, which requires an understanding of the cross-reactions. More recently, the developments of microtiter techniques have permitted larger numbers of strains to be studied (5).

A mathematical analysis of the reciprocal titers between *E. coli* O antisera and the standard antigens has demonstrated that this information provides the basis for a new identification scheme (7). In this paper, we describe the implementation and verification of our new method of serotyping *E. coli* and compare it with the previous method.

MATERIALS AND METHODS

The antisera. Standard strains of *E. coli* representing all O groups from O1 to O163 as listed by Orskov et al. (6) were used for all antiserum production and testing. The antisera were raised in rabbits by a method based on that of Ewing (3) and were stored at −20°C. The sera were diluted for use in 0.85% (wt/vol) saline containing 1:10,000 (wt/vol) thiomersalate as preservative. The diluted sera were stored at +4°C.

TABLE 1. Components of the 15 pools used in the method of mixed pools

Components used in pool:														
A	B	C	D	E	F	G	H	J	K	L	M	N	P	Q
O1	O13	O3	O4	O5	O6	O8	O9	O11	O17	O24	O27	O37	O41	O14
O2	O19	O23	O12	O7	O10	O39	O20	O21	O29	O61	O45	O48	O58	O158
O35	O50	O26	O15	O49	O33	O60	O28	O22	O44	O95	O52	O54	O82	O159
O40	O129	O36	O16	O56	O57	O75	O34	O30	O53	O103	O66	O59	O98	O160
O51	O133	O38	O18ab	O65	O84	O78	O42	O32	O73	O107	O76	O81	O109	O161
O63	O135	O43	O25	O70	O88	O89	O85	O46	O77	O112ab	O79	O86	O111	
O64	O138	O62	O121	O71	O100	O92	O97	O55	O106	O112ac	O80	O87	O118	
O69	O147	O68	O123	O91	O124	O93	O99	O83	O108	O117	O101	O90	O149	
O74	O148	O102	O141	O114	O143	O96	O104	O113	O120	O136	O110	O119	O151	
O150	O155	O139	O142	O130	O146	O105	O115	O125	O132	O137	O116	O127	O163	
O154	O156	O152	O145	O157	O153	O126	O134	O131	O140	O144	O162	O128		

The O antigen suspensions used for all agglutination reactions were made by culturing the strains in nutrient broth at 37°C overnight, steaming for 1 h, and treating with 40% (vol/vol) Formalin to give a final concentration of 0.05% (vol/vol) formaldehyde.

All sera, diluted at 1:50 in thiomersalate, were tested against all *E. coli* standard antigens. They had all been tested by the semi-automatic method (1). Since then, microtiter trays have been found to be more convenient and are now in standard use at the National Health Institute. The sera were retested for this study to provide a consistent up-to-date data set to analyze. Microtiter trays were used for the retesting, since they would be used for the routine tests. Sera giving a positive agglutination with an antigen at a final concentration of 1:100 were then titrated by doubling dilutions against that antigen. Thus a response matrix giving the reciprocal titers of all reactions between the sera and the standard antigens was built up.

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TABLE 2. A selection of the 196 patterns of reactions with the 31 key sera and the associated end groups[a]

Pattern no.	Key sera																
	O1	O2	O3	O4	O5	O13	O15	O19	O22	O25	O50	O53	O54	O57	O60	O68	O74
1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
21	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
22	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
23	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
24	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
35	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
36	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
37	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
38	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
69	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
70	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+	–
71	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+	–
181	–	+	–	–	–	+	–	–	–	–	+	–	–	–	–	+	–
182	–	+	–	–	–	+	–	–	–	–	+	–	–	–	–	+	–
183	–	+	–	–	–	+	–	–	–	–	+	–	–	–	–	+	–
184	–	+	–	–	–	+	–	–	–	–	+	–	–	–	–	+	–
191	+	+	–	–	–	+	–	–	–	–	+	–	–	–	–	+	–
192	+	+	–	–	–	+	–	–	–	–	+	–	–	–	–	+	–
193	+	+	–	–	–	+	–	–	–	–	+	–	–	–	–	+	–
194	+	+	–	–	–	+	–	–	–	–	+	–	–	–	–	+	–
195	+	+	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–
196	+	+	–	–	–	+	–	+	–	–	+	–	–	–	–	–	–

TABLE 2. A selection of the 196 patterns of reactions with the 31 key sera and the associated end groups[a]

Key sera														*P*	Serogroup
O77	O89	O90	O106	O112ac	O118	O125	O131	O133	O136	O147	O148	O157	O162		
−	−	−	−	−	−	−	−	−	−	−	−	−	−		
−	−	−	−	−	−	−	−	−	−	−	−	−	+	0.260	O76
														0.260	O101
														0.221	O116
														0.260	O162
−	−	−	−	−	−	−	−	−	−	−	−	+	−	0.085	O51
														0.388	O56
														0.058	O61
														0.013	O92
														0.456	O157
−	−	−	−	−	−	−	−	−	−	−	+	−	−	0.422	O62
														0.016	O138
														0.562	O148
−	−	−	−	−	−	−	−	−	−	+	−	−	−	0.750	O138
														0.250	O139
−	−	−	−	−	−	−	−	−	−	+	+	−	−	0.750	O138
														0.250	O139
−	−	−	−	−	−	−	−	−	+	−	−	−	−	0.429	O117
														0.571	O136
−	−	−	−	−	−	−	+	−	−	−	−	−	−	0.301	O32
														0.354	O46
														0.045	O61
														0.301	O113
−	−	−	−	−	−	−	+	−	−	−	−	+	−	1.000	O61
−	−	−	−	−	+	−	−	−	−	−	−	−	−	0.192	O38
														0.144	O41
														0.163	O82
														0.144	O111
														0.192	O118
														0.163	O151
−	−	−	−	−	+	−	−	−	−	−	−	+	−	1.000	O70
−	−	+	−	−	−	−	−	−	−	−	−	−	−	0.270	O86
														0.270	O90
														0.230	O127
														0.230	O128
+	−	−	+	−	−	−	−	−	−	−	−	−	−	0.260	O17
														0.221	O44
														0.260	O77
														0.260	O106
−	−	−	−	−	−	−	−	−	−	−	−	−	−	0.058	O26
														0.925	O79
														0.018	O141
−	−	−	−	−	−	−	−	−	−	+	−	−	−	1.000	O139
−	−	−	−	−	−	−	−	−	−	+	+	−	−	1.000	O139
−	−	−	−	−	−	−	−	−	−	−	−	−	−	0.738	O107
														0.262	O138
−	−	−	−	−	−	−	−	−	−	−	+	−	−	0.842	O107
														0.158	O138
−	−	−	−	−	−	−	−	−	−	+	−	−	−	1.000	O138
−	−	−	−	−	−	−	−	−	−	+	+	−	−	1.000	O138
−	−	−	−	−	−	−	−	−	−	+	+	−	−	1.000	O139
−	−	−	−	−	−	−	−	−	−	−	−	−	−	1.000	O26
−	−	−	−	−	−	−	−	−	−	+	−	−	−	1.000	O139
−	−	−	−	−	−	−	−	−	−	+	−	−	−	1.000	O50
−	−	−	−	−	−	−	−	−	−	+	−	+	−	1.000	O50
−	−	−	−	−	−	−	−	−	−	+	+	−	−	1.000	O50
−	−	−	−	−	−	−	−	−	−	+	+	+	−	1.000	O50
−	−	−	−	−	−	−	−	−	−	+	−	−	−	1.000	O50
−	−	−	−	−	−	−	−	−	−	+	−	+	−	1.000	O50
−	−	−	−	−	−	−	−	−	−	+	+	−	−	1.000	O50
−	−	−	−	−	−	−	−	−	−	+	−	−	−	1.000	O129
−	−	−	−	−	−	−	−	−	−	+	−	−	−	1.000	O129

[a] Possible serogroups for the unknown strain if all the results of the day 1 tests are negative are (where asterisks denote antigens that cannot be identified by the 31 key sera) O6, *O9, *O10, O11, *O14, *O20, O21, *O24, *O27, *O28, *O29, *O30, O32, O33, O35, *O37, *O39, *O40, O41, *O42, *O43, *O45, *O49, O51, *O52, *O55, O56, *O58, *O59, O61, O62, *O66, *O69, *O75, *O80, *O81, O82, *O84, *O85, O87, *O88, *O91, O92, *O93, *O95, *O96, *O97, *O98, *O99, *O100, *O103, *O104, *O105, *O108, *O109, *O110, O111, O112ab, O113, O114, *O115, O116, O117, *O119, *O120, O121, O123, *O124, O126, O127, O128, *O130, *O134, *O137, O138, O141, *O142, *O144, *O146, O149, O151, *O153, *O154, *O155, *O156, O158, *O159, O160, *O161, and *O163.

TABLE 3. Arrangement of sera into 11 pools for the second stage of screening

Pool	Sera in pool:										
	A	B	C	D	E	F	G	H	I	J	K
A	O10	O14	O20	O24	O27	O28	O30	O37	O40	O49	O41
B	O14	O55	O58	O59	O66	O69	O75	O80	O81	O84	O111
C	O20	O58	O85	O88	O91	O93	O95	O96	O97	O98	O117
D	O24	O59	O88	O99	O100	O103	O104	O108	O109	O110	O121
E	O27	O66	O91	O100	O115	O119	O120	O124	O130	O134	
F	O28	O69	O93	O103	O119	O137	O142	O144	O146	O153	
G	O30	O75	O95	O104	O120	O142	O154	O155	O156	O159	
H	O37	O80	O96	O108	O124	O144	O155	O161	O163	O29	
I	O40	O81	O97	O109	O130	O146	O156	O163	O43	O62	
J	O49	O84	O98	O110	O134	O153	O159	O29	O62	O52	
K	O41	O111	O117	O121							

The method of mixed pools. Methods involving the use of mixed antiserum pools for the initial screening of an unknown strain are regularly used in many laboratories. On the basis of past experience of the cross-reactions, such a set of antiserum pools was developed at the National Health Institute. Principles similar to the ones previously followed by Bettelheim (K. A. Bettelheim, Ph.D. thesis, University of London, London, England, 1969) were applied. Since the microtiter trays are arranged as 12 rows by 8, 15 pools containing no more than 11 sera each were developed. Two trays could be used to test up to 12 strains against the pools, with row 16 available for control purposes. For each pool that reacted positively with the unknown strain, the strain would be tested against the components of the pool on the next day. Since each pool contained no more than 11 sera, this second phase of screening fitted conveniently onto the 12-by-8 trays by using 11 rows for the components of each pool and allowing row 12 to be used for control purposes. Thus, for example, the components of eight pools could be tested on one tray. The arrangement of pools is shown in Table 1. For convenience, the pools were given letters of the alphabet to distinguish them. This was the current method of *E. coli* identification prior to the introduction of the new method described below.

While many successful investigations have been performed with these pools, it was obvious that their efficiency was very limited. Many unknown strains reacted with more than one pool, the average being around four. Therefore, a very large number of tests and a very large quantity of serum had to be used before an O identification was achieved.

The new method. It was considered that an analysis of the cross-reactions might provide a useful basis from which to build an identification scheme for *E. coli* O antigens. Such a method has recently been developed by Thompson (7). The method involved the use of a computer program for taxonomic identification to identify sera which could be used singly as key sera for an initial screening of the unknown strain. This method made positive use of the cross-reaction information. Strains failing to react in any of the key sera would belong to serogroups with few cross-reactions and hence would be identifiable through a system of intersecting pools.

When applied to the data pertaining to the current *E. coli*

TABLE 4. Arrangement of sera into seven pools for the final stage of screening

Pool	Sera in pool:						
	L	M	N	O	P	Q	R
L	O6	O11	O21	O32	O33	O35	O42
M	O11	O45	O51	O56	O61	O82	O87
N	O21	O51	O92	O112ab	O113	O114	O116
O	O32	O56	O112ab	O126	O123	O127	O128
P	O33	O61	O113	O123	O138	O141	O149
Q	O35	O82	O114	O127	O141	O151	O158
R	O42	O87	O116	O128	O149	O158	O160

TABLE 5. Results of 10 strains reacting in the 31 key sera showing pattern match and subsequent identification

Test strain	Positive agglutination in key sera	Pattern no.	Possible identity	Associated probability	O type confirmed by titration
1	O25, O68, O147, O148	41	O25	1.00	O25
2	O89	17	O78 O89 O92	0.49 0.49 0.03	O78
3	O77	19	O126 O44	0.85 0.15	O126
4	O5	93	O5 O65 O71 O33 O114	0.21 0.21 0.21 0.18 0.18	O33
5	O60	25	O8 O60 O92	0.49 0.49 0.01	O8
6	O13, O19, O133	81	O133	1.00	O133
7[a]	(O4), (O13), (O50), O77, O106	21	O106 O77 O17 O44	0.26 0.26 0.26 0.22	O17
8	O77, O106	21	O106 O77 O17 O44	0.26 0.26 0.26 0.22	O77
9	O3, O68	139	O152 O3 O141	0.47 0.47 0.07	O3
10	O5, O68	96	O7	1.00	O7

[a] For test strain 7, reactions given in parentheses were weak.

TABLE 6. Progress of serotyping by the new method

O type confirmed by titration[a]	No. of strains	Pattern no. from Table 2	Pools from Table 3	Pools from Table 4
O1	9	188		
O2	2	172		
	1	173		
	1	165		
O3	4	139		
O4	1	117		
O6	1	29		
	1			L
O7	10	96		
O8	5	25		
*O9	1		D, G	
O11	1			L, M
O17	1	21		
O20	3		A, C	
O21	1	43		
O23	1	138		
O25	3	42		
	4	41		
O26	1	70		
O32	1	8		
O33	2	93		
O38	1	13		
O41	11		A, K	
O44	3	21		
	1	19		
O50	1	193		
O51	1	32		
O58	3		B, C	
O61	4	9		
O64	2	187		
O66	1		B, E	
O68	1	130		
	2	131		
O70	1	98		
	1	94		
O75	4		B, G	
O77	4	21		
O78	6	17		
O81	1		B, I	
*O82	1	13		
	1		E, H	
O86	1	16		
O88	1		C, D	
O89	2	17		
O92	1	18		
O93	1		C, F	
O101	1	2		
O103	1		D, E	
O107	1	35		
O111	2		B, K	
O112ab	1	15		
O114	1	93		
O116	1	2		
O117	1		C, K	
O118	2	13		
O119	1		E, F	
O120	1		E, G	
O123	1	100		
O125	1	10		
O126	17	19		
O127	1	16		
O128	1			O, R
O129	2	196		
O130	1		E, I	
O131	1	11		
O132	1	100		
O133	1	81		
O134	1	85		
	1		E, J	
O135	1	75		
O137	2		F	
O138	3	38		
O139	7	72		
O141	1	146		
	1		P, Q	
O147	1	88		
O150	3	164		
O153	3		F, J	
O154	2		G	
O157	1	3		
O162	1	2		
O163	2		H, I	

[a] Results marked with an asterisk were identified by means of cross-reactions in day 2 pools.

O antigen collection at the National Health Institute, 31 key sera were found. They are O1, O2, O3, O4, O5, O13, O15, O19, O22, O25, O50, O53, O54, O57, O60, O68, O74, O77, O89, O90, O106, O112ac, O118, O125, O131, O133, O136, O147, O148, O157, and O162. As an outcome of the analysis, a list of 196 possible patterns of reactions against the key sera was generated. For each pattern, a set of possible identifications was given. Some O groups will be identified by more than one pattern of reactions against the 31 key sera. Some patterns will indicate more than one possible solution for the O group. When an end group has more than one member, it is given as a list of possible O groups with their associated probabilities within the end group (7). This system of key sera may identify 100 antigens, not just the 31 whose sera are used. Table 2 presents a selection of the 196 reaction patterns for the purpose of illustrating how the system is used. For example, a strain which gives positive agglutinations in O68 and O13 and negative reactions in all the rest of the key sera would match pattern 70, and this pattern identifies the strain as O26 (Tables 2 and 6). Similarly, a strain which gives positive agglutinations only in O77 and O106 would match pattern 21. In this example Table 2 indicates a set of possibilities for this pattern. The possible O types are O17, O77, O106, and O44, with corresponding probabilities of 0.26, 0.26, 0.26 and 0.22 (i.e., they are all approximately equally likely endpoints for this pattern). Negative reactions in the key sera can be as important diagnostically as positive reactions, as, for example, in pattern 35, which is characterized by a single positive agglutination in O50 and requires definite negative reactions in the remainder of the key sera (Table 2). Note that this does not identify the strain as O50. The list of possibilities for this pattern is O107 with a probability of 0.74 and O138 with a probability of 0.26 (Table 2). In this case, O107 is more likely than O138. Identification of the unknown strain as O50, however, would require positive reactions to O2, O13, O68, and O147 as well as O50 and could also involve some other reactions. There are in fact eight patterns that lead to an identification of O50. Strains which reacted with none of these sera would belong either to O groups given in the footnote to Table 2 or to new, as yet unidentified, groups. The antigens marked with an asterisk in the footnote to

Table 2 are those which cannot be identified by the key sera. The remainder are antigens whose cross-reactions in the key sera are at low levels and hence may fail to be observed in the initial screening.

If all the results of the experiment are negative, a second-stage screening process is required. Two sets of pools of sera were constructed on the principle of the intersecting pools previously applied to H sera (2). The first was constructed from the sera corresponding to antigens which were unlikely to be identified by the key sera. The second set of pools covered all the remaining possibilities. Further details of the pool selection system are contained in reference 7. The components of the two sets of pools are listed in Tables 3 and 4.

The program of identification is thus to first test a strain against the 31 key sera and, if negative, against the set of pools listed in Table 3. If still negative, the unknown strain would be tested against the set of pools listed in Table 4. On the basis of these reactions, a small set of possible identifications will be obtained. The average set size is less than 2 with a range of 1 to 6. The O serotype for the unknown strain is finally found by titration against the sera belonging to each of the members of the set. In the arrangement of pools given in Table 3, there are 59 primary intersections. This system will in fact identify 67 antigens by virtue of the cross-reaction information in the system (7). In both cases, these pools are operated at a 1:400 dilution to conserve serum and to increase accuracy by suppressing the few low-level cross-reactions.

To evaluate this method, a series of strains previously identified by the old methods was subjected to analysis by the new method.

RESULTS

Table 5 presents a selection of typical results for which a pattern match was found with the key sera. Table 6 lists the results of identifying 180 strains, showing the stage of the process at which the small set of possible identifications was obtained. The O types, as subsequently confirmed by titration, are also given. The strain marked with an asterisk in Table 5 gave strong agglutinations in sera O77 and O106 and only weak reactions in sera O4, O13, and O50. The strong reactions indicated pattern 21 and identified the strain, while the other reactions, which were clearly weaker, were probably due to partial roughness. The three sera, O4, O13, and O50, did not react with the standard O17 antigen in the original testing on which the input data matrix was based, and hence a pattern corresponding to these five antigens being positive while all the rest were negative did not occur in the diagnostic table. Of the 180 strains identified, 132 (73%) were ready for titration after 1 day, 45 (25%) needed the second day of screening, and only 3 (2%) needed the day 3 pools. Of the 45 strains requiring screening through day 2 pools, 43 were the obvious intersecting member of the positive pools. The two strains indicated in Table 6 with an asterisk proved to be the second-choice O groups arising from cross-reactions in the positive pools and not the primary intersecting member (for clarification of this process, see reference 7).

DISCUSSION

The implementation of the new O serotyping method proved very straightforward. When verified against 180 previously tested strains, the new method gave 100% correct results. The first stage in an identification program using the old method suffered from a major drawback in addition to being extravagant. It was difficult to maintain effective control over the efficacy of all the sera in all the pools. Each pool had to be regularly tested against the 11 or so different standard antigens, corresponding to the sera it contained. In the new method (7), the efficacy of the sera can be individually controlled with each batch that is studied, since the primary screen consists of 31 single sera. There is also the advantage that in a significant percentage of cases, this first stage of screening with the 31 key sera should be able to provide a short list of the possible O types in only 1 day. This assertion is supported by the results given in Table 6, showing that 73% of the test strains could be given a probable identity in 1 day.

For many epidemiological studies, in which large numbers of strains must be examined and perhaps only the predominant types or some specific types are sought, the results from the first stage of screening may give useful preliminary results in 24 h. This aspect of the new method broadens the fields of application for serotyping *E. coli*. If in a study a serotype is sought which is not listed in the diagnostic table (i.e., it is one of the types marked with an asterisk in the footnote to Table 2), the second stage of screening through the pools listed in Table 3 can be done directly.

We have shown that the new serotyping method can be implemented and verified that it works well. It also offers many advantages over the current method because key sera can be used singly to identify many O types. Other sera with very few cross-reactions can be used efficiently in intersecting pools to identify the remainder.

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