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Evaluation of a Simplified Procedure for Serotyping *Campylobacter jejuni* and *Campylobacter coli* Which Is Based on the O Antigen

S. D. MILLS, R. V. CONGI, J. N. HENNESSY, AND J. L. PENNER*

Department of Microbiology, Banting Institute, University of Toronto, 100 College Street, Toronto, Ontario, Canada M5G 1L5

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A simplified procedure for serotyping *Campylobacter jejuni* and *Campylobacter coli* on the basis of thermostable antigens was developed and tested for its applicability as a routine typing method. The assay involves the sensitization of erythrocytes with an antigenic extract and performance of a slide agglutination assay with specific antisera. In order to simplify the typing system to a greater extent, the standard typing antisera were pooled into nine groups for *C. jejuni* and four groups for *C. coli*. The five antiserum samples allocated to each pool were selected so that pairs or groups of cross-reacting antisera were included in the same pool. When this system was tested with the serotype reference strains, it was found that, in most cases, a strain reacted in only one pool. The specific serotype of that strain could then be further defined by typing in each of the antisera belonging to that pool. To evaluate the specificity of the simplified method, 246 clinical isolates of *C. jejuni* and 57 clinical isolates of *C. coli* were typed at the same time by the standard passive hemagglutination assay and by the rapid slide agglutination system. Although both schemes effectively differentiated isolates and results from both schemes were generally very similar, differences were noted for a few isolates. On the basis of these findings, the simplified procedure may be recommended as an alternative means for serotyping these species for epidemiological purposes.

A serotyping scheme based on the thermostable somatic O antigens has been developed for *Campylobacter jejuni* and *Campylobacter coli* (9, 10). Classical serological techniques could not be used for identifying this class of antigens, because heated cell suspensions tended to autoagglutinate and antisera prepared against them gave numerous nonspecific reactions (9). However, through the use of heat-extracted antigenic material and the passive hemagglutination assay (PHA), isolates of both species could be differentiated, and this made serotyping on the basis of thermostable antigens feasible. Although the original system was developed for both *C. jejuni* and *C. coli* (9), it was later divided into separate schemes for each species, but the original designations of the O serotypes were retained (10). The present serotyping system comprises 42 serogroups of *C. jejuni* and 18 of *C. coli*. With this protocol, the isolates can also be defined according to their titer profiles, i.e., the titers of the reactions they produced in the individual typing antisera. The serotyping procedure has been tested for its applicability in epidemiological studies and has been shown to separate effectively epidemiologically linked isolates from others that were unrelated (8).

Interest in the thermostable antigens has led to their investigation at the molecular level. It has been shown that they possess properties similar to the somatic (O) antigens of other gram-negative bacteria in their wide range of immunological specificities, thermostability, ability to sensitize mammalian erythrocytes, and the methods that can be used for their extraction (1, 9). They occur in the outer membrane (3), antibodies are produced against them in infected patients (5), and evidence has been presented that they are in fact lipopolysaccharide (LPS) in nature (1, 6, 11). However, unlike the serotypes which define the family *Enterobacteriaceae*, approximately two-thirds of the *C. jejuni* serostrains (serotype reference strains) have only low-M_r-type LPS similar to rough or semirough LPS, and only one-third possess high-M_r smooth-type LPS (12). In contrast, all *C. coli* serostrains possess both low-M_r and high M_r LPSs (4). Despite the preponderance of low-M_r-type LPSs, there is evidently sufficient structural diversity to define a large number of serotypes (11).

In order to use this serotyping system in clinical laboratories, the labor-intensive, time-consuming PHA technique must be simplified. In this study, a simpler method to detect the LPS specificities by slide agglutination (SA) was evaluated, and the results that it produced were compared with those obtained by the use of the PHA method.

MATERIALS AND METHODS

Bacterial strains and cultural conditions. The *C. jejuni* and *C. coli* strains used in this study were serostrains of the serotyping system based on heat-stable LPS antigens (10). The clinical isolates were from collections sent by investigators for serotyping. Stock cultures were maintained in a solution consisting of glycerol (40%) and sodium citrate (3%) at −70°C and were grown on blood agar plates (Columbia agar base; Oxoid Ltd., London, England) with 7% oxalated horse blood (Woodlyn Laboratories Inc., Guelph, Ontario, Canada) in an atmosphere containing 7% CO_2 at 37°C for 48 h.

Antisera. The antisera used for passive hemagglutination and SA were prepared previously (9) by injecting rabbits intravenously with saline bacterial suspensions of the 42 *C. jejuni* and 18 *C. coli* serostrains.

PHA. The previously described (9) PHA technique was used. Briefly, antigenic extracts were prepared by harvesting

* Corresponding author.

TABLE 1. Compositions of antiserum pools

Species	Pool	Serotypes of antisera in the pool
C. jejuni	I	1, 44, 8, 17, 27
	II	2, 2, 10, 19, 55
	III	3, 3, 11, 18, 32
	IV	4, 13, 16, 43, 50
	V	5, 31, 33, 35, 38
	VI	6, 7, 12, 40, 41
	VII	9, 15, 21, 42, 45
	VIII	36, 23, 53, 29, 52
	IX	22, 37, 57, 58
C. coli	I	5, 24, 34, 30, 47
	II	20, 25, 28, 46, 49
	III	26, 39, 48, 51, 59
	IV	14, 54, 56

confluent bacterial growth from blood agar plates in saline (0.85% [wt/vol] NaCl; 1 ml per plate), and the suspension was heated for 1 h at 100°C. After cooling to room temperature, the cell suspension was centrifuged (8,000 × *g*, 10 min) and the supernatant containing the thermostable soluble antigen was removed. For titration of the antisera, the supernatant was diluted 1:10 in isotonic phosphate-buffered saline (IPBS; 10 mM Na_2HPO_4 [pH 7.0] in 0.85% [wt/vol] saline) and was then mixed with an equal volume of 1% washed sheep erythrocytes. After incubation at 37°C for 1 h, the sensitized erythrocytes were centrifuged (2,000 × *g*, 10 min), washed three times in the original volume of IPBS, and resuspended in IPBS to a volume equivalent to 0.5% erythrocytes. Twofold dilutions of the antisera were performed with a medimixer (Flow Laboratories, Inc., McLean, Va.) in microtitration plates (Dynatech Laboratories Inc., Alexandria, Va.) with U-shaped wells containing 25 μl of IPBS. Sensitized erythrocytes (25 μl) were added to each well of diluted antisera, and the plates were incubated at 37°C for 1 h and were then stored overnight at 4°C. Agglutination of the erythrocytes was indicative of a positive reaction, and the highest dilution showing agglutination of the erythrocytes was taken as the titer. The initial dilution of the antisera was 1:40.

Slide agglutination of antigen-sensitized erythrocytes. Washed sheep erythrocytes were sensitized with the heated cell extracts for 1 h at 37°C as described above. Without further washings, a volume of 50 μl of the sensitized erythrocytes was mixed on a glass slide with an equal volume of antiserum diluted 1:20 in IPBS. The slides were gently rocked for 5 min and were examined at intervals for agglutination of the erythrocytes.

Antiserum pools. The compositions of the pools for *C. jejuni* and *C. coli* are shown in Table 1. Five unabsorbed antiserum samples diluted 1:5 were included in each pool, except for *C. jejuni* pool IX, which had only four antiserum samples, and *C. coli* pool IV, which had only three antiserum samples. The antisera allocated to each pool were selected so that pairs or groups of antisera that were known to cross-react (i.e., O:23 with O:36, O:1 with O:44) were included in the same pool. The pooled antisera had a shelf-life of at least 6 months if they were maintained at 4°C.

RESULTS

Use of serostrains to test the specificity of SA. Heated cell extracts were prepared from the 60 serostrains and used to sensitize washed sheep erythrocytes (SA reagent) by using the standard technique developed for PHA. Although it is standard protocol in the PHA system to wash the sheep erythrocytes three times after being sensitized, this step was not carried out for the SA test because preliminary experiments indicated that it was unnecessary. Antiserum dilutions of 1:20 were found to cause strong homologous agglutination reactions for all of the *C. jejuni* reference strains (Fig. 1). Antisera O:9 and O:21 reacted very weakly and often unreproducibly with many of the *C. jejuni* serostrains in the SA test (Fig. 1). Corresponding reactions were never observed in the PHA test and could be differentiated readily from the much stronger, more rapid homologous reaction. Known cross-reactions determined by the PHA technique among serostrains O:4, O:13, O:16, O:43, and O:50; between O:6 and O:7; and between O:23 and O:36 were also seen with SA tests (Fig. 1). One cross-reaction between O:29 and O:53 and a unilateral reaction between O:12 and O:40 were observed by the SA but not by the PHA test.

Similar experiments were performed with the serostrains of *C. coli* (Table 2). Homologous reactions were strong for all but two strains, O:28 and O:5. More weak and unreproducible cross-reactions were observed among *C. coli* than among *C. jejuni* strains. These included reactions of O:14 with O:49 and O:47; reactions between O:28 and O:59; reactions of O:30 with O:34 and O:47; reactions of O:54 with O:24, O:25, O:28, and O:59; and reactions between O:59 and O:26.

Since the majority of serostrains were identified correctly by strong reactions in homologous antisera, the SA system for both *C. jejuni* and *C. coli* was examined in further studies for its applicability in serotyping isolates of these two species.

Evaluation of the use of antiserum pools in SA tests. The compositions of the antiserum pools for *C. jejuni* and *C. coli* are given in Table 1. Before use in typing, each pool was tested by slide agglutination against antigens from each of the serostrain's reference strains to ascertain that each reacted only in the corresponding pool. It was found that for some low-titer antisera (antisera O:2 and O:3 in pools II and III, respectively), double concentrations were necessary to produce strong agglutinations. When pooled antisera were used to type serostrains of *C. jejuni*, all of the strains (except O:60, which was not included in this experiment) reacted strongly with the pool containing the homologous antisera (data not shown). Four strains (O:11, O:17, O:18, and O:44) reacted weakly in a second pool but did not react in any of the unpooled antisera belonging to that second pool. These weak reactions were easily distinguished from the strong reactions observed with the pool containing the homologous antisera.

Similar tests were performed with the four pools of *C. coli* antisera and the 18 *C. coli* serostrains (Table 2). Each serostrain agglutinated strongly in the pool containing the homologous antiserum, but seven also agglutinated in two or more pools. Six of the latter serostrains were also agglutinated in heterologous unpooled antisera, but these reactions were weak and the serostrains were readily differentiated from the strong homologous agglutinations with the exception of O:30, which produced strong reactions in two heterologous antisera (Table 2).

With the use of pooled antisera, only 13 to 14 SA tests were required to identify the serotype of a *C. jejuni* isolate and 7 to 9 tests were required for a *C. coli* isolate. In comparison, 42 SA tests for a *C. jejuni* isolate and 18 SA tests for a *C. coli* isolate were required when unpooled

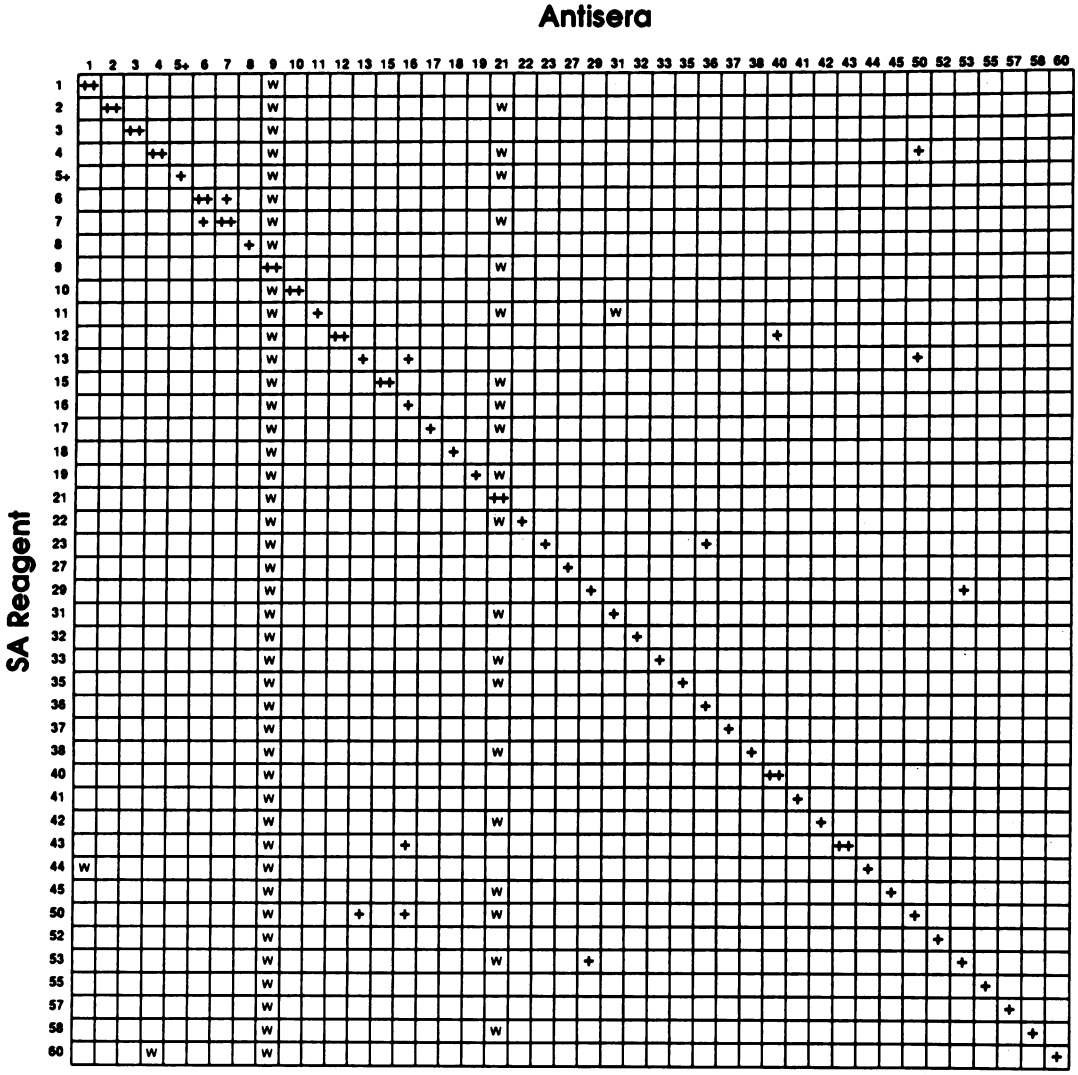

FIG. 1. Results of slide agglutination tests of sheep erythrocytes sensitized with heated cell extracts from serostrains (SA reagent) and then reacted with antisera prepared against whole cells (antisera diluted 1:20). A negative test is indicated by a blank space. ++, very strong agglutination that occurs almost immediately; + strong agglutination that occurs within 5 min; w, weak agglutination.

antisera were used separately. The substantial reduction in time, work load, and volume of antisera are clear advantages of the use of pooled antisera for serotyping these species.

Comparison of SA with PHA for serotyping isolates of *C. jejuni*. Clinical isolates were tested by PHA and then by SA with both pooled antisera and with each of the unpooled antisera that belonged to the pool in which the isolate agglutinated. The results for 246 isolates are shown in Tables 3 and 4. Identical serotypes were obtained by both methods for 205 (83%) of the isolates (Table 3). Of the 17% that were found to give different reactions, more than half (23 of 41) were of a minor nature attributable to weak reactions and cross-reactions (Table 4). These results occurred with antisera that had cross-reacting PHA titers of 1:80 to 1:160 and which failed to produce corresponding positive tests by SA. Approximately half of such results (12 of the 23 isolates) occurred for isolates of serogroups O:4, O:13, O:16, O:43, and O:50 that agglutinated in antiserum pool IV. Two separate isolates, one of O:4 and the other of O:10, which produced PHA titers of 1:320 and 1:640, respectively, were not typeable by SA. Three that were untypeable by PHA agglutinated weakly by slide agglutination—one in both O:6 and O:7 antisera, one with O:18 antiserum, and another with O:19 antiserum. The remaining 13 isolates were agglutinated by SA but did not produce corresponding reactions in PHA. This group included five isolates agglutinated by the O:9 antiserum; two by the O:2 antiserum; and one isolate by each of the O:3, O:21, O:29, O:31, O:38, and O:42 antisera.

Comparison of SA with PHA for serotyping *C. coli* isolates. Fifty-seven clinical isolates of *C. coli* were serotyped by both PHA and SA (Table 5). Of the 57 (79%) isolates, 45 (79%) were determined to be the same serotype by both methods. Of the remaining 12, 2 were typeable by SA but not by PHA, 1 with O:24 antiserum and the other with O:34 antiserum. One isolate showed a weak cross-reaction with O:39 by SA but not by PHA, although two other determinants that cross-reacted with serotypes O:5 and O:24 were detected by both methods. For nine isolates, antigens were detectable by PHA but not by SA, even though the PHA titers were relatively high, ranging from 1:320 to 1:5,120. The O-serotype groups to which these isolates belonged were O:24, O:26, O:30, O:34, O:39, O:47, O:48, O:54,59, and

TABLE 2. Comparison of PHA and SA by using *C. coli* serostrains

Serostrain	Serotype[a]	SA reactions in[b]:	
		Antiserum pools	Unpooled antisera
PC330	5	I_w	5_w
Z2	14	I_w, IV	14, 47_w
MK100	20	II	20
PC66	24	I	24
PC70	25	II	25
PC67	26	III	26
PC262	28	II_w, III	28_w, 59_w
MK219	30	I, IV	30, 34, 47
PC347	34	I	34
PC285	39	III	39
VanH13	46	II	46
Ca72	47	I_w	47
Ca77	48	III_w	48
A1618	49	I, II, III_w, IV	49, 47_w
PC228	51	III, IV	51
PC354	54	I, II_w, IV	54, 24_w, 25_w, 28_w
RO268	56	IV	56
PC349	59	II_w, III_w	59, 26_w

[a] Serotype of serostrain as previously defined by PHA (10).
[b] w, weak reaction.

O:56,59. It was therefore evident that inconsistencies between the two methods were not limited to a few serotypes, as in *C. jejuni*, but were more extensive and included a variety of unrelated serotypes.

DISCUSSION

The classical method for serotyping gram-negative bacteria on the basis of their O antigens involves heating bacterial suspensions to inactivate cell surface components such as flagellar proteins and thermolabile capsular materials before testing for agglutination with specific antisera (2). However, heated cell suspensions of *C. jejuni* tend to autoagglutinate, and therefore, the PHA procedure was adopted for O serotyping *C. jejuni* and *C. coli* (9, 10). The enhanced specificity of this test was due to the receptors on the erythrocyte membrane which bind LPS molecules via the fatty acid moieties of the lipid A component (13). A greater sensitivity was noted, probably because of the larger size of the antigen-carrying erythrocytes, thereby causing more readily visible aggregations (7).

Although PHA can be partially automated, it is still a lengthy and labor-intensive procedure, limiting its use mostly to larger reference centers. To simplify the procedure but to take advantage of its specificity and sensitivity, and therefore to make the practice of serotyping *C. jejuni* and *C. coli* more attractive to clinical laboratories, a procedure was examined in which the titration step was replaced by SA. To reduce the number of SA tests required to define the O-serotype specificity of a strain, the applicability of pooled antisera was also determined.

While evaluating the usefulness of SA by testing each of the serostrains in both homologous and heterologous antisera, it was shown that all serostrains of both *C. jejuni* and *C. coli* are strongly agglutinated by homologous antisera (Fig. 1, Table 2). In the case of *C. jejuni*, the known cross-reactions, for the most part, were also detectable by strong agglutinations, but interestingly, previously unrecognized reactions between serostrains O:29 and O:53 and between O:12 and O:40 were also detected. Two antisera, O:9 and O:21, evidently possess antibody against a common determinant that produced weak agglutinations of all or most of the serostrains. Except for two weak, homologous agglutinations and 13 weak, often unreproducible cross-reactions, the results for *C. coli* serostrains were similar to those obtained for *C. jejuni* serostrains.

Like the PHA serotyping system, the SA method showed that there was an almost complete lack of cross-reactivity between strains of *C. jejuni* and *C. coli* (serotype 5 is common to both). When an organism is received, it is

TABLE 3. Results of serotyping 205 isolates of *C. jejuni* by SA and PHA

No. of isolates	Agglutination in antiserum pool no.:	O serotype[a]	Titer (range)[b]
23	I	1	(640–5,120)
4	I	1, 44	(320–1,280), (320–1,280)
2	I	8	(320–640)
28	II	2	(320–1,280)
2	II	10	(320–1,280)
2	II	19	(320–640)
1	II	55	1,280
12	III	3	(320–640)
2	III	11	5,120
19	IV	4	(320–1,280)
1	IV	4, 13, 16	5,120, 160, 160
1	IV	4, 16	640, 80
1	IV	13, 16, 50	80, 320, 320
1	IV	16	160
1	IV	43	80
2	IV	50	640
6	V	5	(160–5,120)
3	V	5, 31	(40–80), 1,280
1	V	5, 31	640, 80
2	V	31	(320–640)
2	V	33	5,120
1	VI	6	5,120
8	VI	6, 7	(160–5,120), (320–1,280)
28	VI	12, 40	(1,280–5,120), (160–2,560)
1	VI	40	5,120
1	VII	9, 15	160, 160
1	VII	9, 21	5,120, 40
3	VII	15	640
1	VII	15, 42	2,560, 80
3	VII	21	(2,560–5,120)
2	VII	42	(640–2,560)
2	VII	45	640
2	VIII	23	(640–1,280)
14	VIII	23, 36	(160–640), (2,560–5,120)
4	VIII	23, 53	(320–1,280), (160–640)
6	IX	37	5,120
3	II, IV	2, 16	(320–640), (80–160)
3	III, IV	3, 50	80, 80
3	III, VIII	3, 23, 36	(320–640), 1,280, 5,120
3	V, VI	5, 6, 7	160, 5,120, (640–1,280)

[a] The O serogroup was determined by both SA and PHA.
[b] Titers are expressed as reciprocals. Titers of less than 1:40 were considered to be negative.

TABLE 4. Differences in results for serotyping *C. jejuni* isolates by two methods

No. of isolates	O sereotype determined by:		
	SA with antisera[a]		PHA (titer or range of titers)[b]
	Pooled	Single	
6	I	1	1 (640–5,120), 44 (160–640)
1	I	1	1 (1,280), 4 (320), 44 (320)
1	I, II	2, 8_w, 17_w	8 (320), 17 (640)
1	I, II	2_w, 8_w	8 (640)
1	III	3_w	3 (640), 23 (320), 36 (5,120)
1	III, IV	3, 4	4 (640)
3	III, VII	3_w, 9	3 (160–320)
1	III, IV	3_w, 13_w, 16_w	3 (80), 13 (80), 16 (640), 50 (320)
1	III, IV	3, 16	3 (80), 4 (80), 13 (160), 16 (320), 50 (80)
1	IV	4	4 (160), 13 (640), 16 (320)
2	IV	4	4 (320–1,280), 16 (40)
1		NT	4 (640)
4	IV	4	4 (1,280–2,560), 13 (40–80), 43 (40), 50 (40)
1	IV, V	4, 38_w	4 (5,120)
1	VI	6_w, 7_w	NT
1	VI, VII	6, 7, 21	6 (5,120), 7 (160)
1	I	8	8 (320), 17 (640)
1	VII	9_w, 45	45 (5,120)
1	VII, VIII	9, 23_w, 53_w	23 (160), 53 (640)
1		NT	10 (320)
1	IV	13, 50	13 (40), 16 (40), 50 (640)
1	VII, V	15, 31	15 (640)
1	III	18_w	NT
1	II	19_w	NT
1	VII, VIII	21, 29_w	21 (2,560)
2	VIII	23_w, 36	8 (320), 23 (80–320), 36 (2,560–5,120)
1	VII, II	42, 55	55 (640)
1	IV	43, 50	13 (40), 16 (160), 43 (160)
1	IV	43, 50	4 (40), 13 (80), 16 (160), 43 (160), 50 (80)

[a] w, weak reaction; NT, not typeable.
[b] Titers or range of titers are given in parentheses and are expressed as reciprocals. Titers of less than 1:40 were considered to be negative.

TABLE 5. Results of typing 57 isolates of *C. coli* by two methods

No. of isolates	Serotype determined by:			Titer (range)[b]
	SA with antisera[a]		PHA	
	Pooled	Single		
5	I	5	5	(2,560–5,120)
1		5	5, 30	80, 1,280
1		5, 30	5, 26, 30	2,560, 320, 5,120
5		30	30	5,120
2		24	24	2,560
1		24_w	NT	
1		34	34, 48	1,280, 5,120
1		34_w	NT	
1		47	47	640
1	II	25_w	25	1,280
1		28	28, 56, 59	320, 1,280, 5,120
1		28	28, 54, 59	2,560, 1,280, 5,120
5		46	46	5,120
4		49	49	5,120
2	III	26	26, 34	640, 320
1		39	39, 47	2,560, 1,280
3		39	39	320
7		48	48	5,120
3		59	59	640
2	IV	14	14	(2,560–5,120)
1		54	2, 54	320, 1,280
1		56	56	1,280
1	I, II	46, 47_w	46, 47	1,280, 160
2	I, III	24, 39_w	24, 39	640, 160
1		5_w, 24_w, 39_w	5, 24	160, 1,280
3		NT	NT	

[a] w, weak reaction; NT, not typeable.
[b] Titers or ranges of titers are expressed as reciprocals. Titers of less than 1:40 were considered to be negative.

serotyped in sera specific for its species designation. However, if the antigen fails to react, it is subsequently typed in all the remaining *Campylobacter* sera, therefore eliminating the possibility that the strain was identified as the wrong species.

To reduce the number of SA tests for determining the serotype, the use of pooled antisera was first evaluated by using serostrains. It was found that the O-serotype specificities of 41 serostrains of *C. jejuni* were correctly identified by strong agglutinations. Although the 18 serostrains of *C. coli* were also differentiated through the use of pooled antisera and the SA procedure, some unexpected reactions were observed. The majority of these were weak agglutinations, but one strain (O:30) agglutinated strongly in two heterologous antisera. However, in no case was the major O-serotype specificity not detected, and therefore, it was concluded that testing of the simplified procedures with clinical isolates was warranted. In this study, it was found that the same results were obtained by both methods for 83% of the *C. jejuni* (Table 3) and 79% of the *C. coli* (Table 5) isolates. The majority of the discrepancies were either due to weak SA reactions that could not be detected by PHA at antiserum dilutions of 1:80 or due to weak PHA reactions that were not detected by SA. If these weak reactions were discounted, the proportion of correctly identified isolates increased to 93% for *C. jejuni* and 81% for *C. coli*. The remaining differences were due to the inability of one or the other system to detect cross-reacting determinants. However, since there was agreement between the two systems on their identification of the major antigen or major antigens in virtually all cases, the simpler system warranted a recommendation for its use in the clinical laboratory.

In a comparison of the two systems, it is evident that SA coupled with pooled antisera requires considerably less time to perform. If the PHA system is accepted as the standard for reference, the SA technique produces identical results approximately 80% of the time. If the purpose of serotyping is simply to ascertain whether a group of isolates is epidemiologically linked or unrelated, the simpler technique can be recommended with confidence. If isolates from different sources or outbreaks are to be classified for national or international reference, the PHA procedure, which produces titer profiles for each isolate, is advocated. If the simplified system finds use in larger numbers of clinical laboratories and the epidemiology of infections caused by *C. jejuni* and *C. coli* becomes the subject of more detailed investigations, an important objective for developing the simplified system will have been achieved.

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